The ETS1 transcription factor is required for the development and cytokine-induced expansion of ILC2

Erin C. Zook,[1]* Kevin Ramirez,[1]* Xiaohuan Guo,[1] Grant van der Voort,[2] Mikael Sigvardsson,[4] Eric C. Svensson,[3] Yang-Xin Fu,[1,2] and Barbara L. Kee[1,2]

[1]Committee on Immunology, [2]Department of Pathology, and [3]Department of Medicine, Section of Cardiology, The University of Chicago, Chicago, IL 60637
[4]Experimental Hematopoiesis Unit, Department of Clinical and Experimental Medicine, Faculty of Medicine for Health Sciences, Linköping University, 58183 Linköping, Sweden

Group 2 innate lymphoid cells (ILC2s) are a subset of ILCs that play a protective role in the response to helminth infection, but they also contribute to allergic lung inflammation. Here, we report that the deletion of the ETS1 transcription factor in lymphoid cells resulted in a loss of ILC2s in the bone marrow and lymph nodes and that ETS1 promotes the fitness of the common progenitor of all ILCs. ETS1-deficient ILC2 progenitors failed to up-regulate messenger RNA for the E protein transcription factor inhibitor ID2, a critical factor for ILCs, and these cells were unable to expand in cytokine-driven in vitro cultures. In vivo, ETS1 was required for the IL-33–induced accumulation of lung ILC2s and for the production of the T helper type 2 cytokines IL-5 and IL-13. IL-25 also failed to elicit an expansion of inflammatory ILC2s when these cells lacked ETS1. Our data reveal ETS1 as a critical regulator of ILC2 expansion and cytokine production and implicate ETS1 in the regulation of *Id2* at the inception of ILC2 development.

Group 2 innate lymphoid cells (ILC2s) are a subset of ILCs that reside at mucosal surfaces and contribute to immune response against extracellular pathogens, such as helminthes. These cells are present at a low frequency in the lungs of WT mice, but they expand substantially in response to IL-33 and IL-25, which are produced by damaged epithelial cells. ILC2s contribute to pathogen clearance by producing multiple cytokines, including IL-5 and IL-13, which recruit and activate eosinophils and neutrophils as well as amphiregulin, which contributes to the maintenance of the epithelium (McKenzie et al., 2014). Although involved in pathogen clearance, aberrant activation of ILC2s in the lungs leads to eosinophilia and airway inflammation, a hallmark of allergic asthma (McKenzie et al., 2014). Despite the critical role that these cells play in immunity and disease, the key mechanisms controlling ILC2 development and function are just beginning to be revealed.

ILC2s are a subset of ILCs that share properties with T helper type 2 (Th2) cells. In adult mice, ILC2s develop in the BM from a common helper innate lymphoid progenitor (CHILP), which arises from common lymphoid progenitors (CLPs) but has lost adaptive lymphoid (B and T lymphocyte) and NK cell potential (Verykokakis et al., 2014). All of the helper-like ILCs share a requirement for the transcription factors GATA3 and TCF1 and for the E protein transcription factor inhibitor ID2 (Moro et al., 2010; Satoh-Takayama et al., 2010; Yagi et al., 2014; Yang et al., 2015). Downstream of CHILPs, ILC2 differentiation depends on the transcription factors ROR-α, GFI1, and BCL11b (Wong et al., 2012; Spooner et al., 2013; Califano et al., 2015; Walker et al., 2015; Yu et al., 2015). GFI1 promotes ILC2 development by maintaining GATA3, and it represses the expression of the ILC3 cytokine IL-17 (Spooner et al., 2013). BCL11b enforces the expression of GFI1 and similarly controls the development and functional properties of ILC2s (Califano et al., 2015).

We previously demonstrated that the ETS1 transcription factor regulates *Id2* transcription in NK cells (Pereira de Sousa et al., 2012; Ramirez et al., 2012). However, it is not known whether ETS1 plays a role in the transcriptional network that controls the emergence or activation of ILCs. Indeed, there have been few studies of ETS1 function in any cell type because of the high rate of neonatal lethality in mice carrying a germline deletion of *Ets1* (Gao et al., 2010). Here, we report on a novel mouse model for the conditional deletion of ETS1. We demonstrate that BM CHILPs could develop in the absence of ETS1 but are compromised in their fitness and their ability to generate ILC2s. ETS1 functions, at least in part, to promote the up-regulation of *Id2* mRNA that is observed in ILC2s. We also identified a role for ETS1 in the cytokine-induced expansion of lung ILC2s and for their production of IL-5 and IL-13. Our data place ETS1 as a very early regulator in the transcriptional network controlling the emergence and function of ILC2s.

*E. Zook and K. Ramirez contributed equally to this paper.

Correspondence to Barbara L. Kee: bkee@bsd.uchicago.edu

Abbreviations used: CHILP, common helper innate lymphoid progenitor; CLP, common lymphoid progenitor; iILC2, inflammatory ILC2; ILC, innate lymphoid cell; LMC, littermate control; mLN, mesenteric LN; nILC2, natural ILC2; qPCR, quantitative PCR; *Rag1*LMC, *Rag1*$^{-/-}$ LMC; SCF, stem cell factor.

The Rockefeller University Press $30.00
J. Exp. Med. 2016 Vol. 213 No. 5 687–696
www.jem.org/cgi/doi/10.1084/jem.20150851

RESULTS AND DISCUSSION

Lymphoid-specific deletion of *Ets1* mimics germline deletion

We previously demonstrated that ETS1 is required for the development of NK cells (Ramirez et al., 2012). However, our studies were severely hampered by the neonatal lethality of ETS1 deficiency (Bories et al., 1995; Barton et al., 1998; Gao et al., 2010). To overcome this limitation, we created mice in which the *Ets1* gene could be inactivated by Cre-mediated recombination. We flanked the exons coding for the ETS1 DNA–binding domain by loxp sequences such that Cre-mediated recombination results in a mutation analogous to the germline mutation described by Bories et al. (1995), from which no truncated ETS1 protein is produced (Fig. S1 A). We crossed $Ets1^{f/f}$ mice to $Il7ra^{Cre}$ mice, which produce Cre in IL-7 receptor–expressing ($CD127^+$) cells, including CLPs (Schlenner et al., 2010), the progenitors of all lymphoid cells. No ETS1 protein was detected in thymocytes isolated from $Il7ra^{Cre/+}Ets1^{f/f}$ ($Ets1^{\Delta/\Delta}$) mice, consistent with the deletion of *Ets1* exons 8 and 9 in all lymphoid cells (Fig. S1 B).

To confirm that $Ets1^{\Delta/\Delta}$ NK cells phenocopy NK cells in $Ets1^{-/-}$ mice, we performed flow cytometry to examine NK cell numbers and receptor expression. As anticipated, $Ets1^{\Delta/\Delta}$ mice had a decreased frequency and number of mature NK (mNK) cells, and these cells expressed less of the activating receptor NKp46 compared with littermate controls (LMCs; Fig. S1, C–E; Ramirez et al., 2012). These data demonstrate the utility of this $Ets1^{f}$ allele for the analysis of ETS1 function after Cre-mediated recombination.

ETS1 was essential for the generation of BM ILC2s

We noted a dramatic reduction in Lin^-CD127^+ cells in ETS1-deficient mice, which led us to question whether the development of ILC2s requires ETS1, because ILC2s are the major BM cells with this phenotype. Quantitative PCR (qPCR) analysis revealed that *Ets1* mRNA was expressed in BM ILC2s in amounts higher than the mRNA encoding ID2 or ROR-α, both of which are required for ILC2 development (Fig. 1 A; Diefenbach et al., 2014). Analysis of BM cells from $Ets1^{\Delta/\Delta}$ mice revealed a reduced frequency and number of $Lin^-CD127^+SCA1^+$ ILC2s as compared with LMCs (Fig. 1, B and C). The LMC $Lin^-CD127^+SCA1^+$ cells also expressed the IL-33 receptor component IL1RL1 (ST2) and ICOS, and a subset of these cells expressed KLRG1, further confirming that these cells are ILC2s (Fig.1 B and not depicted). We crossed the $Ets1^{\Delta/\Delta}$ mice to $Rag1^{-/-}$ mice to determine whether $Ets1^{\Delta/\Delta}$ T cells influence ILC2 development because these cells have been reported to aberrantly produce cytokines that could affect ILC2s (Garrett-Sinha, 2013). $Rag1^{-/-}Ets1^{\Delta/\Delta}$ ($REts1^{\Delta/\Delta}$) mice also had reduced numbers of BM ILC2s compared with $Rag1^{-/-}$ LMC (*R*LMC) cells (Fig. 1 C). These data demonstrate that ETS1 was required for the development of BM ILC2s.

