The transcription factor Bcl11b is specifically expressed in group 2 innate lymphoid cells and is essential for their development

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Group 2 innate lymphoid cells (ILCs), or ILC2s, are a subset of recently identified ILCs, which play important roles in innate immunity by producing type 2 effector cytokines. Several transcription factors have been found to have critical functions in the development of both ILC2s and T cells. We report here that Bcl11b, a transcription factor essential in T cell lineage commitment and maintenance, is specifically expressed in progenitors committed to the ILC2 lineage and is required for ILC2 development. The *Bcl11b* gene is expressed in ∼28% of ILC progenitors (ILCPs; common helper innate lymphoid progenitors or ILCPs expressing either ID2 or promyelocytic leukemia zinc finger, respectively). Both in vitro and in vivo, these *Bcl11b*-expressing early ILCPs generate only ILC2s. Inactivation of *Bcl11b* causes a complete loss of ILC2 development from hematopoietic progenitors, which is confirmed upon immune challenge with either papain administration or influenza virus infection.

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Abbreviations used: ChILP, common helper innate lymphoid progenitor; CLP, common lymphoid progenitor; IEL, intraepithelial lymphocyte; ILC, innate lymphoid cell; ILCP, ILC progenitor; LPL, lamina propria lymphocyte; LSK, $Lin^{-}Sca1^{+}c\text{-}Kit^{+}$; LTi, lymphoid tissue inducer; MLN, mesenteric LN; qRT-PCR, quantitative RT-PCR; siLP, small intestine lamina propria; Tam, Tamoxifen.

Innate lymphoid cells (ILCs) are important effectors in innate immunity, lymphoid tissue formation, and tissue homeostasis. ILCs are characterized by a lymphoid morphology and the absence of markers for T, B, or myeloid cells and express the IL-7 receptor (IL-7Rα; CD127; Spits et al., 2013; Yagi et al., 2014). ILCs can be divided into ILC1s, ILC2s, and ILC3s based on the effector cytokines produced and the key transcription factors that determine their development and functions (Spits et al., 2013). For example, similar to Th2 cells, group 2 ILCs (ILC2s) produce IL-5 and IL-13 and are found to mediate parasite expulsion, to contribute to regeneration of respiratory tissues after acute influenza virus infection, and to participate in airway inflammation and immune pathologies (Moro et al., 2010; Neill et al., 2010; Price et al., 2010; Chang et al., 2011; Monticelli et al., 2011). All known subsets of ILCs depend on ID2 and cytokine receptor common γ chain for their development (Cao et al., 1995; Yokota et al., 1999; Moro et al., 2010). Transcription factors Rora, Gata3, Tcf1, Nfil3, and Gfi1 have been recently demonstrated to control ILC2 development (Kashiwada et al., 2011; Halim et al., 2012b; Hoyler et al., 2012; Mjösberg et al., 2012; Wong et al., 2012; Klein Wolterink et al., 2013; Spooner et al., 2013; Yang et al., 2013; Geiger et al., 2014; Seillet et al., 2014), and most of them have important roles in T cell development. ILCs are developed from common lymphoid progenitors (CLPs) and early ILC progenitors (ILCPs; Spits et al., 2013). However, it remains unclear how early progenitors become committed to each ILC subset and which transcription factors are involved in this process.

Transcription factor Bcl11b (B cell leukemia/lymphoma 11b) is required for the early T cell progenitors to become committed to the T cell lineage. Inactivation of the *Bcl11b* gene in the mouse causes failure of T cell lineage commitment and

The Rockefeller University Press $30.00
J. Exp. Med. 2015 Vol. 212 No. 6 865–874
www.jem.org/cgi/doi/10.1084/jem.20142318

