TCF-1 upregulation identifies early innate lymphoid progenitors in the bone marrow

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The cellular and molecular events that drive the early development of innate lymphoid cells (ILCs) remain poorly understood. We show that the transcription factor TCF-1 is required for the efficient generation of all known adult ILC subsets and their precursors. Using novel reporter mice, we identified a new subset of early ILC progenitors (EILPs) expressing high amounts of TCF-1. EILPs lacked efficient T and B lymphocyte potential but efficiently gave rise to NK cells and all known adult helper ILC lineages, indicating that they are the earliest ILC-committed progenitors identified so far. Our results suggest that upregulation of TCF-1 expression denotes the earliest stage of ILC fate specification. The discovery of EILPs provides a basis for deciphering additional signals that specify ILC fate.

Innate lymphoid cells (ILCs) lack adaptive antigen receptors but functionally and transcriptionally resemble subsets of effector T cells1–4. They include conventional natural killer (NK) cells and three subsets of cytokine-producing helper cells, ILC1, ILC2 and ILC3 cells5. ILCs are important players in tissue homeostasis, host defense and tumor surveillance6. However, the cellular and molecular events that underlie ILC fate specification and commitment remain poorly understood.

ILCs derive from bone marrow (BM) lymphoid progenitors6–8, but little is known about their subsequent lineage-progression steps. Previous studies identified several candidate ILC progenitor subsets in the BM, but none efficiently give rise to all four ILC lineages at the clonal level9–11. A developmental history of expression of the transcription factor PLZF was observed in several cytokine-producing helper ILC subsets, but not into conventional DX5+ NK cells or CD4+ LTi-like cells11. A similar subset of BM progenitors cells, identified as Id2+Thy-1+IL-7Rααβ7−Lin− cells, are termed common helper innate lymphoid cell progenitors (CHILPs)10. CHILPs consist of both PLZF+ and PLZF− progenitors. CHILPs give rise to all helper ILC subsets, but not to conventional NK cells10. The majority of single BM PLZF+ progenitors or CHILPs give rise to one or two ILC lineages but were found to lack multi-ILC lineage potential when assessed in vitro, which indicates that they may represent more mature ILC progenitor stages10,11. A different progenitor subset defined as CXCR6+IL-7Rαα−α−LP (lymphoid progenitor) cells may give rise to conventional NK cells as well as helper ILC subsets9. However, these CXCR6+IL-7Rαα−α−LP cells are extremely rare (around 50 cells per entire mouse), and only 2.5% of them (1 cell per entire mouse) efficiently give rise to all four ILC lineages at the clonal level9. Thus, physiological early ILC progenitors (EILPs) for all four adult ILC lineages remain to be identified.

T cell factor 1 (TCF-1, encoded by the gene Tcf7) is a sequence-specific high-mobility-group transcription factor. TCF-1 was cloned from T cells12,13, and it has an essential role in T cell lineage specification and differentiation14–19. Recent work by us and others also implicates TCF-1 in the biology of ILCs20–24. Numbers of mucosal ILC2 cells are greatly reduced in Tcf7−/− mice compared with wild-type mice, and therefore these mice cannot clear nematodes or mount innate type 2 immune responses to airway protease challenge20,21. The number of small intestinal lamina propria (SiLP) ILC3 cells is also considerably reduced in Tcf7−/− mice21. In addition, the number of NK cells is modestly reduced in Tcf7−/− mice, and Tcf7−/− NK cells do not express the receptor Ly49a22–24. Thus, TCF-1 is an essential controller in the generation or function of ILCs. The precise role of TCF-1 in early ILC development, however, remains largely unknown.

Here we report that TCF-1 promotes early ILC development in the BM, and we also identify EILPs in the BM that express TCF-1 and efficiently develop into all known adult ILC lineages at the clonal level. We show that TCF-1 is required for the efficient generation of all known adult mature ILC subsets, as well as of CHILPs and NK cell progenitors (NKPs). Using newly generated Tcf7GFP reporter mice, we identified EILPs expressing high levels of TCF-1 but lacking surface markers of adaptive and innate lymphocyte lineages. These EILPs closely resembled BM lymphoid cell progenitors at the transcriptome level but lacked efficient T and B lymphocyte potential. Instead, they
RESULTS

