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# Development and differentiation of early innate lymphoid progenitors

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**Early innate lymphoid progenitors (EILPs) have recently been identified in mouse adult bone marrow as a multipotential progenitor population specified toward innate lymphoid cell (ILC) lineages, but their relationship with other described ILC progenitors is still unclear. In this study, we examine the progenitor–successor relationships between EILPs, all-lymphoid progenitors (ALPs), and ILC precursors (ILCps). Functional, bioinformatic, phenotypical, and genetic approaches collectively establish EILPs as an intermediate progenitor between ALPs and ILCps. Our work additionally provides new candidate regulators of ILC development and clearly defines the stage of requirement of transcription factors key for early ILC development.**

## INTRODUCTION

All innate lymphoid cells (ILCs), like B cells and T cells, have been proposed to arise from all-lymphoid progenitors (ALPs), which contain Ly6D<sup>+</sup> common lymphoid progenitors (CLPs) and IL-7R $\alpha$ -expressing, lymphoid-primed multipotent progenitors (Inlay et al., 2009; Moro et al., 2010; Possot et al., 2011; Yang et al., 2011; Cherrier et al., 2012; Klose et al., 2014; Ghaedi et al., 2016; Ishizuka et al., 2016a). The ILC progenitor potential has been further proposed to reside in the  $\alpha 4\beta 7$ -positive fraction of the CLP population ( $\alpha 4\beta 7^{+}$  CLP), which might represent the first uncommitted ILC progenitor (Seillet et al., 2016).

Because ALPs and most mature ILCs express high levels of IL-7R $\alpha$ , intermediate ILC progenitors were assumed to also express this receptor. This useful assumption, together with reporter mouse models for the transcription factors *Id2* and *Zbtb16* (the gene for PLZF), led to the discovery of several progenitors committed to the ILC lineage that are present in mouse adult bone marrow and fetal liver. A common helper ILC precursor (CHILP) and an ILC precursor (ILCp) were described in mouse bone marrow (Constantinides et al., 2014; Klose et al., 2014). ILCp corresponds to the *Zbtb16*-expressing fraction of CHILP, and a small fraction of this population can differentiate into all helper ILC subsets in single-cell differentiation assays (Constantinides et al., 2014). The *Zbtb16*<sup>+</sup> fraction of CHILP includes lymphoid tissue inducer progenitors and possibly more mature ILC populations that continue to express *Id2* but lose *Zbtb16*, such as ILC2 progenitors (ILC2ps; Hoyler et al., 2012; Constantinides et al., 2014; Klose et al., 2014). Equivalent progenitors have been described in fetal liver (Constantinides et al., 2014; Chea et al., 2016;

Ishizuka et al., 2016a,b; Zook and Kee, 2016). However, these appear less committed to the ILC lineage compared with their adult counterparts because they possess T cell potential at the single-cell level at the ILCp stage (Chea et al., 2016; Ishizuka et al., 2016a).

We previously used a reporter mouse for the gene *Tcf7*, which encodes the transcription factor TCF-1, expressed by all known ILC progenitors. This reporter identified *Tcf7*<sup>+</sup> progenitors with lower levels of IL-7R $\alpha$ , *Zbtb16*, and *Id2* as compared with ILCps. Single-cell differentiation assays showed that this new progenitor population, termed early innate lymphoid progenitors (EILPs), was specified toward the ILC lineage and contained a high frequency of multipotent ILC progenitors (Yang et al., 2015). These properties suggested EILPs are upstream of ILCps. However, many EILPs express low levels of surface IL-7R $\alpha$ , and EILPs also express very low levels of *Il7r* mRNA compared with CLPs (Yang et al., 2015). These results raised the possibility that EILPs do not differentiate from ALPs and challenged their affiliation to the main stream of ILC progenitors (Zook and Kee, 2016).

In this study, we examine whether EILPs represent an intermediate ILC progenitor that transiently down-regulates IL-7R $\alpha$  expression. Using functional, bioinformatic, phenotypical, and genetic approaches, we establish EILP as an intermediate progenitor between ALPs and ILCps. Our work also identifies new candidate regulators of ILC development and better defines the precise stage of requirement for transcription factors that are key for early ILC development.

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## RESULTS

## EILPs differentiate from ALPs

Most EILPs express lower levels of IL-7R $\alpha$  compared with ALPs (Fig. S1, A and B), raising the question of whether EILPs develop from an IL-7R $\alpha$ <sup>+</sup> progenitor such as ALP and transiently down-regulate IL-7R $\alpha$  expression or whether we should consider an alternative progenitor lacking IL-7R $\alpha$ .

We wished to examine whether ALPs are progenitors for EILPs. It is presently not possible to assess the ILC potential of putative upstream ILC progenitors *ex vivo* because of the inefficiency of the differentiation of adult ALPs into ILCs *in vitro* (Seehus and Kaye, 2016). We therefore tested the differentiation potential of ALPs into EILPs *in vivo*. We isolated ALPs and hematopoietic stem cells (HSCs) from *Tcf7<sup>EGFP</sup>* reporter mice and transferred them into lightly irradiated WT recipients. To prevent any contamination of these donor populations by EILPs, GFP<sup>+</sup> cells were excluded from the sort. After 7 d of reconstitution, bone marrow cells were harvested and assessed for the presence of *Tcf7*-expressing cells (Fig. 1 A). Lineage marker-negative (Lin<sup>-</sup>) cells expressing high levels of GFP were detected in all recipients inoculated with ALPs. GFP<sup>+</sup> Thy1<sup>-</sup> cells additionally expressed high levels of  $\alpha\beta\gamma$ , thus resembling EILPs (Fig. 1 B). Later ILC progenitors expressing GFP and Thy1 (likely ILCp and ILC2p; Yang et al., 2015) were also found in these mice (Fig. 1 A). At this relatively early time point, donor-derived EILPs and later ILC progenitors were undetectable in mice inoculated with HSCs (Fig. 1, A and C). ALPs are therefore able to differentiate into EILPs more rapidly than HSCs. To further establish whether EILPs differentiate from an *Il7r*-expressing progenitor such as an ALP, we crossed *Tcf7<sup>EGFP</sup>* reporter mice with an *Il7r* lineage tracer strain in which YFP expression is permanently triggered by *Il7r* expression (*Il7r-iCre R26-stop-YFP*; Schlenner et al., 2010). In these mice, YFP expression is initiated at the ALP stage when cells express IL-7R $\alpha$  (Fig. 1 D). We found that EILPs had a similar history of *Il7r* expression as ALPs (Fig. 1 D). In comparison, ILCps expressed YFP at higher frequency. Importantly, IL-7R $\alpha$ <sup>+</sup> and IL-7R $\alpha$ <sup>low</sup> EILPs expressed similar levels of *Tcf7* and had a similar history of *Il7r* expression, showing that YFP marking likely does not occur at the EILP stage (Fig. 1 E). This result shows that EILPs originate from an *Il7r*-expressing progenitor, which is likely ALP.

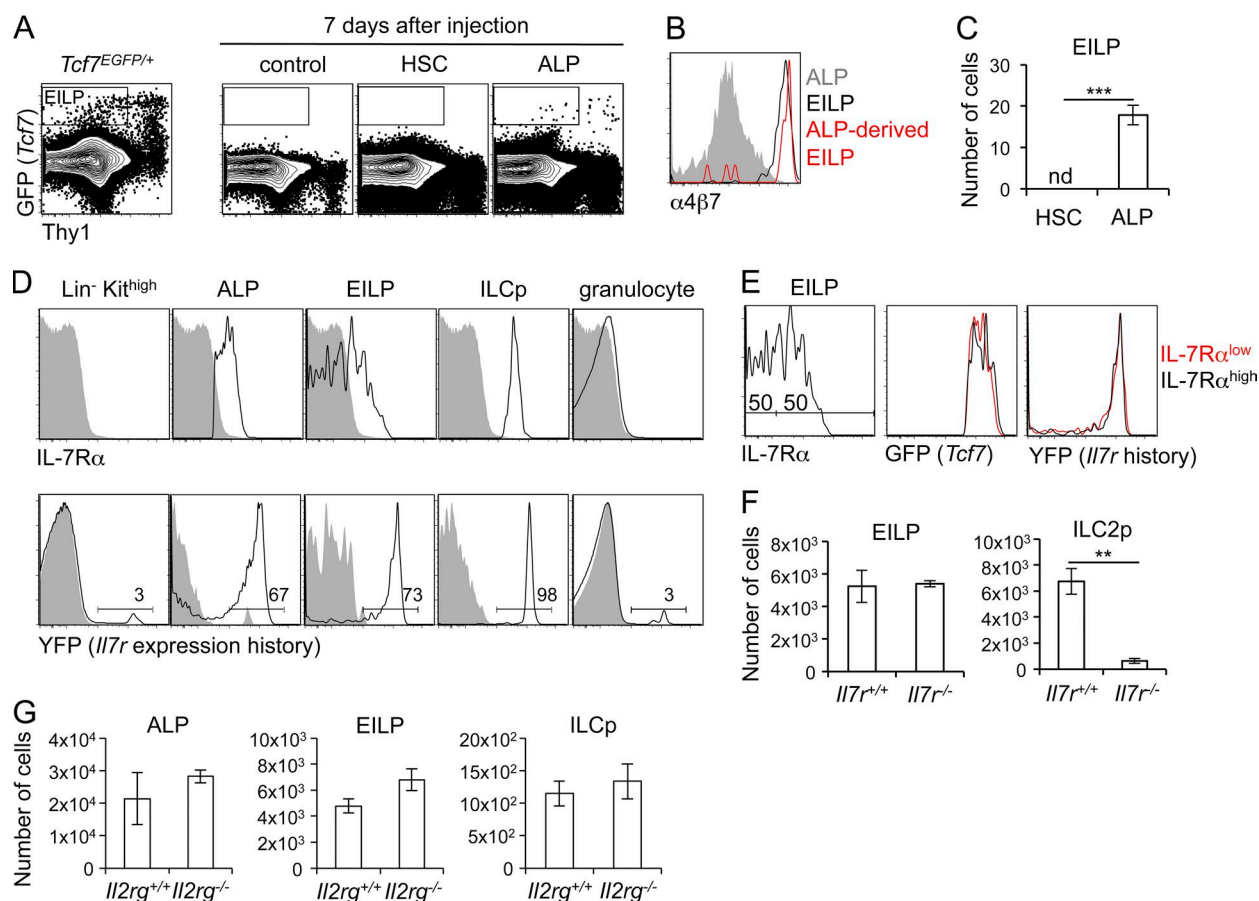
### $\gamma$ c-Dependent cytokines are not required for early ILC development