It is possible that ETS1 was required for the generation of ILC2s because it impacted another lymphoid cell type that was required for ILC2 development, as recently described for NK cell maturation (Kim et al., 2014). To rigorously test whether the requirement for ETS1 was intrinsic to ILC2s or their progenitors, we created chimeric mice in which $CD45.2^+$ $Ets1^{\Delta/\Delta}$ or LMC BM cells were injected into lethally irradiated $CD45.1^+$ hosts along with WT $CD45.1^+$ BM cells. 8 wk later, $CD45.2^+$ cells were equally represented among the CLPs and ILC2s in the BM of *R*LMC:WT chimeras (Fig. 1, D and E). In contrast, in $REts1^{\Delta/\Delta}$:WT chimeras, $CD45.2^+$ cells contributed to the CLP population, but ILC2s were almost exclusively $CD45.1^+$ (Fig. 1, D and E). Therefore, ETS1 was required for the generation of ILC2s even when the hematopoietic environment contained ETS1-sufficient BM cells.

ETS1 was required for the development of LN ILC2s

A recent study revealed that ILC2s in peripheral tissues and secondary lymphoid organs are tissue resident and that under homeostatic conditions, ILC2s are not replenished from BM progenitors (Gasteiger et al., 2015). Therefore, we tested whether ILC2s in mesenteric LNs (mLNs) were present in $Ets1^{\Delta/\Delta}$ mice. We found that the number of mLN ILC2s was substantially reduced in $Ets1^{\Delta/\Delta}$ mice compared with LMCs (Fig. 2, A and B). In contrast, the numbers of ILC2s in the lungs of $Ets1^{\Delta/\Delta}$ and LMC mice were not statistically different (Fig. 2, C and D). Interestingly, similar to what we found in $Ets1^{\Delta/\Delta}$ mice, ILC2s in secondary lymphoid organs and lungs appear to have different requirements for the transcription factor BCL11b (Califano et al., 2015; Yu et al., 2015). Although lung ILC2s are tissue resident, they can expand in response to chronic inflammation (Gasteiger et al., 2015). Therefore, it is possible that mice with ILC2 developmental defects experience more inflammation in the lungs than LMCs and that a few ILC2s that make it to that tissue expand in response to this inflammation. Alternatively, when ILC2 numbers are low, the lung niche may support their proliferation or immigration. Consistent with the tissue-resident status of ILC2s, we were unable to detect significant numbers of *R*LMC or $REts1^{\Delta/\Delta}$ ILC2s in the mLNs or lungs of recipient mice in mixed BM chimeras (Fig. 2 E). Our data demonstrate a requirement for ETS1 for the development of mLN ILC2s.

ETS1 promoted the development of CHILPs in the BM

Ets1 mRNA initiates in lymphoid primed multipotent progenitors (Ramirez et al., 2012) and, therefore, ETS1 could be required for the transition from lymphoid primed multipotent progenitors/CLPs to ILC2s. ILC2s arise from CHILPs, which are phenotypically identified as Lin^-Flt3^- cells that express CD127, α4β7, and SCA1 but not CD25 (Fig. 3 A; Klose et al., 2014). Notably, the frequency and number of CHILPs was similar in $REts1^{\Delta/\Delta}$ and *R*LMC BM (Fig. 3, A and B). In contrast, in $Ets1^{\Delta/\Delta}$ mice, which had an intact *Rag1* gene, there was an ∼23-fold decrease in the number of CHILPs compared with LMC mice (Fig. 3 B). These data raise the possibility that although ETS1 was not required for the development of CHILPs, $Ets1^{\Delta/\Delta}$ CHILPs failed to

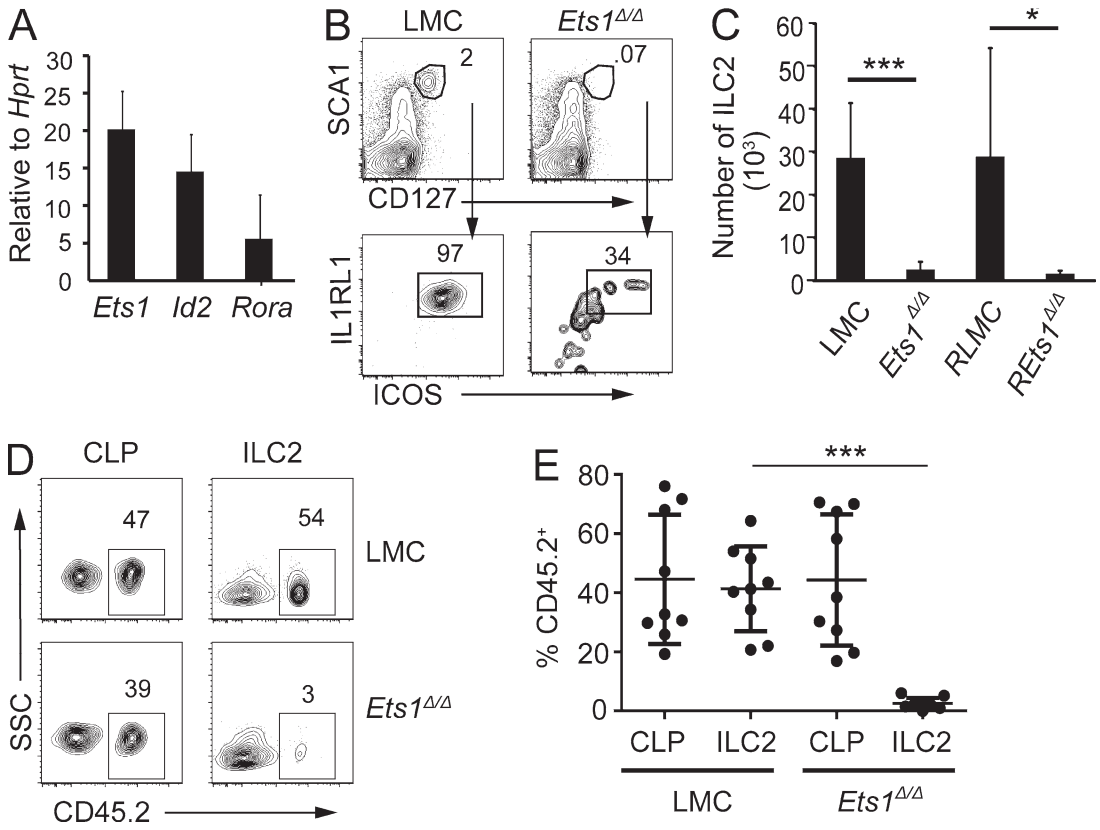

Figure 1. **ETS1 is required for ILC2 development.** (A) *Ets1*, *Id2*, and *Rora* mRNA expression relative to *Hprt* in BM ILC2s from WT mice ± SD. ILC2s were sorted as Lin$^-$SCA1$^+$CD127$^+$ICOS$^+$IL1RL1$^+$ cells. (B) FACS analysis for BM ILC2s in *Ets1*$^{\Delta/\Delta}$ and LMC mice. Lin$^-$ cells are shown. (C) Numbers of Lin$^-$SCA1$^+$CD127$^+$ BM ILC2s in LMC, *Ets1*$^{\Delta/\Delta}$, *R*LMC, and *REts1*$^{\Delta/\Delta}$ mice. (D) FACS analysis showing the representation of *R*LMC or *REts1*$^{\Delta/\Delta}$ (CD45.2) BM cells among CLPs (Lin$^-$CD127$^+$CD117^{int}SCA1^{int}) and ILC2s (Lin$^-$CD127$^+$SCA1$^+$) in mixed BM chimeras. (E) Percentage of CLPs or ILC2s that were CD45.2$^+$ in mixed BM chimeras. Data are from three independent experiments. For E, each circle is one mouse and the bar is the mean. Error bars are SD. *, $P < 0.05$; ***, $P < 0.001$.

compete with RAG1-dependent cells. Alternatively, *Ets1*$^{\Delta/\Delta}$ T cells may have exerted an inhibitory effect on CHILPs, for example, by producing inhibitory cytokines or by occupying a limiting BM niche that supports CHILPs. To address these possibilities, we created mixed BM chimeric mice in which *REts1*$^{\Delta/\Delta}$ and *R*LMC CD45.2$^+$ cells were in competition with *Rag1*$^{+/+}$CD45.1$^+$ cells. Under these conditions, *R*LMC cells were able to generate CLPs and CHILPs efficiently (Fig. 3 C). In contrast, in the *REts1*$^{\Delta/\Delta}$:WT chimeras, *REts1*$^{\Delta/\Delta}$ cells generated CLPs, but they contributed to <5% of CHILPs (Fig. 3 C). These data support the hypothesis that ETS1 deficiency compromised the fitness of CHILPs and that these cells were unable to compete with WT CHILPs or WT B or T lymphocytes.