TCF-1 promotes early ILC development

To understand the specific developmental stage(s) at which TCF-1 acts to promote ILC development, we examined the previously described PLZF-expressing CHILPs10,11 and CD122-expressing NKPs25 in the BM of Tcf7−/− mice (exon VII targeted)19. Numbers of both PLZF+ CHILPs and NKPs were greatly reduced in Tcf7−/− mice compared with wild-type mice, which indicated that TCF-1 was required for early ILC development in the BM (Fig. 1a–c). Frequencies of the previously described pre-NKPs26, refined-purity NKPs26 and pre-pro-NKPs27 were also greatly reduced in Tcf7−/− mice (Supplementary Fig. 1a). Despite the near absence of CHILPs and NKPs in Tcf7−/− mice, some mature NK cells, ILC1 cells and ILC3 cells persisted in the peripheral tissues of these mice12–16 weeks after transplant (Fig. 1d–e). Identification of an early innate lymphoid progenitor

Identification of an early innate lymphoid progenitor

Recent work has described several subsets of BM ILC progenitors expressing interleukin 7Rα (IL-7Rα), but none of them was found to efficiently give rise to all four ILC lineages at the clonal level9–11. To search for TCF-1–expressing progenitors, we generated a Tcf7-GFP reporter allele by inserting GFP-coding sequences into the first intron of the Tcf7 gene (referred to here as Tcf7GFP; Supplementary Fig. 2a–c). In Tcf7GFP mice, GFP was specifically expressed in T lymphocytes that expressed high levels of TCF-1, but not in B lymphocytes that lacked TCF-1 expression (Supplementary Fig. 2d).

Because ILC development does not require the thymus, we initially searched for possible TCF-1+ ILC progenitors in the BM. The Lineage-negative (Lin−) cells in the BM of Tcf7GFP mice contained rare but distinct GFP+ subsets (Fig. 2a). We fractioned the GFP+Lin− BM progenitors on the basis of their surface expression of IL-7Rα and Thy-1. Consistent with previous reports9,11,28, the IL-7Rα+Thy-1+GFP+ subsets included previously described CHILPs and ILC2 cell progenitors (ILC2ps), which expressed TCF-1. We also identified a BM subset that expressed high levels of TCF-1 but had no or low surface expression of IL-7Rα and Thy-1 (Fig. 2a), and thus differed from previously described candidate progenitor populations9,11,25. Unlike previously described α-LPs, NKps, pre-NKps, refined-purity NKps, pre-pro-NKps and CHILPs9,11,25–27, these IL-7Rα−Thy-1−TCF-1+ Lin− cells did not express detectable CD122 or CXCR6 on the cell surface (Fig. 2b) and expressed only low levels of mRNAs for Il7r and efficiently gave rise to various ILC lineages, including conventional NK cells and cytokine-producing helper ILCs at the clonal level, which indicates that they are the earliest ILC progenitors to be identified. Our data implicate TCF-1 in the very early stages of BM ILC specification. Identification of EILPs will provide a basis for understanding the molecular signals that drive early ILC development.

Figure 1 TCF-1 is required for the efficient generation of all known adult ILCs and their progenitors. (a) Flow cytometry analysis of PLZF+ CHILPs in the BM of Tcf7+/+ and Tcf7−/− littermates. (b) Flow cytometry analysis of NK1.1−CD122+ NKps in pre-gated Lin− BM cells from Tcf7+/+ and Tcf7−/− littermates. (c) Quantification of the numbers of PLZF+ CHILPs (left) and NKps (right) per Tcf7+/+ or Tcf7−/− mouse analyzed as in a and b. (d) Flow cytometry analyzing mature ILCs B and T cells in the liver and mesenteric lymph nodes (MLNs) of lethally irradiated chimeric mice (CD45.1) intravenously inoculated with Tcf7−/− or Tcf7+/+ LSK BM cells (CD45.2) equally mixed with wild-type competitor LSK cells (CD45.1) and analyzed 12–16 weeks after transplant. (e) Donor chimerism of mature ILCs B and T cells in the peripheral tissues of recipient mice as assessed as in d. (f) Flow cytometry analyzing BM CHILPs and NKPs in chimeric mice 12–16 weeks after transplant. (g) Donor chimerism of BM CHILPs and NKPs as assessed in f. Numbers adjacent to outlined areas in flow cytometry plots indicate the percentage of cells in the gate. Data are representative of three independent experiments (a,b,d,f) or pooled from three independent experiments with three mice per group (c,e,g) and are presented as mean ± s.e.m. ** P < 0.01.
Zbtb16 (encoding PLZF) (Fig. 2c), which confirmed that they were a previously unrecognized cell subset. These candidate EILPs developed into all four ILC lineages on OP9 stroma, but they lacked efficient B or T cell potential in vitro (Supplementary Fig. 3a–c). EILPs lacked expression of Cd3e, which confirmed that they were not T cells (Fig. 2c); they also lacked expression of B cell lineage transcription factors such as EBF1 (Fig. 2c). However, they expressed high amounts of Nfil3 and Tpx (Fig. 2c), two other genes encoding transcription factors implicated in early ILC development9,29–32, and thus exhibited features of early ILC progenitors.