The Journal of Experimental Medicine

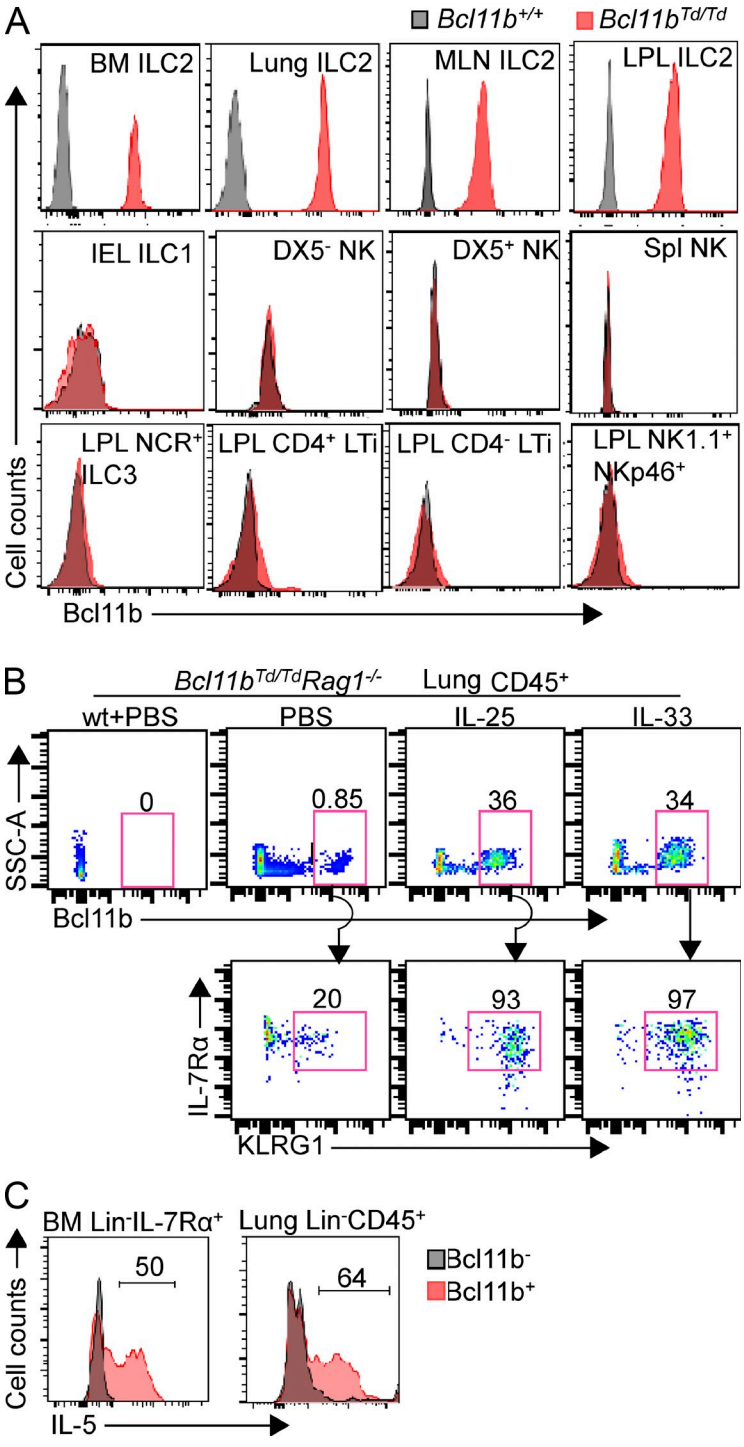

Figure 1. The *Bcl11b* gene is specifically expressed in ILC2s in ILCs. (A) Flow cytometry was performed on ILC subsets to assess *Bcl11b* expression in the *Bcl11b*$^{Td/Td}$ mouse. BM and lung ILC2s were identified as Lin$^-$IL-7Rα$^+$IL-33R$^+$CD25$^+$; MLN ILC2s as Lin$^-$IL-7Rα$^+$Sca1$^+$; LPL ILC2s as Lin$^-$IL-7Rα$^{+/low}$KLRG1$^+$; IEL ILC1s as Lin$^-$NKp46$^+$NK1.1$^+$CD160$^+$; liver DX5$^-$ and DX5$^+$ NK cells as CD3$^-$TCRβ$^-$NK1.1$^+$; LPL NCR$^+$ ILC3s as Lin$^-$NKp46$^+$NK1.1$^-$; LPL CD4$^+$ LTi cells as Lin$^-$KLRG1$^-$IL-7Rα$^+$CD4$^+$CCR6$^+$; LPL CD4$^-$LTi cells as Lin$^-$KLRG1$^-$IL-7Rα$^+$CD4$^-$CCR6$^+$; and Lin as B220, CD19, CD5, CD3, CD8, TCRγδ, TCRβ, CD11b, Gr-1, and Ter119. (B) *Bcl11b*-expressing leukocytes in the lung were elicited by IL-25 or IL-33 cytokine administration and examined by flow cytometry. Wild-type mice treated with PBS (wt+PBS) were used as the negative control for the Tdtomato signal. (C) Production of IL-5 (intracellular staining) by Bcl11b$^+$ cells from ex vivo stimulated BM and lung cells was assessed by flow cytometry. Numbers in plots denote percentages of cells in the indicated areas. ILC gating strategies are noted in Fig. S1. Data in all panels are representative of three to nine mice analyzed in at least three independent experiments.

loss of the T cell identity (Wakabayashi et al., 2003; Ikawa et al., 2010; Li et al., 2010a,b; Avram and Califano, 2014). Two studies indicate that *Bcl11b* expression was detected in ILC2s (Wong et al., 2012; Yang et al., 2013). We thus systematically investigated *Bcl11b* gene expression in ILCs in a *Bcl11b* reporter mouse and identified the essential role of Bcl11b in the development of ILC2s from hematopoietic progenitors.

RESULTS AND DISCUSSION

Bcl11b is specifically expressed in ILC2s

We previously reported that Bcl11b is expressed in all T cells, from DN2 thymocytes to mature T cells, using the *Bcl11b*-Tdtomato reporter mouse (*Bcl11b*$^{Td/Td}$; Li et al., 2010b). The *Bcl11b* gene expression was recently detected in ILC2 in RT-PCR (Wong et al., 2012; Yang et al., 2013). We took advantage of the *Bcl11b*$^{Td/Td}$ reporter mouse and further