ETS1 was required for appropriate *Id2* expression in BM ILC2s

Our data indicate that ETS1 is a component of the early transcriptional network for ILC2 development. In NK cells, ETS1 is required for the increase in *Id2* expression that accompanies NK cell maturation (Ramirez et al., 2012). Therefore, we tested whether ETS1 was required for *Id2* expression in CHILPs or ILC2s using an *Id2*^{GFP} reporter. Expression of GFP from the *Id2*^{GFP} reporter was highly variable in *Ets1*$^{\Delta/\Delta}$ CHILPs, suggesting that ETS1 may be required for stable *Id2* transcription; however, the mean intensity of GFP was similar in both strains (Fig. 3, D and E). In the LMCs, *Id2*^{GFP} expression was higher in ILC2s than in CHILP, but this increase was not observed in the few ILC2s present in *Ets1*$^{\Delta/\Delta}$ mice (Fig. 3, D and E). In contrast to *Id2* mRNA, GATA3 and TCF1 were expressed at similar or higher levels in *Ets1*$^{\Delta/\Delta}$ CHILPs and ILC2s when compared with LMCs (Fig. 3 D). These data indicate that ETS1 was required for stable and high *Id2* mRNA expression in CHILPs as they differentiate into ILC2s.

ETS1 was required for the cytokine-driven expansion of ILC2s

The paucity of ILC2s in the BM of *Ets1*$^{\Delta/\Delta}$ mice impeded an analysis of protein or gene expression. Therefore, we tested whether BM ILC2s could be expanded in vitro. We isolated Lin$^-$CD127$^+$SCA1$^+$ ILC2s from the BM of *R*LMC and *REts1*$^{\Delta/\Delta}$ mice and cultured them in vitro under defined ILC2 conditions for 2 wk: OP9–delta-like ligand 1 (DL1) in the presence of IL-7 and IL-33 or stem cell factor

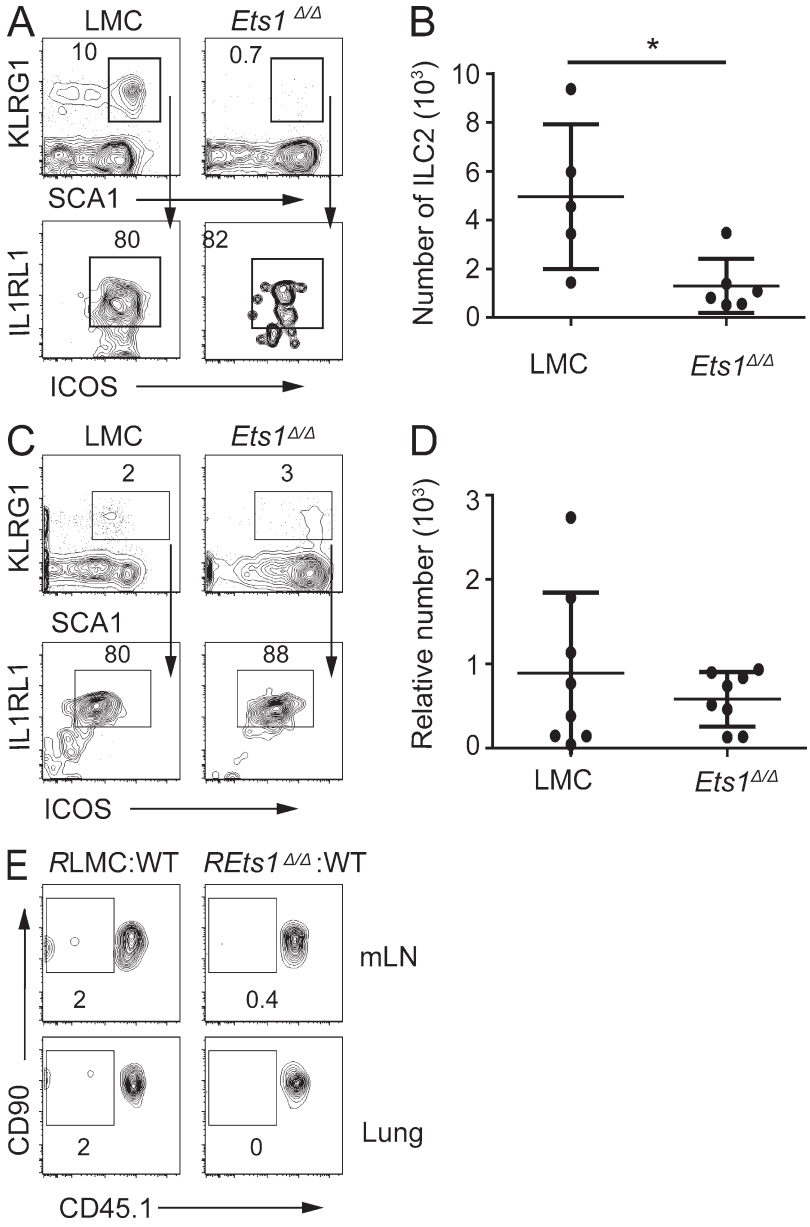

Figure 2. ***Ets1* is required for mLN but not lung ILC2s.** (A and B) FACS analysis for (A) and number of (B) Lin$^-$KLRG1$^+$SCA1$^+$IL1RL1$^+$ICOS$^+$ ILC2s in the mLNs of LMC and *Ets1*$^{\Delta/\Delta}$ mice. (C and D) FACS analysis for (C) and number of (D) ILC2s in the lungs of LMC and *Ets1*$^{\Delta/\Delta}$ mice. (E) FACS analysis showing the contributions of *R*LMC or *REts1*$^{\Delta/\Delta}$ (CD45.2) BM cells to the ILC2 population in the mLNs and lungs of mixed BM chimeras. ILC2s were identified using the staining strategies shown in A and C. For B and D, each symbol is one mouse and each bar is the mean. Error bars are SD. *, $P < 0.05$.

(SCF), IL-7, and IL-2 (Neill et al., 2010; Klose et al., 2014). Under either condition, *R*LMC cells expanded well and generated progeny that were Lin$^-$ICOS$^+$SCA1$^+$CD127$^+$ ILC2s. In contrast, cultures initiated with *REts1*$^{\Delta/\Delta}$ cells generated few ICOS$^+$ cells (Fig. 4 A). Overall, there was a 20-fold decrease in the number of ICOS$^+$ cells generated from *REts1*$^{\Delta/\Delta}$ Lin$^-$CD127$^+$SCA1$^+$ cells compared with *R*LMC cells regardless of the culture conditions used, indicating that ETS1 was required for their in vitro expansion (Fig. 4 B). Identical results were obtained when we isolated the ILC2s from *Rag1*-expressing *Ets1*$^{\Delta/\Delta}$ mice (not depicted). To rule out that a decrease in ILC2 purity contributed to the reduced expansion, we performed similar experiments starting with CLPs. *REts1*$^{\Delta/\Delta}$ CLPs also failed to generate substantial numbers of ILC2s in vitro compared with *R*LMC CLPs (not depicted). Therefore, ETS-deficient ILC2s failed to expand efficiently in vitro.