We compared EILPs with other early hematopoietic progenitors. Like common lymphoid progenitors (CLPs), EILPs expressed low amounts of c-Kit and Sca-1 (Fig. 3a). EILPs also expressed a low level of the receptor tyrosine kinase Flt3 (Fig. 3a). We carried out genome-wide microarray analysis to compare the transcriptome of EILPs with those of other early progenitors, including hematopoietic stem cells, lymphoid-primed multipotent progenitors (LMPPs), Flt3+ common myeloid progenitors (CMPs)33, Flt3− CMPs33, CLPs, early T cell lineage progenitors (ETPs) and double-negative 3 cells (DN3 cells). Genetic profiling indicated that EILPs were closer to CLPs than to other early progenitor populations at the transcriptome level (Fig. 3b and Supplementary Fig. 3d). We next compared LMPPs, CLPs and EILPs in terms of their ability to develop into myeloid and lymphoid cell lineages in vitro. Similar to CLPs, EILPs lacked efficient...
myeloid-differentiation activity (Fig. 3c,d) but rapidly developed into dendritic cells (DCs) in vitro, at an early time point (day 4) when DC differentiation from LMPPs was not yet evident (Fig. 3e,f). Thus, EILPs resembled CLPs in phenotype, gene expression profile and differentiation activity, indicating that EILPs are developmentally closely related to CLPs.

**EILPs are clonogenic progenitors for all known adult ILC subsets**

To determine the *in vivo* lineage potential of EILPs, we intravenously transferred EILPs or TCF-1+ CHILPs together with competitor CLPs into unirradiated *Rag2<sup>−/−</sup>* mice. We analyzed lymphocyte reconstitution 3–6 weeks after transplantation. EILPs failed to generate B lymphocytes and generated very few T cells (Fig. 4a,b). However, they gave rise to all four ILC lineages examined *in vivo* (Fig. 4a,b). The ILC progeny derived from EILPs included liver DX5<sup>+</sup>Eomes<sup>+</sup> NK cells, DX5<sup>+</sup>Eomes<sup>+</sup> ILC1 cells, intestinal KLRG-1<sup>+</sup>Sca-1<sup>+</sup>ICOS<sup>+</sup> ILC2 cells and ROR<sup>γ</sup>δ<sup>+</sup> ILC3 cells, including some CD4<sup>+</sup> LTi-like cells (Fig. 4c). Consistent with previous reports<sup>8,10,11</sup>, TCF-1+ CHILPs efficiently gave rise to ILC1, -2 and -3 cells but not to conventional NK cells, which indicated that they were more likely to be downstream helper ILC progenitors (Fig. 4a,b). Together, these data established that EILPs are ILC-committed progenitors possessing the capability to give rise to all known adult ILC lineages in vivo.

We considered whether the inability of EILPs to reconstitute T lineage cells after intravenous transfer might be due to their inability to home to the thymus, rather than to a lack of T cell potential. To better determine the T cell lineage potential of EILPs, we carried out intrathymic transfer of EILPs or control CLPs. Whereas CLPs generated CD4<sup>+</sup>CD8<sup>+</sup> (double-positive) thymocytes as expected, EILPs lacked T cell lineage potential even when placed inside the thymus (Fig. 4d,e). These data, together with our observation that EILPs did not give rise to T cells on OP-DL1 stroma (Supplementary Fig. 3b), indicated that EILPs lack T cell lineage potential.