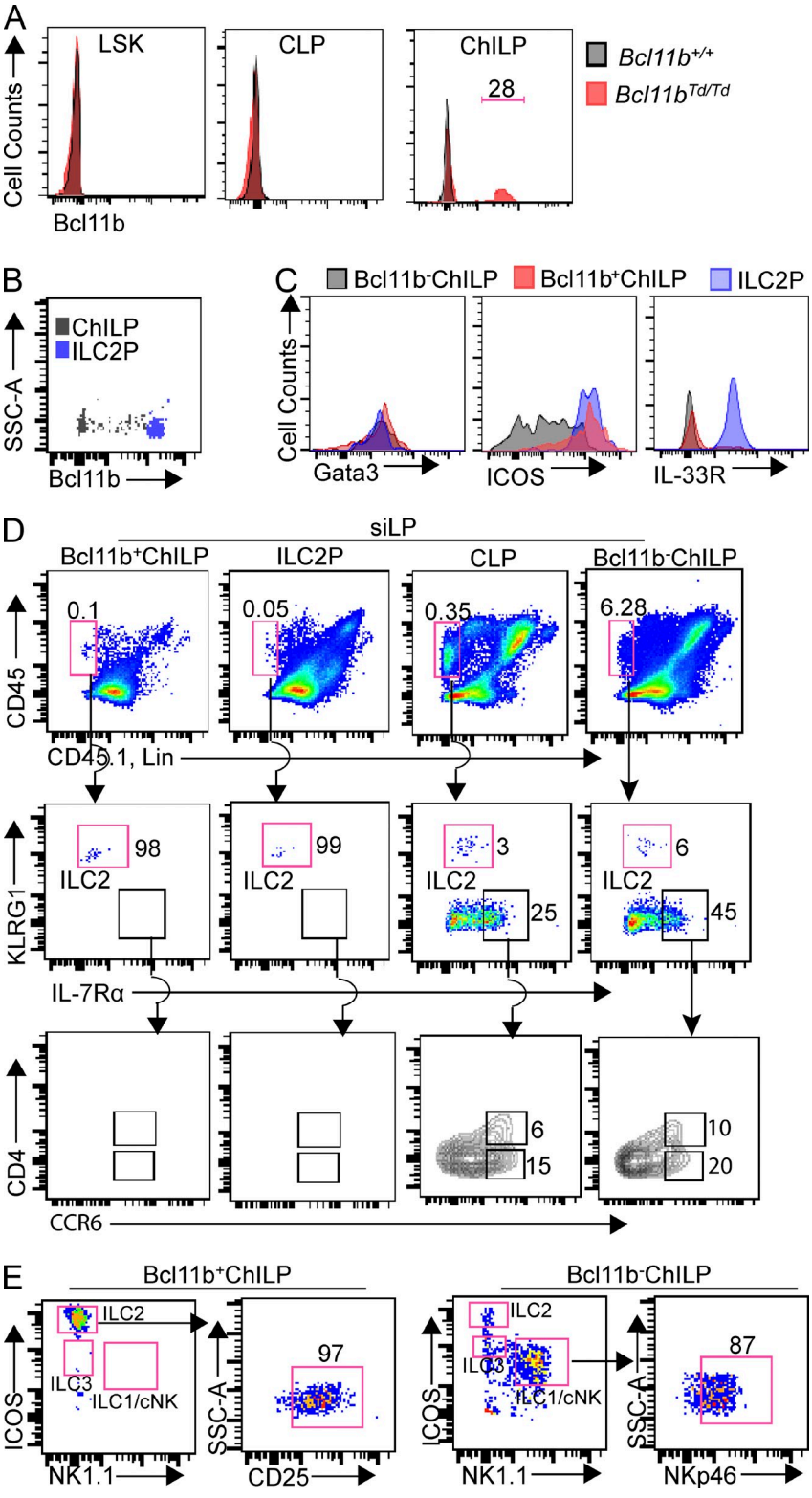

Figure 2. Bcl11b marks early committed ILC2Ps. (A–C) Analysis of expression of Bcl11b, Gata3, ICOS, and IL-33R in the indicated populations of *Bcl11b*$^{Td/Td}$ mice. Bcl11b expression in BM LSKs, CLPs (Lin$^-$Flt3$^+$IL-7Rα$^+$), and ChILPs (A) and in ChILPs and ILC2Ps (B) was assessed by flow cytometry. Gating strategies are noted in Fig. S2. (C) Expression of Gata3, ICOS, and IL-33R in ChILPs and ILC2Ps was analyzed by flow cytometry. (D) The in vivo differentiation potential of Bcl11b$^+$ChILPs, ILC2Ps, CLPs, and Bcl11b$^-$ChILPs was assessed by adaptive transfer followed by flow cytometric analysis. Donor-derived cells (CD45.1$^-$) in the siLP were screened for ILC1, ILC2, and ILC3. (E) The in vitro differentiation potential of Bcl11b$^+$ChILPs was also assessed by flow cytometry. Sorted Bcl11b$^-$ and Bcl11b$^+$ ChILPs were cultured on OP9 cells for 6 d in the presence of 20 ng/ml IL-7 and 20 ng/ml SCF. ILC2 was defined as ICOS^{hi}NK1.1$^-$CD25$^+$, ILC1/conventional NK was defined as ICOS^{low}NK1.1$^-$NKp46$^+$, and ILC3 was defined as ICOS^{int}NK1.1$^-$ as previously reported (Constantinides et al., 2014). Numbers in flow cytometry plots denote percentages of cells in the indicated areas. At least three mice were analyzed in each experiment, and all the experiments were independently repeated at least three times.

systematically explored *Bcl11b* expression in ILC development. At steady state, *Bcl11b* expression was found in all ILC2s in the BM, the lung, the mesenteric LN (MLN), and small intestine lamina propria (siLP; Fig. 1 A and Fig. S1, A–C and G). In contrast, ILC1s and ILC3s, including intraepithelial lymphocyte (IEL) ILC1s, liver DX5$^+$ and DX5$^-$ NK cells, spleen NK cells, lamina propria lymphocyte (LPL) NCR$^+$ILC3s, LPL CD4$^+$ or CD4$^-$ lymphoid tissue inducers