ALPs and ILCps express IL-7R $\alpha$ , raising the possibility that IL-7 might have important functions very early in ILC development. Consistently, ILC2ps are greatly reduced in *Il7r<sup>-/-</sup>* and *Il2rg<sup>-/-</sup>* mice (Hoyler et al., 2012; Robinette et al., 2017). Nevertheless, the requirement for IL-7R $\alpha$  has not been examined at early stages of ILC development, before the ILC2p stage. We therefore assessed whether EILPs might be intact in mice mutant for components of the IL-7 receptor. To analyze EILPs in mutant strains of mice such as *Il7r<sup>-/-</sup>* mice with-

out having to cross each strain to *Tcf7<sup>EGFP</sup>* reporter mice, we tested commercially available anti-TCF-1 antibodies for intracellular staining. We found that a rabbit monoclonal antibody specific for the N-terminal domain of TCF-1 (C63D9) gave the best signal with minimal background staining in the adult mouse bone marrow. This antibody allowed the detection of a population phenotypically similar to EILPs defined in *Tcf7<sup>EGFP</sup>* reporter mice (Fig. S1 C). Quantification of this population demonstrated that TCF-1 intracellular staining identifies similar numbers of EILPs to those obtained using *Tcf7<sup>EGFP</sup>* reporter mice (Fig. S1 D). Hence, TCF-1 intracellular staining is a suitable alternative to the *Tcf7<sup>EGFP</sup>* reporter allele to visualize and quantify EILPs in adult mice. Additionally, using the same strategy as used for the *Tcf7<sup>EGFP</sup>* reporter mouse (Fig. S1 A), TCF-1 intracellular staining can be used to identify ILCps as shown by PLZF intracellular staining (Fig. 4 C). Using TCF-1 intracellular staining, we found that EILPs are present and in normal numbers in *Il7r<sup>-/-</sup>* mice (Fig. 1 F). However, as previously described (Hoyler et al., 2012), ILC2ps (which can be examined without using expression of IL-7R $\alpha$ ) were greatly reduced in *Il7r<sup>-/-</sup>* mice (Fig. 1 F). To examine whether other  $\gamma$ c-dependent cytokines might play a role during early ILC development, we stained for ILC progenitors in *Il2rg<sup>-/-</sup>* mice and found that EILP and ILCp numbers were similarly unaffected (Fig. 1 G). Our results show that IL-7 and other  $\gamma$ c-dependent cytokines are not required for ILC development before the ILC2p stage, and IL-7R $\alpha$  expression itself is not relevant at the EILP stage. We conclude that EILPs originate from IL-7R $\alpha$ -expressing progenitors such as ALP, but they do not require IL-7 or other  $\gamma$ c-dependent cytokines for their development.

### EILP is a progenitor for ILCp

We wished to compare the properties of EILPs and ILCps in more detail and examine a possible relationship between the two progenitors. Consistent with EILPs and ILCps being closely related, the frequencies of the three mature ILC subtypes derived from these two progenitors were similar at day 8 in ILC differentiation conditions *in vitro* (Fig. S2, A and B). We further examined earlier time points of these cultures to analyze the early steps of differentiation of EILPs and ILCps. At day 4, ALPs, EILPs, and ILCps gave rise to a similar number of cells (Fig. 2 B). However, whereas ILCp-derived cells were predominantly differentiated ILCs, as shown by the expression of inducible T cell co-stimulator (ICOS; expressed by ILC2 and ILC3) or natural killer (NK) 1.1 (expressed by ILC1 and NK cells), most progeny of EILPs lacked expression of these markers (Fig. 2 A). Because EILPs are known to possess dendritic cell potential (Yang et al., 2015), we further examined the ICOS<sup>-</sup> NK1.1<sup>-</sup> cells in these cultures for markers expressed by such cells. EILPs, but not ILCps, gave rise to a large population of *Tcf7<sup>-</sup>* Mac-1<sup>+</sup> cells in numbers similar to ALPs (Fig. 2, C and D). EILP cultures additionally contained *Tcf7<sup>+</sup>* cells expressing Thy1 and resembling ILCps (Fig. 2 C). Undifferentiated cells from ILCp cultures nearly all expressed

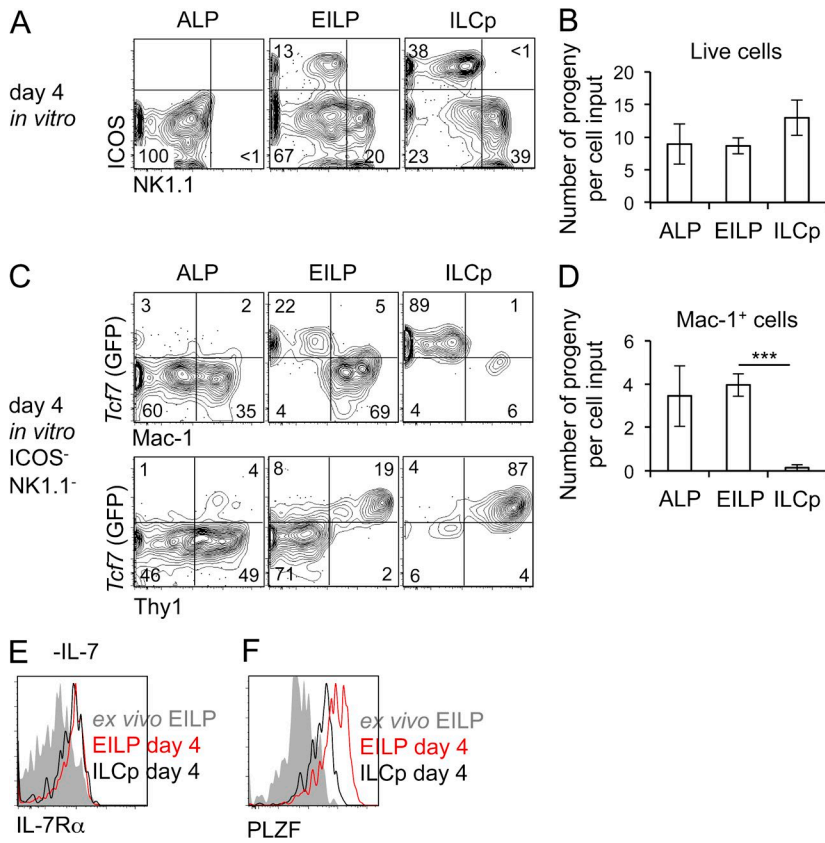


**Figure 1. ALPs differentiate into EILPs. (A–C)** 20,000 HSCs defined as CD150<sup>+</sup> Flt3<sup>-</sup> LSK cells and 30,000 ALPs isolated as GFP<sup>-</sup> from *Tcf7<sup>EGFP/+</sup>* mice were injected into irradiated (150 rads) WT mice. Bone marrow cells were analyzed by flow cytometry after 7 d of reconstitution. **(A)** Lin<sup>-</sup> Kit<sup>high</sup> CD122<sup>low</sup> bone marrow cells from an untreated *Tcf7<sup>EGFP/+</sup>* mouse, or WT mice injected with PBS, HSCs, or ALPs. **(B)**  $\alpha 4 \beta 7$  expression on ALPs (gray), EILPs from a *Tcf7<sup>EGFP/+</sup>* mouse (black), and ALP-derived EILPs from A (red). **(C)** Quantification of EILPs recovered per mouse as shown in A. Data are representative of three independent experiments and are presented as mean  $\pm$  SEM for  $n = 3$  mice per group. **(D)** Flow cytometric analysis of IL-7R $\alpha$  and YFP on the indicated bone marrow populations in an *Il7r-Cre R26-stop-YFP Tcf7<sup>EGFP/+</sup>* mouse. Lin<sup>-</sup> Kit<sup>high</sup> cells were used as a negative control for IL-7R $\alpha$  expression (gray). Corresponding populations from a *Tcf7<sup>EGFP/+</sup>* mouse are used as a negative control for YFP expression (gray). Data are representative of three independent experiments. **(E)** GFP and YFP expression by IL-7R $\alpha$ <sup>low</sup> and IL-7R $\alpha$ <sup>high</sup> EILPs from an *Il7r-Cre R26-stop-YFP Tcf7<sup>EGFP/+</sup>* mouse as gated on the left histogram. **(D and E)** Numbers indicate the percentage of cells in each gate. **(F)** Flow cytometric analysis of bone marrow cells from *Il7r*<sup>-/-</sup> mice and *Il7r*<sup>+/+</sup> littermates. Quantification of EILPs and ILC2ps defined as Lin<sup>-</sup> Kit<sup>high</sup> Sca-1<sup>+</sup> CD25<sup>+</sup> Thy1<sup>+</sup> cells. **(G)** Quantification of ALP, EILP, and ILCp numbers in the bone marrow of *Il2rg*<sup>-/-</sup> and *Il2rg*<sup>+/+</sup> littermates. **(F and G)** Data are representative of two independent experiments and are presented as mean  $\pm$  SEM for  $n = 3$  mice per group. A two-tailed unpaired Student's *t* test was performed to determine significance. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.005$ ; nd, not detectable.