Despite the reduced expansion of *Ets1*$^{\Delta/\Delta}$ ILC2s in vitro, we were able to generate more ILC2s than were available in vivo. In an attempt to gain insight into the requirements for ETS1 in ILC2s, we performed a microarray analysis on mRNA isolated from the ICOS$^+$ cells that expanded from LMC and *Ets1*$^{\Delta/\Delta}$ ILC2s cultured on OP9-DL1 with IL-7 and IL-33. There were 986 probe sets that were differentially expressed by at least 1.5-fold ($P < 0.05$), of which 418 were decreased (1.9% of LMC expressed probes) and 568 were increased (2.4% of *Ets1*$^{\Delta/\Delta}$ expressed probes) in *Ets1*$^{\Delta/\Delta}$ cells (Fig. 4 C). As expected, the expression of *Ets1*

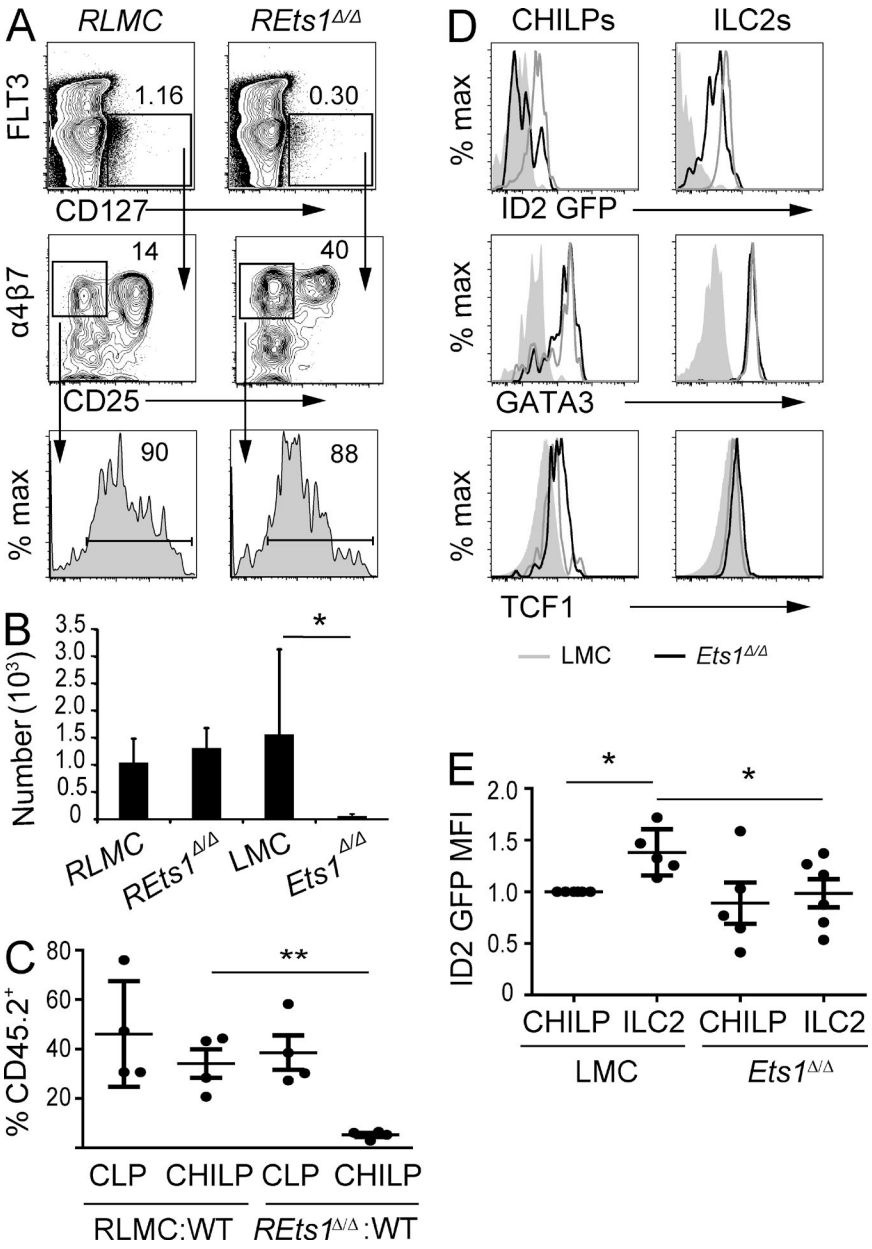

Figure 3. **ETS1 regulates CHILP fitness and expression of *Id2*.** (A) FACS staining strategy for BM CHILPs in Lin$^-$ cells from *Ets1*$^{\Delta/\Delta}$ and LMC mice. (B) Number of BM CHILPs as determined in A. (C) Percentage of CLPs or CHILPs that were CD45.2$^+$ in mixed *R*LMC:WT or *REts1*$^{\Delta/\Delta}$:WT chimeras. (D) *Id2*^{GFP}, GATA3, and TCF1 expression in *R*LMC and *REts1*$^{\Delta/\Delta}$ CHILPs and ILC2s. (E) *Id2*^{GFP} mean fluorescence intensity in LMC and *Ets1*$^{\Delta/\Delta}$ CHILPs and ILC2s. For C and E, each symbol is one mouse and each bar is the mean. Error bars are SD. *, $P < 0.05$; **, $P < 0.01$.

mRNA was lower in *Ets1*$^{\Delta/\Delta}$ cells (Fig. 4 E). In addition, *Klrg1* and *Areg*, genes associated with mature ILC2s, were decreased in the *Ets1*$^{\Delta/\Delta}$ cells, and KLRG1 protein was not detected on the *Ets1*$^{\Delta/\Delta}$ cells by flow cytometry (Fig. 4, E and G). An analysis of Gene Ontology terms revealed that 54 of the differentially expressed genes encoded proteins involved in cell proliferation ($P < 1.5 \times 10^{-8}$), including *Ccnd2*, 34 were involved in the regulation of phosphorylation ($P < 3.4 \times 10^{-7}$), and 71 encoded proteins involved in intracellular signaling cascades ($P < 1.7 \times 10^{-6}$; Table S1; Fig. 4 D). GSEA revealed that LMC cells had higher expression of genes associated with T effector cell differentiation than *Ets1*$^{\Delta/\Delta}$ cells (Fig. 4 D). Therefore, despite selection for cells that can grow in vitro, genes involved in cell proliferation and signaling were dysregulated in the absence of ETS1, and no KLRG1$^+$ ILC2s were generated.

In addition to the obvious failure to generate mature ILC2s, a KEGG pathway analysis revealed dysregulation of cytokine–cytokine receptor signaling ($P = 4.5 \times 10^{-6}$). Interestingly, the signature cytokines for ILC2s, IL-5 and IL-13, were not highly expressed in LMC or *Ets1*$^{\Delta/\Delta}$ in vitro–expanded cells. In contrast, *Il24* and *Il6* were among the most differentially expressed transcripts, and we confirmed that mRNA encoding these cytokines was increased in *Ets1*$^{\Delta/\Delta}$ ILC2s expanded in vitro by qPCR (Fig. 4, E and G). *Cd160*, which encodes a cell surface protein involved in mucosal barrier function and cytokine production from NK cells, was also more highly expressed in *Ets1*$^{\Delta/\Delta}$ cells compared with