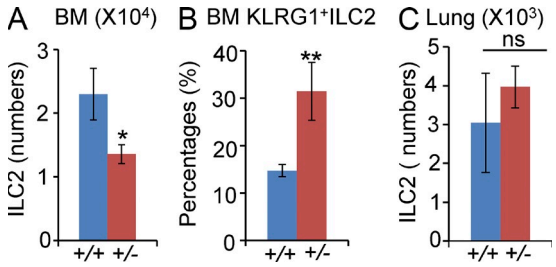

Figure 3. Bcl11b has a dose-dependent role in ILC2 development. ILC2s in the adult (14 wk old) wild-type mice (+/+) and *Bcl11b* mutant heterozygotes (+/−) were analyzed by flow cytometry and quantitated. (A) Cell numbers of ILC2s ($Lin^-IL\text{-}7R\alpha^+IL\text{-}33R^+CD25^+$) in the BM. (B) Percentages of $KLRG1^+$ cells in BM ILC2s. (C) Lung ILC2s ($Lin^-IL\text{-}7R\alpha^+IL\text{-}33R^+CD25^+$). Error bars indicate the SD. In all panels, $n = 4$ mice per genotype were analyzed, and each experiment was independently repeated at least three times. Statistical significance is indicated as follows: *, $P < 0.05$; **, $P < 0.01$. ns, not significant. Gating strategies are noted in Fig. S3.

(LTi's), or LPL $NKp46^+NK1.1^+$ cells (including conventional NK cells, ILC1s, and ILC3s; Klose et al., 2014), did not have detectable *Bcl11b* expression (Fig. 1 A and Fig. S1, D–G).

We next examined *Bcl11b* expression in ILC2s in the presence of IL-25 and IL-33, as recent studies suggest that ILC2s are elicited by these cytokines (for example, Neill et al., 2010). To minimize the background of the adaptive immune responses and to eliminate potential contamination with T cells, we crossed $Bcl11b^{Td/Td}$ reporter mouse to the $Rag1^{-/-}$ knockout mouse to obtain $Bcl11b^{Td/Td}Rag1^{-/-}$ mice. In $Bcl11b^{Td/Td}Rag1^{-/-}$ mice, 0.85% of the lung leukocytes expressed *Bcl11b*, and 20% of them expressed the mature ILC2 marker KLRG1 (Fig. 1 B; Hoyler et al., 2012). Once IL-25 or IL-33 was administered, *Bcl11b*-expressing lung leukocytes increased to 34% and 36%, respectively (Fig. 1 B). Notably, almost all of the expanded cells expressed KLRG1 (Fig. 1 B).

Similar to Th2 cells, ILC2s secrete type 2 cytokines such as IL-5 and IL-13 (Spits et al., 2013). We next purified $Lin^-Bcl11b^+$ leukocytes from the BM and the lung of $Bcl11b^{Td/Td}Rag1^{-/-}$ mice and briefly stimulated these cells ex vivo with PMA and Ionomycin for IL-5 production (Hoyler et al., 2012). Many $Lin^-Bcl11b^+$ cells produced IL-5 (Fig. 1 C). In contrast, the $Lin^-Bcl11b^-$ cells from both the BM and the lung did not have detectable IL-5 production (Fig. 1 C). These results thus demonstrate that *Bcl11b* is specifically expressed in ILC2s in ILCs.