*Tcf7<sup>+</sup>* and Thy1 and so appeared to be still at the ILCp stage (Fig. 2 C). To establish whether EILP-derived *Tcf7<sup>+</sup>* Thy1<sup>+</sup> cells were ILCps, we examined their expression of additional markers normally up-regulated from EILPs to ILCps. We first wished to examine whether expression of surface IL-7R $\alpha$  was up-regulated from ex vivo EILPs to EILP-derived *Tcf7<sup>+</sup>* Thy1<sup>+</sup> cells. Because IL-7 can reduce surface expression of its receptor by receptor internalization as well as transcriptional inhibition (Fig. S2 C; Park et al., 2004), we established cultures without IL-7 supplementation. In accordance with our finding that  $\gamma$ -dependent cytokines are not required for ILCp development (Fig. 1 G), *Tcf7<sup>+</sup>* Thy1<sup>+</sup> cells still developed from EILPs in these cultures. IL-7R $\alpha$  expression was higher

in these cells compared with ex vivo EILPs and similar to ILCps cultured in the same conditions (Fig. 2 E). Finally, we found that EILP-derived TCF-1<sup>+</sup> Thy1<sup>+</sup> cells recapitulated the phenotype of ILCp as they up-regulated PLZF expression (Fig. 2 F). Interestingly, EILP-derived ILCps expressed PLZF at even higher levels than cultured ILCps (Fig. 2 F). This result is coherent with the transient nature of *Plzf* expression at the ILCp stage (Constantinides et al., 2014).

Together, these results show that upon short-term culture in ILC differentiation conditions, EILPs differentiate into cells phenotypically resembling ILCps. In addition, they reveal that the ability to access non-ILC lineages remains evident in ALPs and EILPs, but it is greatly attenuated in ILCps.



**Figure 2. EILPs differentiate into ILCps.** ALPs, EILPs, and ILCps were isolated from *Tcf7<sup>EGFP/+</sup>* mice by cell sorting and cultured for 4 d. **(A–D)** Cultures were supplemented with IL-7. Data are representative of three independent experiments. **\*\*\***,  $P < 0.005$ . **(A)** ICOS<sup>-</sup> and NK1.1<sup>-</sup> expression analyzed by flow cytometry. **(B)** Quantification of total cells. **(C)** GFP, Mac-1, and Thy1 expression of ICOS<sup>-</sup> NK1.1<sup>-</sup> cells from A. **(A and C)** Numbers indicate the percentage of cells in each gate. **(D)** Quantification of Mac-1<sup>+</sup> cells from C. **(B and D)** Data are presented as mean  $\pm$  SD for triplicate wells. A two-tailed unpaired Student's *t* test was performed to determine significance. **(E)** Flow cytometric analysis of IL-7R $\alpha$  expression on ex vivo EILPs or NK1.1<sup>-</sup> ICOS<sup>-</sup> *Tcf7<sup>+</sup>* *Thy1<sup>+</sup>* cells from EILPs and ILCps cultured without IL-7. **(F)** Flow cytometric analysis of PLZF expression measured by intracellular staining by ex vivo EILPs, in vitro EILP-derived NK1.1<sup>-</sup> TCF-1<sup>+</sup> cells, and ILCp-derived NK1.1<sup>-</sup> TCF-1<sup>+</sup> cells cultured with IL-7. **(E and F)** Data are representative of two independent experiments.

EILPs and ILCps therefore appear to be two successive yet functionally distinct stages of ILC development.

### Transcriptional analysis places EILP as an intermediate between ALP and ILCp

We transcriptionally profiled early ILC progenitors found in adult mouse bone marrow. Because of the rarity of EILPs and the difficulty extracting them from bone marrow in sufficient numbers, previous transcriptional profiling was limited to a single microarray. This only allowed limited analysis and, further, was not performed along with ILCps (Yang et al., 2015). We used a library preparation method optimized for small samples to perform RNA sequencing analysis on 500–1,000 cells. We isolated seven replicates of ALP (ALP.1–7), seven replicates of EILP (EILP.1–7), and three replicates of ILCp from *Tcf7<sup>EGFP</sup>* mice (ILCp.1–3) as gated in Fig. S1 A. We additionally isolated two ILCp samples (ILCp.4 and 5) from *Zbtb16<sup>GFPcre</sup>* mice as previously described (Constantinides et al., 2014). We first analyzed the clustering of the different sample replicates in an unsupervised way (Fig. S3 A). Replicates of each sample clustered together, and ILCps isolated from *Tcf7<sup>EGFP</sup>* and *Zbtb16<sup>GFPcre</sup>* clustered together. Further comparison of these two types of ILCp showed that they were almost indistinguishable (see gene expression analysis and surface phenotype such as PD-1 and DNAM-1 expression in Fig. 3, Fig. 4 A, and Fig. S3). RNA sequencing replicates were next used to construct an unsupervised clus-

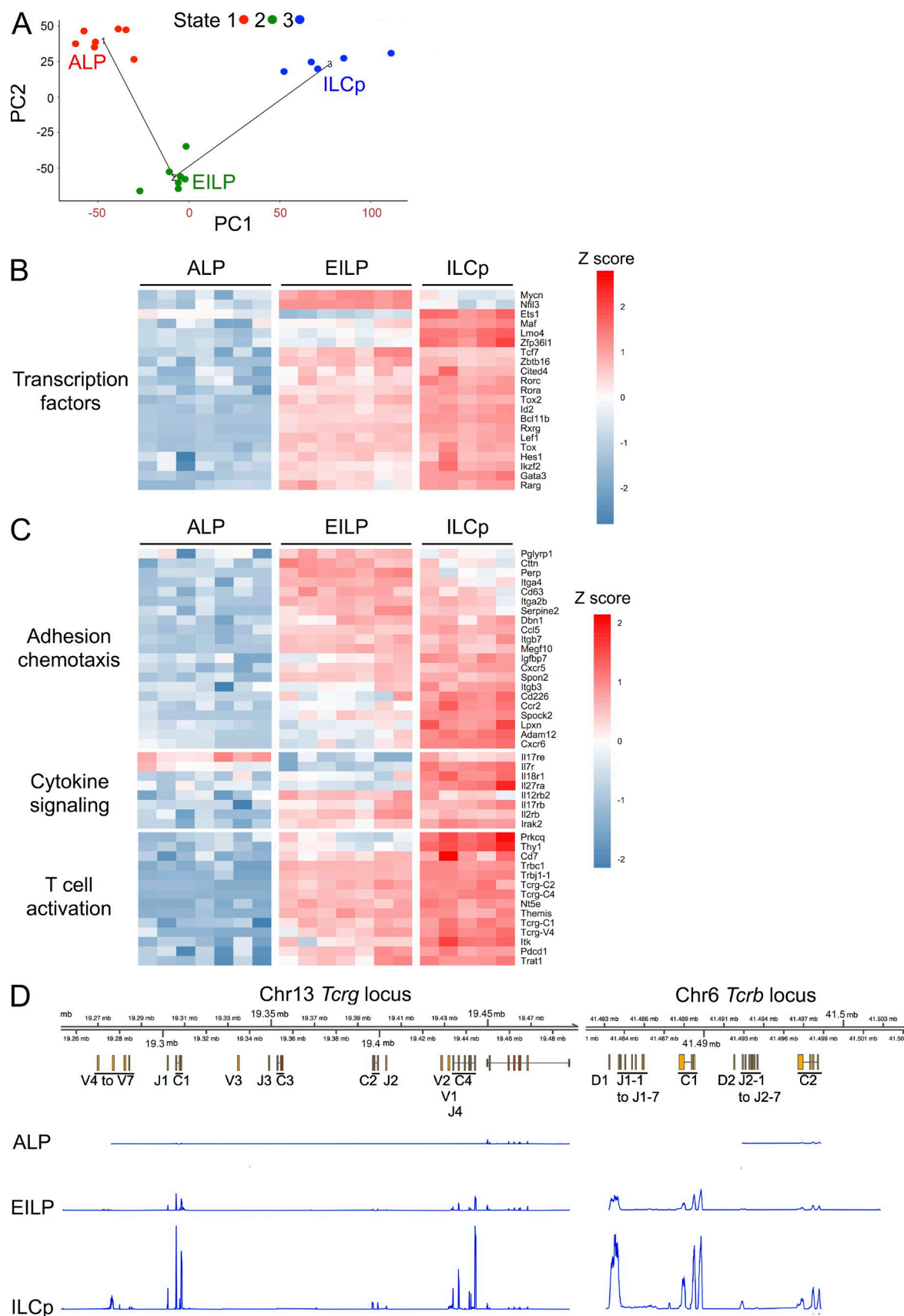
ter-based minimum spanning tree on 13,917 well-expressed genes. Replicates for each subset clustered together again, and, as predicted from our functional data, a pseudotemporal path generated by TSCAN (Ji and Ji, 2016) positioned EILP as an intermediate state between ALP and ILCp (Fig. 3 A).