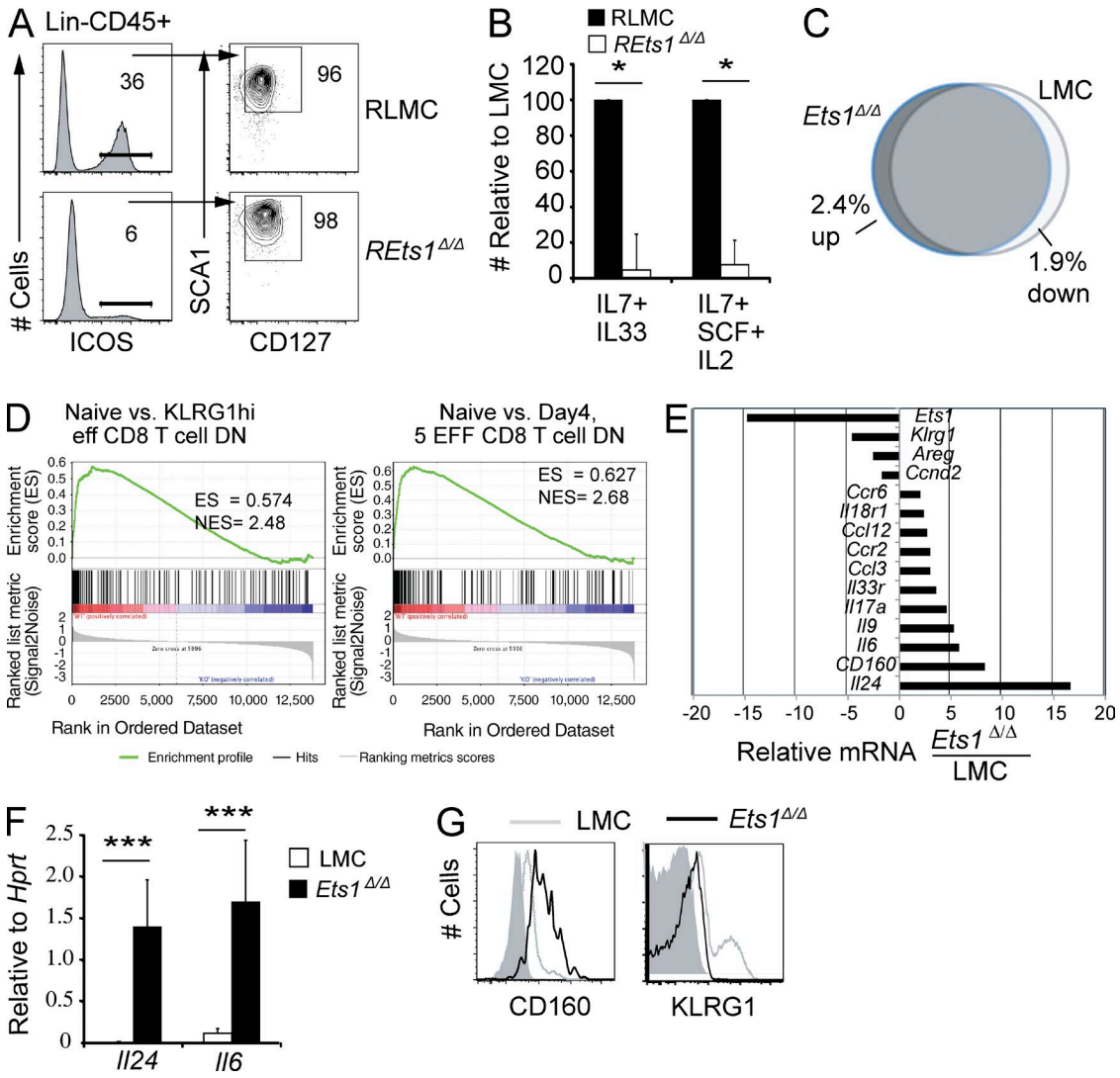

Figure 4. ***Ets1*$^{\Delta/\Delta}$ BM ILC2s expand poorly in vitro and have altered gene expression.** (A) FACS analysis of BM ILC2s from *R*LMC and *REts1*$^{\Delta/\Delta}$ mice after in vitro expansion for 2 wk on OP9-DL1 with IL-33 and IL-7. Lin$^-$CD45$^+$ cells were analyzed for ICOS, Sca1, and CD127. (B) Number of Lin$^-$CD45$^+$ICOS$^+$CD127$^+$ ILC2s recovered from LMC and *Ets1*$^{\Delta/\Delta}$ cells after culture with IL-7 and IL-33 or SCF, IL-2, and IL-7 + SD. (C) Representation of overlap in probe sets from microarray analysis of LMC and *Ets1*$^{\Delta/\Delta}$ ILC2s after in vitro expansion on OP9-DL1, IL-7, and IL-33. (D) GSEA of the microarray dataset revealed an enrichment of genes in the LMCs that were down in the gene sets from naive versus KLRG1^{hi} effector CD8$^+$ T cells or day 4/5 effector CD8$^+$ T cells. (E) Relative mRNA expression as determined by microarray analysis for a subset of genes in *Ets1*$^{\Delta/\Delta}$ compared with LMC ILC2s. (F) qPCR for *Il24* and *Il6* mRNA relative to *Hprt* in cultured LMC and *Ets1*$^{\Delta/\Delta}$ ILC2s + SD. (G) CD160 and KLRG1 on *Ets1*$^{\Delta/\Delta}$ and LMC cultured ILC2s as gated as in A. *, P < 0.05; ***, P < 0.001.

LMC cells (Fig. 4 F; Shui et al., 2012; Tu et al., 2015). Because no mature ILC2s were generated in our in vitro cultures, we considered the possibility that CD160 was a marker of immature ILC2s. However, BM KLRG1$^-$ or KLRG1$^+$ ILC2s did not express CD160, indicating that CD160 was not a marker of this ILC2 progenitor population. Collectively, our data demonstrate that ETS1 was required for the in vitro generation of mature ILC2s and that the cells generated in vitro from *Ets1*$^{\Delta/\Delta}$ Lin$^-$ICOS$^+$ cells differ from LMC ILC2s in the expression of genes involved in intracellular signaling as well as cytokine expression.

ETS1 was required for the cytokine-induced expansion of lung ILC2s and their production of cytokines

Given that lung ILC2 numbers were not severely affected by ETS1 deficiency, we tested whether ETS1 was required for ILC2 expansion after systemic administration of the alarmin IL-33. Intravenous injection of IL-33 into LMC mice resulted in an approximately eightfold expansion of ILC2s within 72 h (Fig. 5, A and B). In contrast, in *Ets1*$^{\Delta/\Delta}$ mice, IL-33 injection resulted in only a 3.8-fold expansion of ILC2s (Fig. 5, A and B). Therefore, similar to what was observed in vitro, ETS1 was required for the appropriate expansion of lung ILC2s in response to IL-33.