We next sought to determine the lineage potential of EILPs at the clonal level *in vitro*. We tested a number of different cytokine conditions based on previous reports<sup>8–11,13,34</sup> to identify the cytokine combination (SCF, IL-2 and IL-7) with which EILPs displayed the greatest proliferative expansion (Supplementary Fig. 4a). We plated single EILPs isolated by cell sorting on OP9 stromal cells in each well of 96-well plates. Plating efficiencies in these experiments were approximately 50% (Supplementary Fig. 4b). The majority of positive
Figure 5  EILPs efficiently give rise to all four ILC lineages at the clonal level. (a) Clonogenic differentiation assay showing the emergence of ILC progeny derived from single EILPs cultured on OP9 stroma at one cell per well in the presence of IL-2, IL-7 and SCF for 10 d. Each column represents one well, with detected ILC lineages in gray. Only wells with positive ILC growth are shown. (b) Flow cytometry analyzing the daughter cells derived from EILP single-cell culture as assessed in a. Numbers in outlined areas indicate the percentage of cells in the gate. (c) Quantification of the number of ILC progeny derived from EILPs that were cultured on OP9 or OP9-DL1 in the presence of SCF, IL-2 and IL-7 for 10 d. Data are pooled or are representative of five independent experiments (b) or are from three independent experiments (c). Data are presented as mean and s.e.m. *P < 0.05.

wells contained all four ILC lineages, including ROR\(\gamma\)+ ILC3 cells, GATA-3+ ILC2 cells, and cells with the phenotypes of conventional NK cells (NK1.1+NKp46+DX5+)11.35–37 and ILC1 cells (NK1.1+NKp46+DX5−)11.35–37 (Fig. 5a,b). The remaining wells contained a wide variety of combinations of ILC progeny, including a few ILC1–3 cell trilineage helper ILC progenitors that may overlap with the previously described PLZF+ progenitors10,11 (Fig. 5a). The downstream TCF-1+ CHILPs chiefly gave rise to one or two lineages in vitro, consistent with previous work, which suggested that they were likely a mixture of intermediate stages of progenitors10,11 (data not shown). The earlier progenitors—LMPPs, CLPs and ETPs—did not efficiently give rise to ILCs under these conditions, which indicated that additional signals are required in vitro for ILC fate specification and commitment (Supplementary Fig. 4c). Together, these results establish that EILPs efficiently develop into all four adult ILC lineages in vivo and in vitro at the clonal level and indicate that they likely represent the earliest ILC progenitors identified so far.

Previous reports have indicated that Notch signaling promotes the earliest stages of ILC development but is dispensable for ILC maturation at later stages6,10,20,34. Our experiments indicated that EILPs do not require Notch signals for further maturation in vitro, probably because they already express TCF-1 and possibly other Notch target genes. To examine whether Notch can modulate ILC development after the EILP stage, we compared the differentiation of EILPs on OP9 and OP9-DL1 stroma. The Notch ligand DL1 enhanced the generation of ILC2 cells but repressed the development of NK cells from EILPs (Fig. 5c). Nevertheless, all ILC subsets clearly developed on OP9 stroma in the absence of Notch signals, which indicated that Notch signaling is not strictly required for the further development of EILPs into ILC lineages (Fig. 5c).

Our identification of EILPs in the BM indicated that the earliest stages of ILC specification and commitment occur at this site. To understand whether the thymus might also be a suitable site for early ILC development, we searched for EILPs in the thymus. A few thymocytes displayed the phenotype of EILPs (Lin−TCF-1+Thy-1−IL-7R+ROR\(\gamma\)+), Supplementary Fig. 4d), but they failed to efficiently generate ILCs in vitro (Supplementary Fig. 4e). Thus, functional EILPs are rare in the thymus, if they exist at all. These data indicate that although upregulated expression of TCF-1 occurs during both early ILC development in the BM and early T cell development in the thymus, there must be other important differences in early lymphocyte development at these sites.

Id2 is dispensable for the generation of EILPs

In addition to TCF-1, the transcriptional inhibitor of E proteins Id2 is also required for the generation of all known ILCs38–40. However, previous studies suggested that ILC fate specification might be initiated independently of Id2 (refs. 9,10,41). Indeed, compared with numbers in wild-type mice, the number of NKPs remained constant in Id2−/− mice41 but was greatly reduced in TCF-1−/− mice, which suggested that TCF-1 might act before Id2 during early ILC development (Fig. 1b,c). EILPs expressed Id2 mRNA, but the levels were lower than those in
CHILPs and ILC2 cell progenitors (Fig. 2c). To determine whether Id2 was required for EILP generation, we generated Id2−/−Tcf7GFP mice. EILPs clearly persisted in Id2−/− mice, although their numbers were 50% lower than those in Id2+/+ mice (Fig. 6a,b). CHILPs and ILC2ps were absent, as expected10. Id2−/− EILPs expressed Nfil3 and TOX at slightly lower levels than observed in Id2+/+ EILPs (Fig. 6c). Thus TCF-1 is expressed before Id2 dependence develops during the emergence of the earliest progenitors for ILCs in early BM hematopoiesis.