### Evidence of ILC specification and ongoing commitment at the EILP stage

We used the newly established progenitor–successor relationship between ALPs, EILPs, and ILCps to analyze the transcriptional changes occurring during early ILC development. Analysis of transcription factors highly up-regulated from ALPs to either EILPs or ILCps identified factors known to be important for early ILC development, such as *Nfil3*, *Tox*, *Zbtb16*, *Id2*, *Tcf7*, *Gata3*, and *Bcl11b* (Ishizuka et al., 2016b; Zook and Kee, 2016), and also identified factors with unknown function in ILC development (Fig. 3 B). These transcriptional regulators represent new candidate controllers of ILC development. Interestingly, *Nfil3* was highly expressed in EILPs compared with ALPs and ILCps (Fig. 3 B). This is consistent with its transient requirement during early ILC development (Geiger et al., 2014; Xu et al., 2015; Seillet et al., 2016) and highlights the distinct transcriptional profile of EILPs compared with other ILC progenitors.

Several genes important for stem cell properties or differentiation into alternative hematopoietic lineages were down-regulated from ALPs to EILPs, indicating ongoing





commitment to the ILC lineage (Fig. S3 B). As an example, expression of the recombination-activating genes was highly down-regulated from ALPs to EILPs, indicating active repression of adaptive lymphocyte fate (Fig. S3 C). However, expression of the stem cell transcription factors *Runx1* and *Sox4*, as well as the myeloid transcription factors *Spi1* and *Irf8*, was maintained from ALPs to EILPs and down-regulated in ILCps (Fig. S3 B). This delayed repression is correlated with and is likely important for the dendritic cell potential observed in EILPs as well as ALPs (Fig. 2, C and D; Yang et al., 2015). Collectively, our examination of transcription factor expression indicates ILC specification and ongoing commitment at the EILP stage.

#### ILC functional properties are imprinted from the EILP stage

We further examined the biological processes enriched among genes whose expression significantly changed between the two stages of ILC development ( $P < 0.05$ ; fold change  $\geq 2$ ; Fig. S3 D). Genes up-regulated during ILC development were enriched for genes linked to adhesion and chemotaxis (Fig. S3 D and Fig. 3 C). Within this category were genes known to be expressed during ILC development and important for the migration of ILCs into tissues, such as *Itga4* and *Itgb7* (encoding  $\alpha 4\beta 7$ ), *Cxcr5*, and *Cxcr6*. In addition, we found adhesion molecules important for ILC tissue homing, but not previously described on ILC progenitors such as *Ccr2*, as well as adhesion molecules not yet known to be important for ILCs, such as *Itga2b*, *Itgb3*, *Cd63*, and *Cd226* (Fig. 3 C; Kim et al., 2016; Seillet et al., 2016; Yu et al., 2016). We extended the analysis to other homing receptors important for ILCs (Fig. S3 E; Kim et al., 2016). Several of them were expressed from the ALP stage onward (*Selp*, *Cxcr4*, *Cx3cr1*, *Ccr1*, *Ccr7*, and *Ccr9*). *Ccr4*, *Ccr8*, and *Cxcr3* were up-regulated from the ILCp stage. Other molecules such as *Itga2* or *Itgae* were not yet highly expressed by ILCps and were likely up-regulated on subsets of ILCp or more mature ILCs (Fig. S3 E). Several genes associated with cytokine signaling were highly up-regulated during ILC development (Fig. S3 D and Fig. 3 C). *Il17rb* and *Il2rb* are known to be important for ILCs, whereas roles for *Il17re*, *Il18r1*, and *Il12rb2* have not been reported. *Il7r* appeared transiently down-regulated from ALPs to EILPs and highly reexpressed at the ILCp stage (Fig. S3 D), concordant with the surface phenotype of these subsets for IL-7R $\alpha$  expression (Fig. S1 B). Genes up-regulated during ILC development were also enriched for T cell activation molecules such as signaling molecules that are generally associated with TCR signaling (*Themis*, *Prkcg*, and *Itk*), T cell interaction molecules (*Thy1*

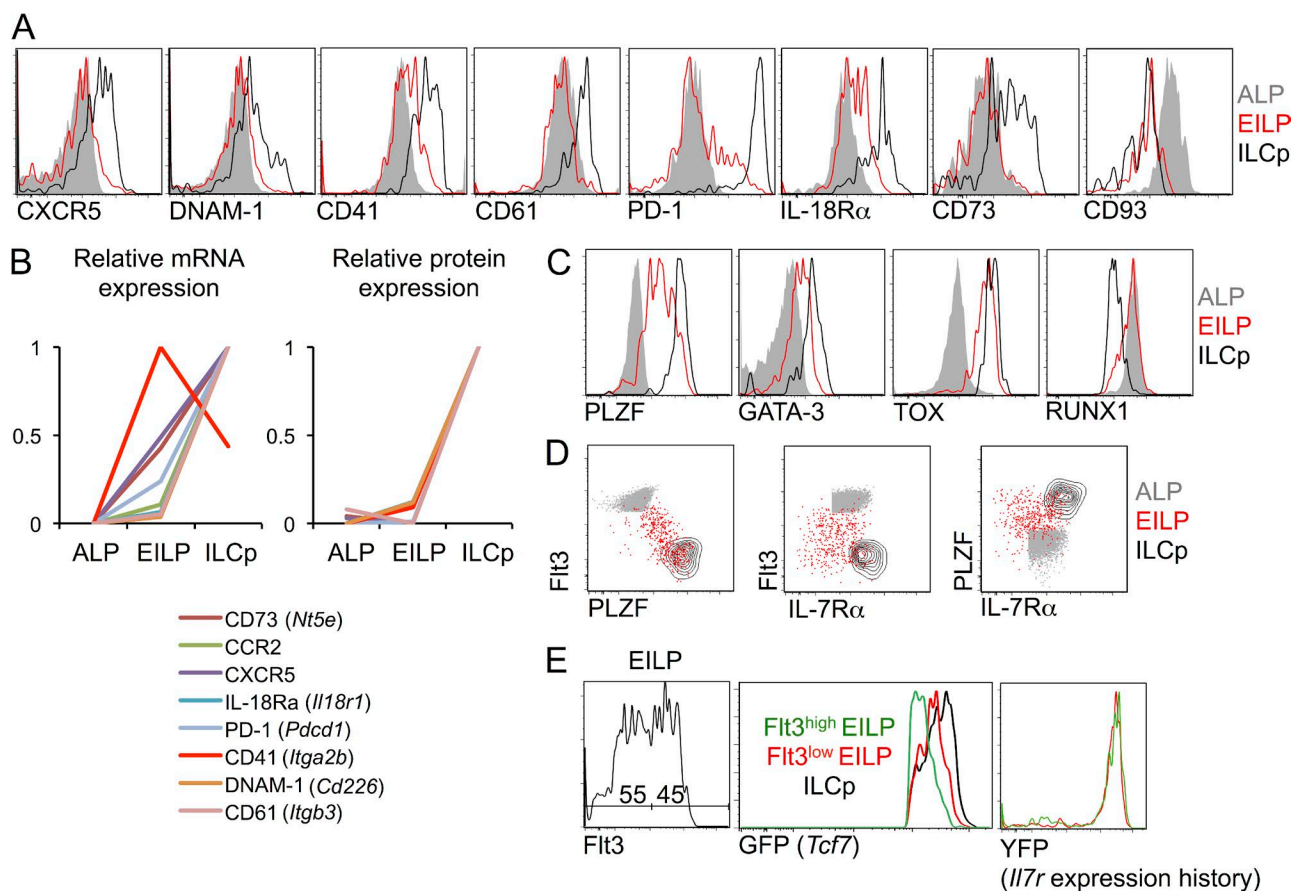
and *Cd7*), and molecules induced by TCR activation such as *Pdcd1* or *Nt5e* (Fig. S3 D and Fig. 3 C; Seillet et al., 2016; Yu et al., 2016). Interestingly, several TCR- $\beta$  and - $\gamma$  genes were expressed (Fig. 3 C; Yu et al., 2016). In particular, many genes encoding constant regions of the TCR- $\beta$  and - $\gamma$  were highly expressed (Fig. 3 D). TCR-associated molecules such as TRIM (encoded by *Trat1*), and genes encoding for CD3 subunits were also expressed (Fig. 3 B and not depicted). Importantly, coherent with the down-regulation of recombination-activating genes at the EILP stage (Fig. S3 C), the TCR loci did not appear rearranged in early ILC progenitors (not depicted). This also confirms that T lineage gene expression in EILPs is not caused by T cell contamination. It is not known whether these genes have functions in ILCs or whether their expression is a byproduct of the expression of T cell transcription factors such as *Tcf7*, *Gata3*, or *Bcl11b* in ILCs (Fig. 3 B).