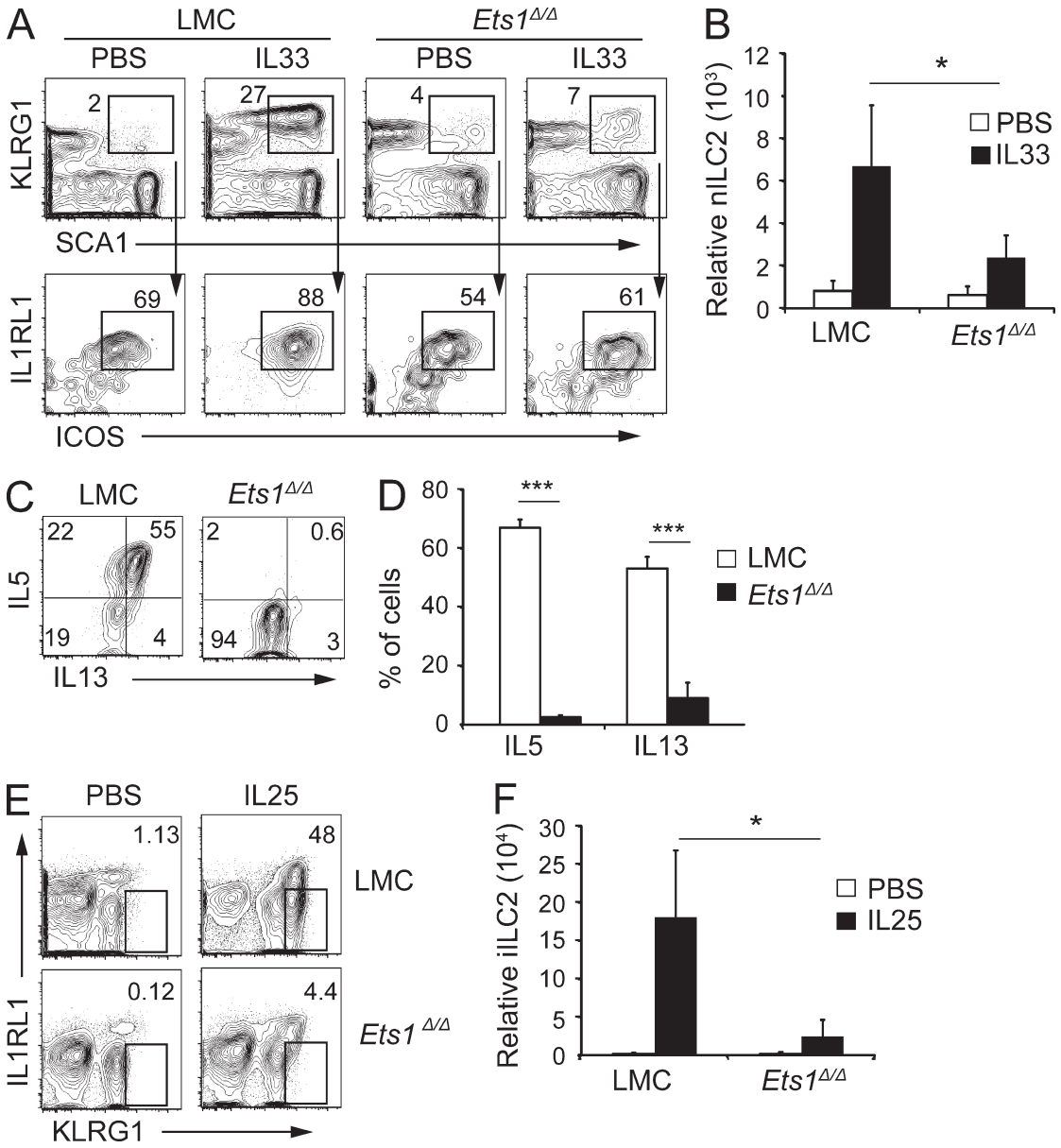

Figure 5. **Lung ILC2s from *Ets1*$^{\Delta/\Delta}$ mice show reduced expansion in vivo after exposure to IL-33 and IL-25.** (A) FACS analysis for ILC2s in the lungs of LMC and *Ets1*$^{\Delta/\Delta}$ mice injected i.p. with PBS or IL-33. Plots are gated on Lin$^-$ cells. (B) Relative number of lung ILC2s in PBS or IL-33–injected mice + SD. (C) Intracellular IL-5 and IL-13 production in lung ILC2s from IL-33–treated LMC and *Ets1*$^{\Delta/\Delta}$ mice. ILC2s are gated as Lin$^-$SCA1$^+$KLRG1$^+$CD90$^+$IL1RL1$^+$. (D) Percentage of lung ILC2s that produce IL-5 and IL-13 after IL-33 stimulation + SD. (E) FACS analysis for lung iILC2s in LMC and *Ets1*$^{\Delta/\Delta}$ mice treated with PBS or IL-25. The plots show IL1RL1 and KLRG1 on Lin$^-$ cells. (F) Total number of iILC2s isolated from the lungs of LMC and *Ets1*$^{\Delta/\Delta}$ mice + SD. *, $P < 0.05$; ***, $P < 0.001$.

We next tested whether the few lung ILC2s that expanded in response to IL-33 in vivo could produce the Th2 cytokines IL-5 and IL-13. Upon stimulation, ∼70% of LMC ILC2s were able to produce IL-13 and >50% could produce IL-5 (Fig. 5, C and D). In contrast, only 3% of ILC2s from *Ets1*$^{\Delta/\Delta}$ lungs produced IL-13 and <7% produced IL-5 (Fig. 5, C and D). *Ets1*$^{\Delta/\Delta}$ ILC2s also produced less IL-6 compared with LMCs, in contrast to what was observed in the in vitro–expanded BM ILC2s (not depicted). Collectively, our data indicate that ETS1 was required for cytokine production from lung ILC2s after exposure to IL-33.

ETS1 was required for the generation of inflammatory ILC2s (iILC2s)

Recently, iILC2s were described in the lungs that express KLRG1 but lack the receptor for IL-33, IL1RL1/ST2 (Huang et al., 2014). In contrast to natural ILC2s (nILC2s) that respond to IL-33, iILC2s proliferate and become acti-

vated in response to IL-25. After activation, iILC2s were proposed to differentiate into nILC2s. To determine whether IL-25 could elicit the expansion of *Ets1*$^{\Delta/\Delta}$ iILC2s, we injected IL-25 into *Ets1*$^{\Delta/\Delta}$ and LMC mice and quantified the number of iILC2s 3 d later. iILC2s could not be detected in the lungs of untreated LMC or *Ets1*$^{\Delta/\Delta}$ mice (Fig. 5, E and F). 3 d after the injection of IL-25, there was a 115-fold expansion in the number of iILC2s in the lungs of LMC mice. In contrast, at the same time point, iILC2s remained near the limit of detection in the lungs of *Ets1*$^{\Delta/\Delta}$ mice (Fig. 5, E and F). Our data demonstrate that in addition to being required for ILC2 development in the BM and expansion of nILC2s by IL-33, ETS1 was required for the expansion of iILC2s in response to IL-25.

In conclusion, we have created a conditional allele for *Ets1* that allowed us to demonstrate a role for ETS1 in the regulation of *Id2* in ILC2s, the fitness of CHILPs, and the development of ILC2s in the BM and mLNs. Despite the presence of ILC2s in the lungs, ETS1 was required for their expansion and for the expression of IL-5 and IL-13 in response to IL-33 and for the expansion of iILC2s in response to IL-25. Although we could expand a small number of ILC2s in vitro from *Ets1*$^{\Delta/\Delta}$ BM ILC2s, these cells failed to express critical markers of mature ILC2s, and they expressed genes that were not typical of ILC2 progenitors such as *Cd160*. These findings raise the possibility that the cells we expanded in vitro are not bone fide ILC2s; they could be rare ILC2 progenitors, ILC2 progenitors with dysregulated gene expressions, or distinct cell types. The cells do not appear to be NK cells, ILC1s, or ILC3s because they did not express critical transcription factors for these lineages such as *Tbx21*, *Eomes*, or *Rorc*, but they did express *Rora*, *Gata3*, and *Id2*, suggesting that they had entered the ILC2 program. Because these cells expressed *Id2* and had altered expression of numerous signaling proteins, we propose that they were selected for alterations that allow them to overcome the most severe requirements for ETS1 in ILC2 expansion, thus masking the critical targets of ETS1. One candidate factor that could drive the survival of these cells is IL-9, which is highly expressed in the in vitro–expanded *Ets1*$^{\Delta/\Delta}$ cells (Turner et al., 2013). Future studies in which ETS1 is removed after the expansion of ILC2s may allow for more insight into the direct targets of ETS1 in these cells.

In some important aspects, the requirements for ETS1 in ILC2s parallel those in NK cells. Although ETS1 was required for the up-regulation of *Id2* mRNA during NK cell and ILC2 development, the requirements for ETS1 in both lineages is likely to extend beyond *Id2*. Dysregulation of multiple surface receptors and intracellular signaling pathways also occur in ETS1-deficient NK cells and other lymphoid cells (Ramirez et al., 2012). However, the NK cells and B cells that develop in *Ets1*$^{-/-}$ mice show hyperreactivity to cytokines and increased activation through some receptors, whereas *Ets1*$^{\Delta/\Delta}$ ILC2s appear less activated and have reduced cellular expansion. Whether these distinctions reflect unique functions for ETS1 in ILC2s or distinct selective pressures placed on these cells in vivo remains to be determined. The availability of a conditional allele for *Ets1* makes it feasible to test whether the hyperresponsiveness of NK cells and B cells in the absence of ETS1 is a consequence of selection or an intrinsic function for ETS1 in these mature cell types.

MATERIALS AND METHODS

Mice. *Ets1*$^{f/f}$ mice were generated in the University of Chicago Transgenic Core Facility using 129/SvJ embryonic stem cells. The offspring were backcrossed onto the C57BL/6 background for >12 generations. In brief, a targeting vector containing the floxed sites along with exons 8 and 9 containing the *Ets1* binding domain was generated and introduced to germline DNA through homologous recombination. After backcrossing, the *Ets1*$^{f/f}$ mice were crossed to *Il7ra*^{cre} mice, which had also been crossed onto the C57BL/6 background for >12 generations (Schlenner et al., 2010). C57BL/6 *Rag1*$^{-/-}$ mice and CD45.1^{+} C57BL/6 mice were purchased through The Jackson Laboratory. All mouse lines were housed at the University of Chicago Animal Resource Center in accordance with the guidelines of the Institutional Animal Care and Use Committee.