To determine whether Id2 might control the lineage commitment of EILPs, we cultured Id2−/− EILPs under B or T lymphocyte differentiation conditions with OP9 or OP9-DL1 stroma. Id2−/− EILPs failed to give rise to B or T cells in vitro, which indicated that other molecules can repress T and/or B lymphocyte potential in EILPs (Supplementary Fig. 5a,b). We examined the expression of other Id proteins in Id2−/− EILPs. In the absence of Id2, Id1 expression was increased threefold and Id3 expression was increased 11-fold in EILPs relative to expression in Id2+/+ EILPs (Fig. 6c). Thus Id2 might participate in ILC fate commitment, but the expression of other Id proteins may be upregulated to compensate for the loss of Id2.

Together, our data suggest a model in which EILPs represent the earliest BM ILC progenitors identified so far, prior to the stages of NKPs and CHILPs (Supplementary Fig. 6).

**DISCUSSION**

The present study identifies an early ILC progenitor. EILPs express TCF-1 and are clonogenic progenitors for NK cells and all known adult helper ILC lineages. EILPs were identified in the BM, persisted in the absence of Id2 and were most closely related to CLPs at the transcriptome level. Together these findings indicate that TCF-1 might have a role in the earliest steps of BM ILC development, before the requirement of Id2. NKPs and CHILPs were nearly absent in adult Tcf7−/− mice, which indicated that these innate progenitors are likely to arise downstream of EILPs, and that TCF-1 is required for their generation.

Although CHILPs and NKPs were nearly completely lacking in adult Tcf7−/− mice, many mature ILCs developed and were present in these mice in the absence of competition20,21,24. However, the remaining ILCs in Tcf7−/− mice may be functionally compromised20,21,24. The remaining NK cells in Tcf7−/− mice do not express Ly49a24, the remaining Tcf7−/− ILC2 cells do not make IL-13 in response to in vivo intranasal protease challenge20,21, and the development of Peyer’s patches is severely compromised in Tcf7−/− mice despite a near-normal number of CD4+ LTI-like cells21. How these abnormal ILCs develop in Tcf7−/− mice remains to be determined; compensatory upregulation of Lef-1 expression is one possible mechanism14,20,42. Nevertheless, our data from competitive LSK chimeras indicated that the TCF-1–dependent pathway is the predominant pathway by which normal, functionally intact ILCs are replenished in adult mice.

EILPs are closely related to CLPs at the transcriptome level, but EILPs lack efficient T and B lymphocyte potential and express very low levels of IL-7Rα. ETPs, the earliest identified T cell–lineage progenitors, also show low Il7r mRNA expression, but most ETPs are marked with a history of IL-7Rα expression in Il7r-Cre/Rosa YFP mice3,44. Future investigations could explore whether EILPs also have a history of IL-7Rα expression and what controls the dynamic expression of IL-7Rα during early ILC and T cell lineage development. One possibility is that downregulation of IL-7Rα expression might help quench B cell–lymphoid fate to allow entry into the ILC and T cell lineages45.

Previous studies indicated that NK cell fate specification is initiated before the requirement for Id2 in NK cell development9,10,41. Consistently, we observed that Id2 was dispensable for the formation and lineage commitment of EILPs. However, Id2−/− EILPs exhibited increased expression of Id1 and Id3, which indicated that other Id proteins may compensate for the loss of Id2 during early ILC development. The role of Id proteins in the earliest stages of ILC fate specification and commitment, as well as the implication of other molecular signals, warrants further investigation.