Our transcriptional analysis of early ILC progenitors indicates that most of the genes up-regulated during ILC development are already expressed at the EILP stage and either maintained at the ILCp stage or further up-regulated. A few adhesion molecules (*Pglyrp1*, *Ctnn*, and *Perp*) were transiently expressed at the EILP stage and might indicate migration properties unique to EILPs.

#### EILPs are a transitional stage between ALPs and ILCps

We next analyzed the cell surface phenotype of ILC progenitors to confirm protein expression encoded by genes that were highly transcriptionally up-regulated during ILC development, such as *Cxcr5*, *Cd226* (gene for DNAM-1), *Itga2b* (gene for CD41), *Itgb3* (gene for CD61), *Pdcd1* (gene for PD-1), *Il18r1* (gene for IL-18R $\alpha$ ), and *Nt5e* (gene for CD73; Fig. 3 C). Surprisingly, these markers showed little or no expression on EILPs compared with ILCps (Fig. 4 A). Importantly, ILCps identified in *Tcf7<sup>EGFP</sup>* mice expressed high surface levels of PD-1 and low levels of DNAM-1 similarly to *Plzf*-expressing ILCps (Fig. 4 A; Yu et al., 2016). Overall, these up-regulated molecules showed a delayed expression at the protein level compared with RNA (Fig. 4 B), and EILPs appeared phenotypically more similar to ALPs than ILCps. In contrast, CD93 was down-regulated from ALP to EILP at the RNA and protein level (Fig. S3 B and Fig. 4 A). This analysis indicates that EILPs are phenotypically distinct from both ALPs and ILCps. Additionally, delayed protein expression for genes transcriptionally up-regulated from ALPs to EILPs supports the progenitor-successor relationship we previously established between ALPs, EILPs, and ILCps. We next examined transcription factor expression by intracellular staining. Confirming our earlier transcriptional analysis

**Figure 3. Transcriptional analysis of early ILC progenitors. (A)** TSCAN analysis using RNA sequencing data of ALPs, EILPs, and ILCps. **(B)** Heat map of gene expression quantified by RNA sequencing for all transcription factors highly expressed in at least one of the three subsets of ILC progenitors and significantly up-regulated from ALPs to EILPs or from EILPs to ILCps. **(C)** Heat map of gene expression quantified by RNA sequencing for structural genes highly expressed in at least one of the three subsets of ILC progenitors, significantly up-regulated from ALPs to EILPs or from EILPs to ILCps, and in the indicated biological processes ( $P < 0.01$ ; fold change  $\geq 4$ ). **(D)** RNA sequencing traces of TCR- $\gamma$  and TCR- $\beta$  loci for ILC progenitors.



**Figure 4. Phenotypic analysis of early ILC progenitors. (A)** Cell surface expression of the indicated protein on ALPs, EILPs, and ILCps from *Tcf7<sup>EGFP/+</sup>* mice analyzed by flow cytometry. **(B)** Relative mRNA expression of the indicated molecules quantified by RNA sequencing for ALPs, EILPs, and ILCps. Relative protein expression of the indicated molecules quantified by flow cytometry for ALPs, EILPs, and ILCps from *Tcf7<sup>EGFP/+</sup>* mice as shown in A. Data are presented as mean of expression.  $n = 7$  for ALPs and EILPs;  $n = 5$  for ILCps and are presented as geometric mean fluorescence intensity minus fluorescence minus one. **(C)** Flow cytometric analysis of ALPs, TCF-1<sup>+</sup> EILPs, and TCF-1<sup>+</sup> ILCps by intracellular staining for the indicated transcription factors. **(D)** Flow cytometric analysis of ALPs, EILPs, and ILCps. Cell surface expression of Flt3 and IL-7R $\alpha$  and intracellular staining of PLZF are shown. **(E)** Flt3 expression on EILPs, and Flt3<sup>low</sup> and Flt3<sup>high</sup> gating used for the histograms on the right. GFP expression on ILC progenitors from a *Tcf7<sup>EGFP/+</sup>* mouse. YFP expression on ILC progenitors from an *Il7r-Cre R26-stop-YFP Tcf7<sup>EGFP/+</sup>* mouse. Flt3<sup>low</sup> EILPs and Flt3<sup>high</sup> EILPs as gated on the left histogram are compared with ILCps. All data are representative of three independent experiments.

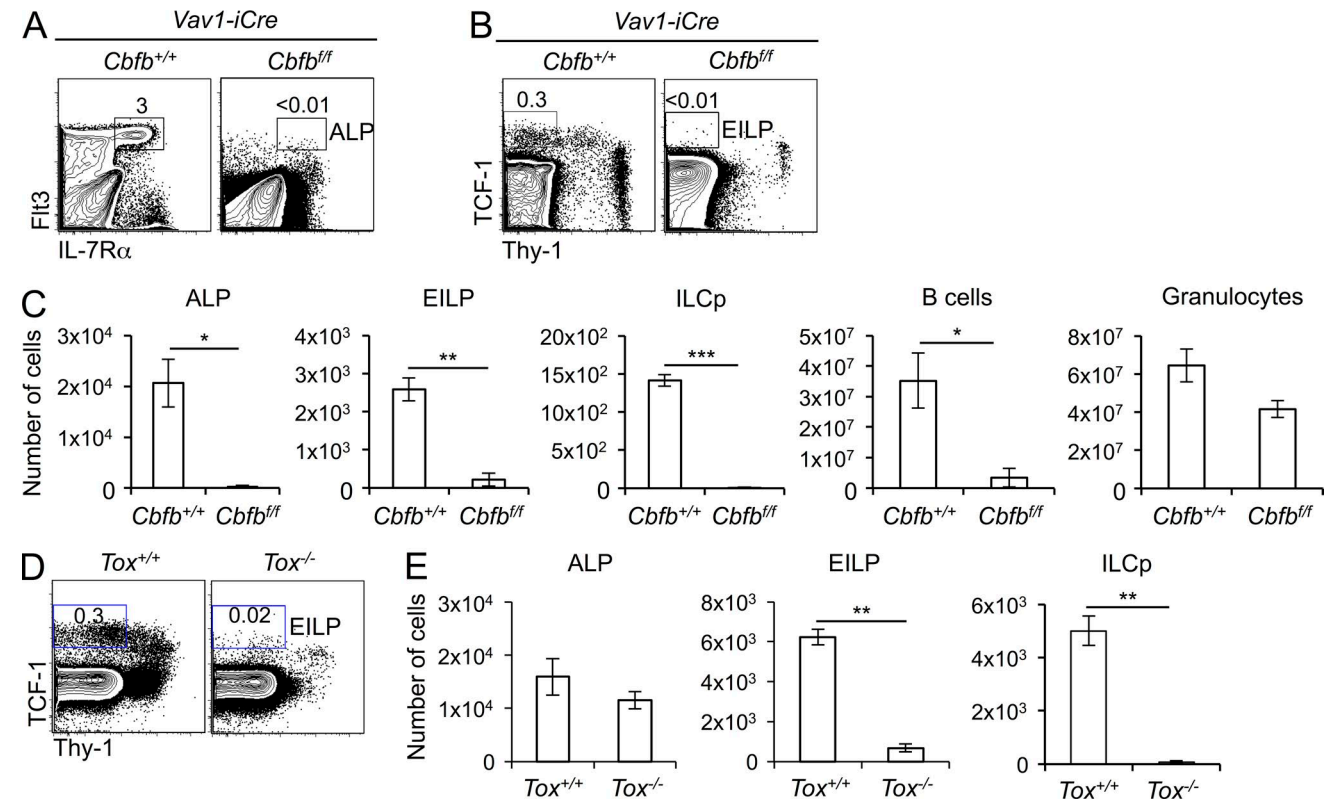
(Fig. 3 B), we found that PLZF and GATA-3 were expressed in EILPs at levels intermediate between ALPs and ILCps, TOX was highly up-regulated from ALPs to EILPs, and RUNX1 was expressed in EILPs at a level similar to ALPs, but down-regulated in ILCps (Fig. 4 C).

Our single-cell analysis by flow cytometry further supports the progenitor–successor relationship we established between ALPs, EILPs, and ILCps. Importantly, most markers examined were homogeneously expressed on EILPs. Nevertheless, the variegated expression of some factors such as Flt3, IL-7R $\alpha$  (Fig. S1 B), and PLZF (Fig. 4 C) at the EILP stage prompted us to examine a possible heterogeneity within the EILPs. Analysis of the coexpression of these three proteins in ALPs, EILPs, and ILCps by flow cytometry revealed that IL-7R $\alpha$  expression was not correlated with either PLZF or Flt3, but Flt3 and PLZF expression were inversely correlated

in EILPs at the single-cell level (Fig. 4 D). We further examined whether EILPs might progressively down-regulate Flt3 while up-regulating PLZF during differentiation. Coherent with a gradual loss of Flt3 at the EILP stage, *Tcf7* expression, as examined by GFP expression in *Tcf7<sup>EGFP</sup>* mice, appeared up-regulated from Flt3<sup>high</sup> EILPs to Flt3<sup>low</sup> EILPs and further to the ILCp stage (Fig. 4 E). Importantly, Flt3<sup>high</sup> EILPs and Flt3<sup>low</sup> EILPs had a similar history of *Il7r* expression as examined by YFP expression in an *Il7r-Cre R26-stop-YFP Tcf7<sup>EGFP/+</sup>* mouse (Fig. 4 E), which confirms that YFP marking likely does not occur during EILP maturation, but instead reflects the differentiation of EILPs from an IL-7R $\alpha$ -expressing progenitor such as ALP.