Antibodies and flow cytometry. Cells suspensions were incubated with an unlabeled purified CD16/32 (2.4G2.1) blocking antibody before the addition of any biotinulated or flourochrome-conjugated antibodies (FITC, PE, APC, PECY7, Percp-cy5.5, AF780, and Brilliant violet 421). Antibodies were purchased from eBioscience, BD, or BioLegend unless noted otherwise. Lineage cocktails for ILC2s included CD3 (145-2C11), CD4 (GK1.5), CD8 (2.43.1), TCR-β (H57-597), TCR-γδ (UC7-13D3), CD11c (N418), NK1.1 (PK136), GR1 (RB6-8C5), B220 (RA3-6B2), CD19 (1D3), and Ter119 (TER-119). ILC2s were identified using a combination of antibodies against SCA1 (D7), CD127 (A7R34), ICOS (7E.17G9), ST2 (DJ8; MD Biologicals), KLRG1 (2F1), and GATA3 (TWAJ). ILC2s were also stained for the expression of CD160 (7H1). To stain for CHILPs, BM was depleted using B220, Gr1, and Ter119. Cells were then stained for lineage markers (B220, Gr1, Ter119, CD3, CD19, and NK1.1), Flt3 (A2F1D), CD127, SCA1, α4β7 (LPAM1), and CD25 (PC61.5). NK cells and CLPs were stained as previously described (Ramirez et al., 2012).

qPCR. BM ILC2s were sorted from WT mice. Total RNA was isolated using the RNeasy Micro kit (QIAGEN). cDNA was synthesized using Superscript III (Invitrogen). qPCR was performed using the following primers: *Hprt*, forward (5′-ACCTCTCGAAGTGTTGGATA-3′) and reverse (5′-CAACAAACTTGTCTGGA-3′); *Ets1*, forward (5′-CTGACCTCAACAAGGACAAGCC-3′) and reverse (5′-TTCCAGAAGAAACTGCCACAGC-3′); *Id2*, forward (5′-CACAGAGTACTTTGCTATCATTCG-3′) and reverse (5′-CCTGAACACGGACATCAGC-3′); *Rora*, forward (5′-TGATCGGACCAGCAGAAA-3′) and reverse (5′-CTT

GGACATCCGACCAAAC-3′); *Il24*, forward (5′-GCCCAG TAAGGACAATTCCA-3′) and reverse (5′-ATTTCTGCA TCCAGGTCAGG-3′); and *Il6*, forward (5′-CCGGAGAGG AGACTTCACAG-3′) and reverse (5′-GGAAATTGGGGT AGGAAGGA-3′). Samples were run in duplicate or triplicate and expression was measured relative to HPRT (hypoxanthine-guanine phosphoribosyltransferase).

BM chimeric mice. CD45.2 $Ets1^{\Delta/\Delta}$, LMC, $R Ets1^{\Delta/\Delta}$, or RLMC BM cells were mixed with CD45.1 $Rag1^{+/+}$ BM cells at a 1:1 ratio. A total of 5×10^6 BM cells were injected retroorbitally into lethally irradiated CD45.1 hosts. At 8 wk after reconstitution, the frequency of $CD45.2^+$ cells to the CLP, ILC2, or CHILP populations was determined by flow cytometry.

Cytokine administration and analysis of lung ILC2s. Mice were injected with 0.4 µg IL-33 (BioLegend), 0.4 µg IL-25 (R&D Systems), or PBS for three consecutive days. On the fourth day, the mice were sacrificed and the lungs were analyzed for ILC2s. The lung tissue was minced into small pieces and placed into 10 ml DMEM supplemented with 10% FBS, 1× pen/strep, and DNase I. 1,500 U collagenase I (Gibco) was added to the media, and tissue was digested by shaking at 37°C for 30 min. Tissue was further digested by mechanical disruption to break up the remaining pieces before staining. Lymphocytes were isolated using a Percoll gradient and analyzed by flow cytometry from which the frequency or relative or total number of lung ILC2s or iILC2s was calculated.

ILC2 stimulation and cytokine production. $Ets1^{\Delta/\Delta}$ and LMC mice were treated with IL-33 or PBS for three consecutive days before isolating the lungs as described in the previous section. Lymphocytes were stimulated in vitro with 1 µg/ml ionomycin and 20 ng/ml PMA in the presence of brefeldin A for 5 h followed by staining for ILC2 cell surface markers. Cells were then fixed and permeabilized using a cytofix/cytoperm kit (BD) and then stained for IL-13 (ebio13A) and IL-5 (TRFK5) before analysis by flow cytometry.

Cell culture and microarray. Sorted ILC2s were cultured for 2 wk on OP9-DL1 with 10 ng/ml each of IL-7 and IL-33 or on OP9-DL1 with 10 ng/ml of SCF and IL-7 and 2,000 U/ml of IL-2. OP9-DL1 was provided by J.C. Zuniga-Pflucker (University of Toronto, Toronto, ON, Canada). RNA from 100,000 cultured ILC2s was isolated using the RNeasy Micro kit, and cDNA was prepared to probe Affymetrix MOE 430_2 arrays (GEO accession number GSE79742) as previously described (Dias et al., 2008). Raw data were normalized using RMAExpress.

Statistical analysis. Statistical differences between groups were calculated using Student's *t* test, with p-values <0.05 considered significant. All statistics were performed using Prism 5 (GraphPad Software). All error bars are SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Online supplemental material. Fig. S1 shows that ETS1 was required for the development of BM ILC2s. Table S1 is provided as an Excel file and shows the gene expression in LMC and $Ets1^{\Delta/\Delta}$ Lin^-ICOS^+ cells expanded in vitro. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20150851/DC1.

ACKNOWLEDGMENTS

We would like to thank the members of the Kee, Fu, and Bendelac laboratory for their helpful discussions.

This work was funded by National Institutes of Health grants R01 AI106352 and R21 AI115388 (to B.L. Kee) and R01 HL071063 (to E.C. Svensson) and by the Rafael Rivera Asthma Research Fund (to K. Ramirez and B.L. Kee), as well as National Institutes of Health training grants T32AI007090 (to E.C. Zook and K. Ramirez) and F32CA177235 (to E.C. Zook). B.L. Kee was supported by the Leukemia and Lymphoma Society.

The authors declare no competing financial interests.

Author contributions: E.C. Zook, K. Ramirez, X. Guo, and G. van der Voort performed experiments. M. Sigvardsson performed the microarray. E.C. Svensson designed and generated the *Ets1* conditional mice. E.C. Zook, K. Ramirez, Y.-X. Fu, and B.L. Kee designed, analyzed, and interpreted the experiments. E.C. Zook and B.L. Kee wrote the manuscript.

Submitted: 19 May 2015
Accepted: 29 February 2016

REFERENCES

Barton, K., N. Muthusamy, C. Fischer, C.N. Ting, T.L. Walunas, L.L. Lanier, and J.M. Leiden. 1998. The Ets-1 transcription factor is required for the development of natural killer cells in mice. *Immunity.* 9:555–563. http://dx.doi.org/10.1016/S1074-7613(00)80638-X

Bories, J.C., D.M. Willerford, D. Grévin, L. Davidson, A. Camus, P. Martin, D. Stéhelin, and F.W. Alt. 1995. Increased T-cell apoptosis and terminal B-cell differentiation induced by inactivation of the Ets-1 proto-oncogene. *Nature.* 377:635–638. http://dx.doi.org/10.1038/377635a0

Califano, D., J.J. Cho, M.N. Uddin, K.J. Lorentsen, Q. Yang, A. Bhandoola, H. Li, and D. Avram. 2015. Transcription factor Bcl11b controls identity and function of mature type 2 innate lymphoid cells. *Immunity.* 43:354–368. http://dx.doi.org/10.1016/j.immuni.2015.07.005

Dias, S., R. Månsson, S. Gurbuxani, M. Sigvardsson, and B.L. Kee. 2008. E2A proteins promote development of lymphoid-primed multipotent progenitors. *Immunity.* 29:217–227. http://dx.doi.org/10.1016/j.immuni.2008.05.015