TCF-1 expression is high in EILPs in the BM and in ETPs14 in the thymus, yet EILPs and ETPs differ significantly in their lineage potentials and gene expression profiles. Thus other differences must exist between early ILC development and early T cell development. EILPs differ from ETPs in their expression of several key transcription factors, such as Id2 and Nfil3, that may collaborate with TCF-1 to specify ILC fate. How their expression is elicited in the BM but not in the thymus is presently unknown. One possibility is that expression of other key transcriptional controllers in EILPs is elicited by signals distinct from those that induce TCF-1 expression. Our discovery and characterization of EILPs will facilitate the identification of these signals and aid in deciphering the mechanisms underlying innate lymphoid specification.

In summary, our results implicate TCF-1 in early stages of ILC development and suggest that it promotes the development of all known adult ILC subsets and precursors. We show that TCF-1 upregulation is an identifying characteristic of the earliest known innate lymphoid progenitors in the BM. Comparison of EILPs with adaptive lymphocyte-biased progenitors may shed light on the unique pathways that determine the fate of each lymphocyte lineage.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Accession codes.** Microarray data were deposited at GEO with accession number GSE69789.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

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**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. C57BL/6 (B6, CD45.2) and B6-Ly5.2 (CD45.1) mice were from the National Institutes of Health or the Jackson Laboratory. Rag2−/− Il2rg−/− mice were from Taconic. We also used Tcf7−/− mice13, Tcf7Il2rg−/− mice and Id2+ Tcf7Il2rg−/− mice. All mice used were on the B6 background. Mice used were 6–12 weeks old and of either sex. Sample sizes were empirically determined. No statistics were used to predetermine the sample sizes. No samples were excluded from analysis. No randomization or blinding was used. All animal experiments were conducted according to protocols approved by the Institutional Animal Care and Use Committee policies at the University of Pennsylvania, the University of Iowa and the US National Institutes of Health.

Generation of TcF7Il2rg−/− reporter mouse strain. Targeting-construct design. The Tcf7 gene is expressed in multiple isoforms due to the use of different promoters and alternative splicing. The full-length TCF-1 isoforms (p45 and p42) are transcribed from an upstream promoter using exon 1, and the short isoforms (p33 and p30) are transcribed from exon 3 (Supplementary Fig. 2a). To generate a TcF7Il2rg−/− reporter allele, we inserted an enhanced green fluorescent protein (EGFP) expression cassette and a neomycin-resistant gene (neo') cassette into the least conserved region in the first intron of TcF7. The EGFP expression cassette contains a strong En2 gene-splicing acceptor to facilitate splicing of TcF7 exon 1 to the reporter, an internal ribosome entry site to facilitate independent translation of EGFP; and the EGFP cDNA followed by a polyadenylation sequence. Two Frt sites were inserted to flank the EGFP and neo' cassettes. One loxp site was inserted immediately downstream of the second Frt site in intron 1, and another loxp site was inserted into the least conserved region in intron 2. These features, although not used in this study, were designed to remove the EGFP and neo' cassettes with Flippase, converting the TcF7-EGFP reporter allele to a TcF7 exon 2–floxed allele, so as to conditionally target the long TCF-1 isoforms.

The targeting construct was assembled via the recombineering approach. All sequences surrounding the insertion sites were verified to ensure that no unwanted mutations had occurred. The targeting construct was electroporated into C57BL/6 embryonic stem (ES) cells, and the ES cell clones with expected homologous recombination were screened by Southern blotting (Supplementary Fig. 2b,c). Blastocyst injection of the ES cells was done at the Transgenic Animal Model Core of the University of Michigan. The chimeras containing the targeted allele were crossed with C57BL/6 mice to achieve germline transmission.

Isolation of hematopoietic cells in peripheral tissues. For isolation of lung hematopoietic cells, we perfused lungs by injecting 10 ml PBS into the right ventricle of the heart. Lungs were carefully cut into small fragments and digested in HBSS containing 0.025 mg/ml Liberase D (Roche Diagnostics) and 10 U/ml DNase I (Roche Diagnostics). Cells were filtered with a cell strainer.

For isolation of intestinal lymphocytes, Peyer's patches were removed from the small intestines. The small intestines were opened and emptied of their contents. The small intestines were then cut into 1-cm pieces and shaken at 37 °C for 30 min in RPMI 1640 medium containing 1% FCS (Invitrogen), 1 mM EDTA (Intravitrogen), and 1 mM DTt (Sigma-Aldrich). The tissues were washed and subsequently digested with HBSS containing 0.025 mg/ml Liberase D (Roche Diagnostics) and 10 U/ml DNase I (Roche Diagnostics) with shaking at 37 °C for 30 min. The digested tissues were filtered. Lamina propria lymphocytes were isolated with 40% Percoll.