Our analysis supports the emergence of EILPs from ALPs (Flt3<sup>high</sup> PLZF<sup>−</sup>) and their progressive differentiation toward ILCps (Flt3<sup>−</sup> PLZF<sup>high</sup>; Fig. 4 D). However, variation





**Figure 5. CBF- $\beta$  and TOX are required before the EILP stage.** (A–C) Flow cytometric analysis of bone marrow cells from *Vav1-iCre Cbfb<sup>fl/fl</sup>* mice and *Vav1-iCre Cbfb<sup>+/+</sup>* littermate controls. Profiles of ALPs (A) and EILPs (B) are shown after gating on Lin<sup>−</sup> Kit<sup>+</sup> CD122<sup>low</sup> cells. (C) Quantification of ALP, EILP, ILCp, B cell, and granulocyte numbers. (D and E) Flow cytometric analysis of bone marrow cells from *Tox<sup>−/−</sup>* mice and *Tox<sup>+/+</sup>* littermate controls. (D) Profiles of EILPs are shown after gating on Lin<sup>−</sup> Kit<sup>+</sup> CD122<sup>low</sup> cells. (E) Quantification of ALP, EILP, and ILCp numbers. Numbers indicate the percentage of cells in each gate. All data are representative of at least two independent experiments and are presented as mean  $\pm$  SEM for  $n = 3$  mice per group. A two-tailed unpaired Student's *t* test was performed to determine significance. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.005$ .

in IL-7R $\alpha$  expression did not appear to be related to the degree of maturation of EILPs. Overall, this analysis supports the idea that EILPs are a transitional stage of ILC development between ALPs and ILCps.

#### CBF- $\beta$ and TOX are required for the generation of EILPs

We examined whether key transcription factors expressed at the EILP stage were required for the generation and differentiation of EILPs (Fig. 4 C). *Runx1* and *Runx2* are expressed in EILPs and down-regulated in ILCps (Fig. S3 B). We examined the requirement for these factors for the generation of ILC progenitors by deleting CBF- $\beta$  (encoded by *Cbfb*), which is required for RUNX activity and highly expressed in all hematopoietic progenitors (not depicted). We crossed mice carrying conditional knockout alleles for *Cbfb* (*Cbfb<sup>fl/fl</sup>*) with mice possessing the *Vav1-iCre* transgene that is active in all hematopoietic cells (de Boer et al., 2003). As previously described, lymphoid-primed multipotent progenitors, ALPs, and downstream mature lymphocyte populations known to develop from these progenitors, namely B cells, T cells, and NK cells, were not detectably present (Fig. 5, A and C; Guo et

al., 2008; Satpathy et al., 2014). Consistent with their differentiation from ALPs, EILPs and ILCps were absent in *Vav1-iCre Cbfb<sup>fl/fl</sup>* mice (Fig. 5, B and C). In contrast, the CBF- $\beta$  defect only presented a mild effect on granulocyte numbers (Fig. 5 C; Talebian et al., 2007; Satpathy et al., 2014). RUNX activity is therefore required for development of lymphoid progenitors and ILC progenitors.

We next investigated a role for TOX in early ILC development. We examined early ILC progenitors by TCF-1 intracellular staining in *Tox<sup>−/−</sup>* mice and found that EILP numbers were reduced at least 10-fold (Fig. 5, D and E). ILCps were also absent as previously reported (Fig. 5 E; Seehus et al., 2015). To ensure that the defect in EILP generation seen in *Tox<sup>−/−</sup>* mice was not simply caused by a defect in TCF-1 expression by *Tox<sup>−/−</sup>* EILPs, we examined additional markers of EILPs in these mice. Similarly to ALPs and ILCps, EILPs expressed a high level of 2B4 (Fig. S4 A). Because TCF-1<sup>+</sup> EILPs remaining in *Tox<sup>−/−</sup>* EILPs expressed normal levels of 2B4 and  $\alpha 4\beta 7$  (Fig. S4 B), we could use these two markers to examine the EILPs in *Tox<sup>−/−</sup>* mice. We found that the proportion of  $\alpha 4\beta 7^{\text{high}}$  2B4<sup>high</sup> cells was greatly reduced in *Tox<sup>−/−</sup>*

mice, and a reduced but clearly detectable fraction of these cells expressed TCF-1 at levels comparable to WT EILPs (Fig. S4 C). Our results therefore indicate that TOX is unlikely to be solely required for TCF-1 expression in otherwise intact populations of EILPs; rather, TOX is required for the efficient generation of EILPs and later ILC populations.

### GATA-3 is required at the EILP stage and for further ILC development

*Gata3* is required for the development of all helper ILC subsets (Hoyler et al., 2012; Klose et al., 2014; Serafini et al., 2014; Yagi et al., 2014), suggesting the possibility that *Gata3* is required for the development of ILCps (Serafini et al., 2014). GATA-3 has important functions in HSCs (Ku et al., 2012), and the absolute numbers of ALPs are reduced in the *Vav1-iCre Gata3<sup>f/f</sup>* mouse (Fig. S5 A). To examine the effect of GATA-3 deficiency on ILC development after the ALP stage, we examined EILPs and ILCps by TCF-1 intracellular staining and used ALP absolute numbers to normalize EILP and ILCp numbers in *Vav1-iCre Gata3<sup>+/+</sup>* and *Vav1-iCre Gata3<sup>f/f</sup>* mice. EILPs were detectable and expressed normal levels of  $\alpha 4\beta 7$  in *Vav1-iCre Gata3<sup>f/f</sup>* mice (Fig. 6, A and B). However, they were reduced by twofold compared with *Vav1-iCre Gata3<sup>+/+</sup>* mice, and ILCp appeared absent (Fig. 6 C). Similar to what was described for GATA-3-deficient mature ILCs (Yagi et al., 2014; Zhong et al., 2016), GATA-3-deficient EILPs had reduced expression of IL-7R $\alpha$  (Fig. 6 B). This observation raised the possibility that ILCps were still present in *Vav1-iCre Gata3<sup>f/f</sup>* mice but lacked IL-7R $\alpha$ . We therefore examined ILCps using alternative markers and found that ILCps as defined by expression of PLZF and  $\alpha 4\beta 7$  were also greatly reduced in GATA-3-deficient bone marrow (Fig. 6 D). Hence, in the absence of GATA-3, EILPs continue to be generated, albeit in reduced numbers, but this factor is required for further differentiation into ILCps.

### PLZF and Bcl11b are required after the ILCp stage

PLZF (encoded by *Zbtb16*) has been proposed to play important functions during early ILC development (Constantinides et al., 2015). However, competitive chimeras only revealed a requirement for PLZF in ILC2 and liver ILC1 development (Constantinides et al., 2014). This result suggested that PLZF is not required for the development of ILCps, but because these cells were previously defined using PLZF expression (Constantinides et al., 2014), their development was not examined in mice lacking PLZF. Using *Tcf7* reporter, we investigated early ILC development in *Zbtb16<sup>-/-</sup>* mice. ALP numbers were significantly reduced in these mice (Fig. S5 B), perhaps as a result of skeletal or HSC defects (Barna et al., 2000; Vincent-Fabert et al., 2016). To examine the effect of PLZF deficiency on ILC development after the ALP stage, we thus again used ALP absolute numbers to normalize ILC progenitor numbers in *Zbtb16<sup>+/+</sup>* and *Zbtb16<sup>-/-</sup>* mice (Fig. 6 E). We found that EILPs and ILCps were not significantly reduced in *Zbtb16<sup>-/-</sup>* mice. We further examined