Diefenbach, A., M. Colonna, and S. Koyasu. 2014. Development, differentiation, and diversity of innate lymphoid cells. *Immunity.* 41:354–365. http://dx.doi.org/10.1016/j.immuni.2014.09.005

Gao, Z., G.H. Kim, A.C. Mackinnon, A.E. Flagg, B. Bassett, J.U. Earley, and E.C. Svensson. 2010. Ets1 is required for proper migration and differentiation of the cardiac neural crest. *Development.* 137:1543–1551. http://dx.doi.org/10.1242/dev.047696

Garrett-Sinha, L.A. 2013. Review of Ets1 structure, function, and roles in immunity. *Cell. Mol. Life Sci.* 70:3375–3390. http://dx.doi.org/10.1007/s00018-012-1243-7

Gasteiger, G., X. Fan, S. Dikiy, S.Y. Lee, and A.Y. Rudensky. 2015. Tissue residency of innate lymphoid cells in lymphoid and nonlymphoid organs. *Science.* 350:981–985. http://dx.doi.org/10.1126/science.aac9593

Huang, Y., L. Guo, J. Qiu, X. Chen, J. Hu-Li, U. Siebenlist, P.R. Williamson, J.F. Urban Jr., and W.E. Paul. 2014. IL-25-responsive, lineage-negative $KLRG1^{hi}$ cells are multipotential 'inflammatory' type 2 innate lymphoid cells. *Nat. Immunol.* 16:161–169. http://dx.doi.org/10.1038/ni.3078

Kim, T.J., V. Upadhyay, V. Kumar, K.M. Lee, and Y.X. Fu. 2014. Innate lymphoid cells facilitate NK cell development through a lymphotoxin-

mediated stromal microenvironment. *J. Exp. Med.* 211:1421–1431. http://dx.doi.org/10.1084/jem.20131501

Klose, C.S., M. Flach, L. Möhle, L. Rogell, T. Hoyler, K. Ebert, C. Fabiunke, D. Pfeifer, V. Sexl, D. Fonseca-Pereira, et al. 2014. Differentiation of type 1 ILCs from a common progenitor to all helper-like innate lymphoid cell lineages. *Cell.* 157:340–356. http://dx.doi.org/10.1016/j.cell.2014.03.030

McKenzie, A.N., H. Spits, and G. Eberl. 2014. Innate lymphoid cells in inflammation and immunity. *Immunity.* 41:366–374. http://dx.doi.org/10.1016/j.immuni.2014.09.006

Moro, K., T. Yamada, M. Tanabe, T. Takeuchi, T. Ikawa, H. Kawamoto, J. Furusawa, M. Ohtani, H. Fujii, and S. Koyasu. 2010. Innate production of T_H2 cytokines by adipose tissue-associated c-Kit$^+$Sca-1$^+$ lymphoid cells. *Nature.* 463:540–544. http://dx.doi.org/10.1038/nature08636

Neill, D.R., S.H. Wong, A. Bellosi, R.J. Flynn, M. Daly, T.K. Langford, C. Bucks, C.M. Kane, P.G. Fallon, R. Pannell, et al. 2010. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature.* 464:1367–1370. http://dx.doi.org/10.1038/nature08900

Pereira de Sousa, A., C. Berthault, A. Granato, S. Dias, C. Ramond, B.L. Kee, A. Cumano, and P. Vieira. 2012. Inhibitors of DNA binding proteins restrict T cell potential by repressing Notch1 expression in Flt3-negative common lymphoid progenitors. *J. Immunol.* 189:3822–3830. http://dx.doi.org/10.4049/jimmunol.1103723

Ramirez, K., K.J. Chandler, C. Spaulding, S. Zandi, M. Sigvardsson, B.J. Graves, and B.L. Kee. 2012. Gene deregulation and chronic activation in natural killer cells deficient in the transcription factor ETS1. *Immunity.* 36:921–932. http://dx.doi.org/10.1016/j.immuni.2012.04.006

Satoh-Takayama, N., S. Lesjean-Pottier, P. Vieira, S. Sawa, G. Eberl, C.A. Vosshenrich, and J.P. Di Santo. 2010. IL-7 and IL-15 independently program the differentiation of intestinal CD3$^-$NKp46$^+$ cell subsets from Id2-dependent precursors. *J. Exp. Med.* 207:273–280. http://dx.doi.org/10.1084/jem.20092029

Schlenner, S.M., V. Madan, K. Busch, A. Tietz, C. Läufle, C. Costa, C. Blum, H.J. Fehling, and H.R. Rodewald. 2010. Fate mapping reveals separate origins of T cells and myeloid lineages in the thymus. *Immunity.* 32:426–436. http://dx.doi.org/10.1016/j.immuni.2010.03.005

Shui, J.W., A. Larange, G. Kim, J.L. Vela, S. Zahner, H. Cheroutre, and M. Kronenberg. 2012. HVEM signalling at mucosal barriers provides host defence against pathogenic bacteria. *Nature.* 488:222–225. http://dx.doi.org/10.1038/nature11242

Spooner, C.J., J. Lesch, D. Yan, A.A. Khan, A. Abbas, V. Ramirez-Carrozzi, M. Zhou, R. Soriano, J. Eastham-Anderson, L. Diehl, et al. 2013. Specification of type 2 innate lymphocytes by the transcriptional determinant Gfi1. *Nat. Immunol.* 14:1229–1236. http://dx.doi.org/10.1038/ni.2743

Tu, T.C., N.K. Brown, T.J. Kim, J. Wroblewska, X. Yang, X. Guo, S.H. Lee, V. Kumar, K.M. Lee, and Y.X. Fu. 2015. CD160 is essential for NK-mediated IFN-γ production. *J. Exp. Med.* 212:415–429. http://dx.doi.org/10.1084/jem.20131601

Turner, J.E., P.J. Morrison, C. Wilhelm, M. Wilson, H. Ahlfors, J.C. Renauld, U. Panzer, H. Helmby, and B. Stockinger. 2013. IL-9-mediated survival of type 2 innate lymphoid cells promotes damage control in helminth-induced lung inflammation. *J. Exp. Med.* 210:2951–2965. http://dx.doi.org/10.1084/jem.20130071

Verykokakis, M., E.C. Zook, and B.L. Kee. 2014. ID'ing innate and innate-like lymphoid cells. *Immunol. Rev.* 261:177–197. http://dx.doi.org/10.1111/imr.12203

Walker, J.A., C.J. Oliphant, A. Englezakis, Y. Yu, S. Clare, H.R. Rodewald, G. Belz, P. Liu, P.G. Fallon, and A.N. McKenzie. 2015. Bcl11b is essential for group 2 innate lymphoid cell development. *J. Exp. Med.* 212:875–882. http://dx.doi.org/10.1084/jem.20142224

Wong, S.H., J.A. Walker, H.E. Jolin, L.F. Drynan, E. Hams, A. Camelo, J.L. Barlow, D.R. Neill, V. Panova, U. Koch, et al. 2012. Transcription factor RORα is critical for nuocyte development. *Nat. Immunol.* 13:229–236. http://dx.doi.org/10.1038/ni.2208

Yagi, R., C. Zhong, D.L. Northrup, F. Yu, N. Bouladoux, S. Spencer, G. Hu, L. Barron, S. Sharma, T. Nakayama, et al. 2014. The transcription factor GATA3 is critical for the development of all IL-7Rα-expressing innate lymphoid cells. *Immunity.* 40:378–388. http://dx.doi.org/10.1016/j.immuni.2014.01.012

Yang, Q., F. Li, C. Harly, S. Xing, L. Ye, X. Xia, H. Wang, X. Wang, S. Yu, X. Zhou, et al. 2015. TCF-1 upregulation identifies early innate lymphoid progenitors in the bone marrow. *Nat. Immunol.* 16:1044–1050. http://dx.doi.org/10.1038/ni.3248

Yu, Y., C. Wang, S. Clare, J. Wang, S.C. Lee, C. Brandt, S. Burke, L. Lu, D. He, N.A. Jenkins, et al. 2015. The transcription factor Bcl11b is specifically expressed in group 2 innate lymphoid cells and is essential for their development. *J. Exp. Med.* 212:865–874. http://dx.doi.org/10.1084/jem.20142318