Flow cytometry and cell sorting. All antibodies used in this study were purchased from eBioscience. Clones for antibodies can be found in Supplementary Table 1. Flow cytometric analysis was done on an LSR-II (BD Biosciences). Intracellular staining was done with the Fix/Perm Kit (eBioscience) according to the manufacturer’s instructions.

Adoptive transfer. For the creation of competitive LSK chimeras, 5 × 10^3 sorted BM LSK progenitors from donor mice (CD45.2) were mixed with 5 × 10^3 competitor LSK cells from wild-type mice (CD45.1) and injected into lethally irradiated (950 rad) wild-type recipient mice (CD45.1). Reconstitution of ILCs in the recipient mice was examined at 12–16 weeks post-transfer.

For adoptive transfer of purified BM progenitors, 1 × 10^3 EILPs or TCF-1−/− CHILPs from TcF7Il2rg−/− mice were mixed with BM-equivalent numbers (around 2 × 10^4) of competitor CLPs (CD45.1) and transferred intravenously into Rag2−/− Il2rg−/− mice. Reconstitution of lymphocytes was examined at 3–6 weeks post-transfer.

Cell culture. BM progenitors were sorted by flow cytometric cell sorting. For ILC-differentiation assays, progenitors were cultured in DMEM containing 20% FCS, 30 ng/ml SCF, 30 ng/ml IL-7 and 30 ng/ml IL-2 on OP9 stromal cells. Cytokines were purchased from PeproTech or R&D Systems. Progenitor differentiation was examined at 7–14 d after culture. For B cell–differentiation assays, progenitors were cultured with OP9-DL1 stromal cells in the presence of IL-7 (10 ng/ml) and Flt3L (5 ng/ml) for 7 d. For T cell–differentiation assays, progenitors were cultured with OP9-DL1 stromal cells in the presence of IL-7 (1 ng/ml or 10 ng/ml) and Flt3L (5 ng/ml) for 7 d. For myeloid differentiation, progenitors were cultured with OP9 psp in the presence of IL-7 (1 ng/ml) and Flt3L (5 ng/ml) for 4 d as described46,47. For assessment of DC differentiation, progenitors were cultured in suspension with IL-1β (2 ng/ml), IL-3 (400 ng/ml), IL-7 (10 ng/ml), SCF (10 ng/ml), Flt3L (100 ng/ml) and TNF (1 ng/ml) for 4 d as described48.

Microarray. BM hematopoietic stem cells (Flt3−CD150−Lin−Sca-1−c-Kit+), LMPPs (Flt3Lin−Fca-1−c-Kit+), CLPs (Lin−c-KitSca-1−Flt3Il2−7R−), Flt3+ CMPs (Flt3Lin−Sca-1−c-Kit+FcYRI CD150 CD34+33), Flt3− CMPs (Flt3Lin−Sca-1−c-Kit+FcYRI CD150 CD34+33), and EILPs (LinTcF1Thy1− IL-7Rα−b8) and thymus ETPs (Lin−c-Kit+b2DC25+) and DN3 cells (Lin−c-KitCD25+) were isolated by flow cytometric cell sorting. Microarrays were conducted as described in the Affymetrix GeneChip Expression Analysis Technical Manual by the UPENN Microarray Core Facility. RNA was extracted with Trizol and amplified with the Nugen PicoV2 kit (Nugen), and the quality of the RNA was tested on a bioanalyzer. Biotinylated cDNA was prepared with the Encore Biotech Module kit (Nugen) from 5.5 μg RNA according to the manufacturer's instructions. 2.5 μg of total RNA was hybridized for 16 h at 45 °C on an Affymetrix Mouse Gene 2.0 ST Array. The microarrays were then washed and stained with streptavidin-phycocerythin. GeneChips were scanned using the GeneArray Scanner 3000 7G. The data were analyzed with Affymetrix Expression Console with the default analysis settings. Gene-signal values for the arrays were normalized and log2-transformed. Heat maps were generated with the heatmap R package (version 1.0.2) (ref. 49).

Statistics. Statistical analysis was performed on groups with limited variance. Comparison between two groups was done via two-sided Student's t-test. Differences with a P value less than 0.05 were considered significant.

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