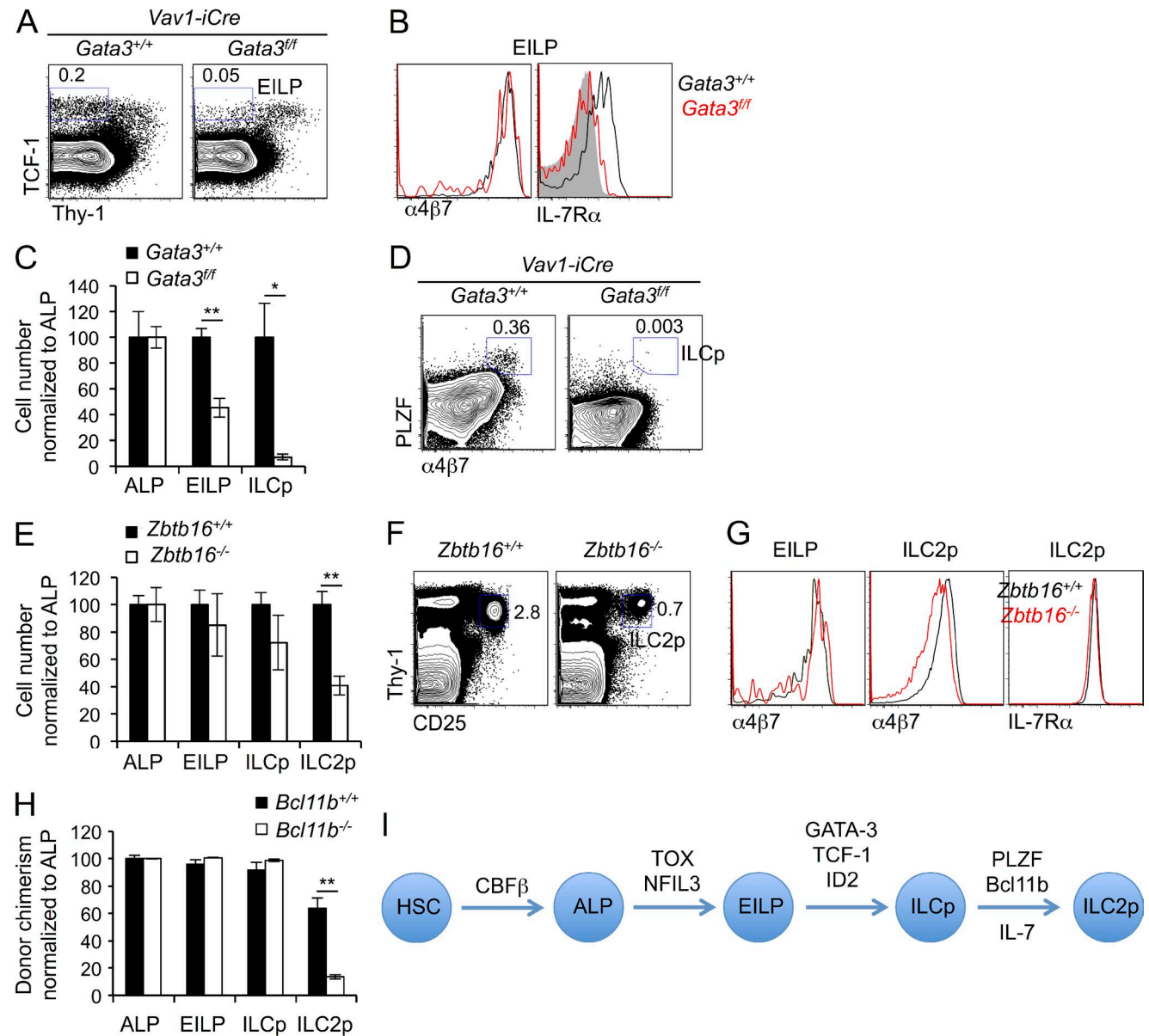
ILC2p numbers in *Zbtb16<sup>-/-</sup>* mice. Because PLZF was proposed to regulate IL-7R $\alpha$  expression (Constantinides et al., 2014, 2015), we excluded this marker from the definition of ILC2p. ILC2ps, as defined as Lin<sup>-</sup> Kit<sup>-</sup> CD122<sup>low</sup> Thy1<sup>+</sup> CD25<sup>+</sup> bone marrow cells, were reduced by more than twofold (Fig. 6, E and F). The phenotype of the remaining ILC2ps was also affected, as shown by their reduced  $\alpha 4\beta 7$  expression, but their IL-7R $\alpha$  expression was comparable to that of *Zbtb16<sup>+/+</sup>* ILC2p (Fig. 6 G).

Finally, we examined the requirement for Bcl11b during early ILC development. Bcl11b is required for ILC2 development, but not ILC3 or NK development (Califano et al., 2015; Walker et al., 2015; Yu et al., 2015). Bcl11b is expressed in ILCps committed to the ILC2 lineage (Yu et al., 2015) and is required for ILC2p generation (Walker et al., 2015; Yu et al., 2015, 2016). However, stages of ILC development before ILC2p were not examined in these earlier studies, and frequencies of ILCps or earlier precursors were not assessed. Because *Bcl11b* germline deficiency is lethal before birth, we generated long-term bone marrow chimeras using *Bcl11b<sup>-/-</sup>* or *Bcl11b<sup>+/+</sup>* fetal liver Lin<sup>-</sup> Kit<sup>high</sup> Sca-1<sup>+</sup> (LSK) cells in competition with WT congenic bone marrow LSK cells. We found that chimerism of *Bcl11b<sup>-/-</sup>* cells was comparable to *Bcl11b<sup>+/+</sup>* cells at the EILP and ILCp stages (Fig. 6 H). Therefore, consistent with recent transcriptional analysis of Bcl11b-deficient ILC progenitors (Yu et al., 2016), Bcl11b is not required for the generation of ILCp but plays an important role at the transition from ILCp to ILC2p.

### DISCUSSION

We characterized the recently described EILP and examined its relationship with other early ILC progenitors. Using short-term differentiation assays *in vivo* and *in vitro* and an *Il7r* lineage tracing mouse strain, we show that EILP is an intermediate between ALP and ILCp. Pseudotemporal modeling based on the transcriptional profiling of these three early ILC progenitors confirmed this relationship, and single-cell flow cytometric analysis additionally suggested that EILP is a transitional subset between ALP and ILCp. Comparison of these populations using *in vitro* culture confirmed that EILPs were specified but not committed to ILC lineages, whereas development of non-ILC lineages from ILCps was greatly reduced. EILPs are thus functionally distinct from both ALPs and ILCps.

Because IL-7R $\alpha$  is expressed at the ALP and ILCp stages but appears transiently down-regulated by most EILPs, we analyzed the relevance of IL-7 and other  $\gamma$ c-dependent cytokines during early ILC development. We validated intracellular staining for TCF-1 to visualize and quantify EILPs and ILCps and examined these progenitors in mouse models mutant for *Il7r* and *Il2rg*. We found that EILP and ILCp numbers were unaffected in the absence of  $\gamma$ c-dependent cytokine signaling. Thus, we have not identified a requirement for IL-7R $\alpha$  in early ILC development, and the mechanism and relevance (if any) of *Il7r* down-regulation at the



**Figure 6. GATA-3, PLZF, and Bcl11b are required after the EILP stage.** (A–D) Flow cytometric analysis of bone marrow cells from *Vav1-iCre Gata3*<sup>ff</sup> mice and *Vav1-iCre Gata3*<sup>+/+</sup> littermate controls. (A) Profiles of EILPs after gating on Lin<sup>−</sup> Kit<sup>+</sup> CD122<sup>low</sup> cells. (B)  $\alpha 4\beta 7$  and IL-7R $\alpha$  expression of GATA-3-sufficient (black) and -deficient (red) EILPs as gated in A. Kit<sup>+</sup> Lin<sup>−</sup> cells were used as a negative control for IL-7R $\alpha$  expression (gray). (C) Quantification of ALP, EILP, and ILCp numbers normalized using ALPs (Fig. S5). (D) Profiles of ILCps as defined as PLZF<sup>+</sup>  $\alpha 4\beta 7$ <sup>+</sup> after gating on Lin<sup>−</sup> Kit<sup>+</sup> Flt3<sup>−</sup> cells. (E and G) Flow cytometric analysis of bone marrow cells from *Zbtb16*<sup>−/−</sup> mice and *Zbtb16*<sup>+/+</sup> littermate controls. (E) Quantification of ALP, EILP, ILCp, and ILC2p numbers normalized using ALPs (Fig. S5). (F) Profiles of ILC2p after gating on Lin<sup>−</sup> Kit<sup>+</sup> CD122<sup>low</sup> cells. (A, D, and F) Numbers indicate the percentage of cells in each gate. (G)  $\alpha 4\beta 7$  and IL-7R $\alpha$  expression for PLZF-sufficient (black) and -deficient (red) EILPs or ILC2ps gated in F. (H) ILC development analyzed by flow cytometry in the bone marrow of long-term competitive chimeras reconstituted for 10–12 wk with *Bcl11b*<sup>+/+</sup> or *Bcl11b*<sup>−/−</sup> fetal liver LSK cells (CD45.2) mixed with equal numbers of WT bone marrow LSK cells (CD45.1). ILC2ps are gated as Lin<sup>−</sup> Kit<sup>+</sup> Sca1<sup>high</sup> Flt3<sup>−</sup> IL-7R $\alpha$ <sup>+</sup> cells. Donor chimerism of the indicated population was normalized to bone marrow ALP chimerism for individual mice. (C, E, and H) Data are representative of at least two independent experiments and are presented as mean  $\pm$  SEM for  $n = 3$  mice per group. A two-tailed unpaired Student's *t* test was performed to determine significance. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ . (I) Breakdown of progenitors during early ILC development. Progenitor–successor relationships between cell types are indicated by arrows. The requirements for key transcription factors at developmental transitions are specified.

EILP stage remain to be resolved. One possibility is that *Il7r* is actively down-regulated in response to IL-7 signaling (Park et al., 2004). A speculation is that EILPs might occupy niches in close proximity to ALPs and early B cell precursors: down-regulation of *Il7r* might serve to maintain IL-7 availability and allow IL-7-dependent B cell precursors to develop in the same microenvironment as ILCs, similar to the altruistic sharing of IL-7 previously described for T cells responding to IL-7 (Park et al., 2004). Another possibility is that transient *Il7r* down-regulation could be a consequence of a switch in expression of *Il7r* controllers as lymphoid progenitors differentiate into committed ILC progenitors.

By transcriptional profiling, we found that many genes related to adhesion, chemotaxis, and cytokine signaling are up-regulated from ALPs to EILPs, indicating that ILC migration and cytokine responsiveness programs start being imprinted at this stage. However, most of these genes were only expressed at the protein level in ILCps. This delayed expression supports a progenitor–successor relationship between EILPs and ILCps and suggests that ILCps possess tissue-homing properties. Consistently, ILC progenitors resembling ILCps have been described in blood and multiple tissues in adult humans (Scoville et al., 2016; Lim et al., 2017) as well as mouse fetal intestine (Bando et al., 2015). Additionally, deficiency in CXCR6, which is expressed by ILCps, results in ILC progenitor accumulation in the bone marrow and reduction in circulating ILC progenitors and tissue-resident ILCs in mice (Chea et al., 2015).

Transcriptional profiling also revealed expression of many transcription factors in EILPs that were previously identified as important for ILC development. We examined early ILC development in WT and mutant mouse models for key transcription factors and found that RUNX is required upstream of ALP, TOX seems important for ALPs to differentiate into EILPs, GATA-3 is required at the EILP stage and for further differentiation into ILCps, and PLZF and Bcl11b are important after ILCps, but before the appearance of ILC2ps (Fig. 6 I). Such analyses necessarily have the caveat that the expression of molecules used to stage differentiation may themselves be gene targets of the transcription factors being studied. Where feasible, we assessed alternative definitions of progenitor populations to mitigate this concern. Overall, the stage-specific defects we report are consistent with requirements previously surmised for these factors (Ishizuka et al., 2016b; Zook and Kee, 2016). Our work supplements previous work showing that NFIL3, which we find transiently expressed at the EILP stage, is required for the development of  $\alpha 4\beta 7^+$  CLP and thus likely EILPs (Seillet et al., 2016), whereas TCF-1 and Id2 are required at or after the EILP stage for the development of ILCps (Fig. 6 I; Yang et al., 2015; Jeevan-Raj et al., 2017).

Interestingly, TCF-1, GATA-3, and Bcl11b are also important during early T cell development (Yui and Rothenberg, 2014). We speculate that these shared transcription factors play similar functions during early T cell and ILC de-

velopment. Consistently, we find many T cell genes expressed in early ILC progenitors, including TCR genes that appear unlikely to have functions in ILCs. An interesting possibility is that these early controllers program some of the functional similarities noted between mature T cells and ILCs (Eberl et al., 2015). How these shared factors and other factors unique to early ILC development together impose innate lymphocyte identity is a fascinating topic for future studies.

In summary, this study places EILPs in the main stream of ILC development and establishes this population as intermediate between ALPs and ILCps.

## MATERIALS AND METHODS

### Mice

B6-Ly5.2 (CD45.1) mice were from the Jackson Laboratory. *Tcf7<sup>EGFP</sup>* (Yang et al., 2015), *Zbtb16<sup>GFPcre</sup>* (Constantinides et al., 2014), *Tcf7<sup>-/-</sup>* (Verbeek et al., 1995), *Il2rg<sup>-/-</sup>* (Cao et al., 1995), *Il7r<sup>-/-</sup>* (Peschon et al., 1994), *Tox<sup>-/-</sup>* (Aliahmad and Kaye, 2008), *Cbfb<sup>fllox</sup>* (Naoe et al., 2007), *Gata3<sup>fllox</sup>* (Pai et al., 2003), *Zbtb16<sup>-/-</sup>* (Barna et al., 2000), *Bcl11b<sup>-/-</sup>* (Wakabayashi et al., 2003), and *Vav1-iCre* (de Boer et al., 2003) mice have previously been described. The *Il7r-iCre R26-stop-YFP* mouse strain was provided by H.R. Rodewald (Division of Cellular Immunology, German Cancer Research Center, Heidelberg, Germany; Schlenner et al., 2010). Mice used were 7–10 wk old and of either sex. Animal procedures were approved by relevant National Institutes of Health Animal Care and Use Committees.

### Antibodies and flow cytometry

Bone marrow cell suspensions were incubated with a mix of purified rat, mouse, and hamster IgG before addition of specific antibodies. Antibodies specific for Kit (2B8), Thy-1.2 (53–2.1),  $\alpha 4\beta 7$  (DATK32), IL-7R $\alpha$  (A7R34), Sca-1 (D7), CD150 (mShad150), ICOS (C398.4A), CD25 (PC61.5), CD73 (TY/11.8), CD93 (AA4.1), CXCR5 (SPRCL5), 2B4 (eBio244F4), CD45.2 (104), CD45.1 (A20), TOX (TXRX10), PLZF (Mags.21F7), GATA-3 (TWAJ), and RUNX1 (RXD MC) were from eBioscience. Anti-CD122 (TM- $\beta 1$ ), DNAM-1 (TX42.1), CD41 (MWRreg30), CD61 (2C9G2), and PD-1 (29F1A12) were from Biolegend. Anti-Flt3 (A2F10) was from BD, and anti-TCF-1 (C63D9) was from Cell Signaling. The bone marrow lineage cocktail was a mix of the following antibodies from eBioscience: anti-Ly-6D (49H4), B220 (RA3-6B3), CD19 (1D3), Mac-1 (M1/70), Gr-1 (8C5), CD11c (N418), Ter119 (TER119), NK1.1 (PK136), CD3- $\epsilon$  (2C11), CD8- $\alpha$  (53–6.72), CD8- $\beta$  (H35–17.2), CD4 (GK1.5), TCR- $\beta$  (H57), and TCR- $\gamma\delta$  (GL-3). Intracellular staining was performed using eBioscience's transcription factor staining buffer set according to the manufacturer's instructions. LIVE/DEAD discrimination was performed by staining with DAPI or LIVE/DEAD Fixable blue (Invitrogen). Samples were acquired using a flow cytometer (LSRFortessa; BD) and analyzed using FlowJo software (Tree Star). All analyses were presented on singlet live cells. GFP/YFP separation by flow



cytometry was achieved using the filters 509/21, 505LP and 530/30, 525LP. Bone marrow progenitors were sorted using an Aria flow cytometer (BD). Absolute cell numbers were obtained using an Accuri C6 PLUS flow cytometer (BD).

### Cell culture

100 bone marrow progenitors were seeded in 24-well plates on irradiated OP9 stromal layers in  $\alpha$ -MEM supplemented with 20% FBS, glutamine, penicillin, streptomycin, stem cell factor, and Flt3-L (30 ng/ml). 30 ng/ml IL-7 was added when indicated. CD45.2<sup>+</sup> cells were considered for analysis of hematopoietic progeny.

### RNA sequencing and analysis

Seven ALP (ALP.1-7), seven EILP (EILP.1-7), and three ILCp samples (ILCp.1-3) were isolated from *Tcf7<sup>EGFP/+</sup>* mice according to the gating strategy shown in Fig. S1 A. Two additional ILCp samples (ILCp.4 and 5) were isolated from *Zbtb16<sup>GFPcre</sup>* mice as previously described (Constantinides et al., 2014). Replicates for each subset were isolated from individual mice in four or more independent experiments (independent cell isolation, RNA extraction, library preparation, and RNA sequencing; Fig. S3 A). RNA was extracted using the RNeasy plus micro kit (Qiagen) according to the manufacturer's instructions. Quality control was performed by Bioanalyzer (Agilent), and RNA samples with an RNA integrity number >9 were subsequently used. mRNA sequencing libraries were prepared using the SMARTer Ultra Low Input RNA kit v3 (Clontech) and Nextera XT DNA library preparation kit (Illumina). Paired-end sequence reads of 126 bp were generated by a HiSeq2500 sequencer (Illumina). The raw RNA-Seq FASTQ reads were aligned to the mouse genome (mm10) using STAR (v.2.4.0h) on two-pass mode with mouse Gencode (release 4) gene transfer format. Genes were subsequently counted using Rsubread and analyzed for gene expression changes using limma-voom with quantile normalization. The gene- and sample-specific normalization factors were then used to correct counts. Pseudotime reconstruction of the different isolated cell populations was performed using TSCAN on 13,917 well-expressed genes (log cpm  $\geq 1$ ; Ji and Ji, 2016), so 75% of all expressed genes. Biological process enrichment was performed using protein analysis through evolutionary relationships (Mi et al., 2013). Visualization was done using R (R Development Core Team, 2014).

### Accession codes

The GEO accession no. for RNA sequencing data is GSE81530.

### Statistics

Statistical analysis was performed on groups with limited variance. Differences between groups of mice were determined by a two-tailed unpaired Student's *t* test. *P* < 0.05 was considered significant. Sample sizes were empirically determined, no samples or animals were excluded from the analysis, and no randomization or blinding was used.

### Online supplemental material

Fig. S1 describes the flow cytometric gating strategies used to define ALPs, EILPs, and ILCps. Fig. S2 shows the profiles of ALPs, EILPs, and ILCps after in vitro culture. Fig. S3 presents RNA sequencing analyses of ALPs, EILPs, and ILCps. Fig. S4 shows that TCF-1<sup>+</sup> EILPs are present in *Tox<sup>-/-</sup>* mice. Fig. S5 shows that ALP numbers are reduced in mice deficient for GATA-3 or PLZF.

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Author contributions: All authors helped design research; C. Harly performed experiments; C. Harly, M. Cam, and A. Bhandoola analyzed data; J. Kaye provided materials; and C. Harly and A. Bhandoola wrote the paper. All authors read and commented on the manuscript.

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