Recent studies indicate that ILCs are differentiated from progenitors, which, in turn, originate from CLPs in the BM (Spits et al., 2013; Constantinides et al., 2014; Klose et al., 2014). Analysis of reporter mouse lines has identified two overlapping ILCP populations: common helper innate lymphoid progenitor (ChILP) cells that express ID2 (Klose et al., 2014) and ILCPs, which are characterized by the expression of promyelocytic leukemia zinc finger (Constantinides et al., 2014). Both progenitor populations can give rise to three types of ILCs but are heterogeneous as revealed in in vitro single-cell developmental fate assays. Notably, ∼28% of cells in the ChILP or ILCP compartment were reported to generate only ILC2s in vitro (Constantinides et al., 2014; Klose et al., 2014). High levels of *Bcl11b* expression mark the commitment of the T cell lineage in the thymus (Li et al., 2010a,b; Yu et al., 2012). We asked whether Bcl11b is also involved in early development of ILC2s. Consistent with previous studies, *Bcl11b* expression was not detected in either the $Lin^-Sca1^+c\text{-}Kit^+$ (LSK) compartment or CLP (Fig. 2 A; Li et al., 2010b). However, in the ChILP compartment defined by $Lin^-Flt3^-IL\text{-}7R\alpha^+\alpha_4\beta_7^+CD27^+CD25^-CD244^+$ (Fig. S2), coincidentally, *Bcl11b* expression was found in ∼28% of ChILPs (Fig. 2 A). Expression of Bcl11b appeared to increase from $Bcl11b^+$ChILPs to ILC2 progenitors (ILC2Ps; Fig. 2 B and Fig. S2). Moreover, $Bcl11b^+$ChILPs expressed other ILC2 genes such as Gata3 and ICOS, but little IL-33R (Fig. 2 C). To test whether these *Bcl11b*-expressing ChILPs overlap with those ones that generate only ILC2s (Constantinides et al., 2014; Klose et al., 2014), we intravenously injected purified $Bcl11b^+$ChILPs into sublethally irradiated alymphoid $Rag2^{-/-}Il2rg^{-/-}$ mice to examine their in vivo developmental potential. 6–8 wk after transplantation, siLP cells from recipients were harvested for ILC analysis. $Bcl11b^+$ChILPs produced only ILC2s, similar to what ILC2Ps ($Lin^-Flt3^-IL\text{-}7R\alpha^+\alpha_4\beta_7^+CD27^+CD25^+CD244^-$) did (Fig. 2 D; Hoyler et al., 2012; Klose et al., 2014). On the other hand, both CLPs and $Bcl11b^-$ChILPs produced $CD4^+$ and $CD4^-$ LTi cells besides ILC2s (Fig. 2 D). These results indicate that these $Bcl11b^+$ChILPs are already committed to the ILC2 lineage and may represent an earlier progenitor population ($CD25^-IL\text{-}33R^-$) than ILC2P ($CD25^+IL\text{-}33R^+$). We subsequently performed short-term in vitro fate assay, which again demonstrated that $Bcl11b^+$ChILPs only produced ILC2 (Fig. 2 E). Therefore, Bcl11b expression in ChILPs marks early committed ILC2Ps.

Bcl11b has critical and cell-autonomous functions in early ILC2 development

We started investigating Bcl11b functions in ILC2 development by examining the *Bcl11b* heterozygous germline loss of function mutant mice (+/−) because the homozygous mutants die at birth (Wakabayashi et al., 2003). Compared with the wild-type control mice (+/+), the heterozygotes had ∼59% of ILC2s ($Lin^-IL\text{-}33R^+IL\text{-}7R\alpha^+CD25^+$) in the BM (Fig. 3 A and Fig. S3 A), indicating that Bcl11b has a dose-dependent role in ILC2 development. Surprisingly, compared with the wild-type control mice, the heterozygotes had significantly higher percentages of mature ILC2s in the BM defined by $CD25^+KLRG1^+$ (Fig. 3 B and Fig. S3 A), indicating that Bcl11b might also normally prevent production of mature ILC2s from progenitors. On the other hand, ILC2 numbers in the lung of the heterozygotes were not significantly reduced (Fig. 3 C and Fig. S3 B).

We performed further analysis of Bcl11b in ILC2 development using the $Bcl11b^{flox/flox}Rosa26^{CreERT2/CreERT2}$ conditional knockout mice (Li et al., 2010b). These mice were grossly normal and fertile as previously reported (Li et al., 2010b).

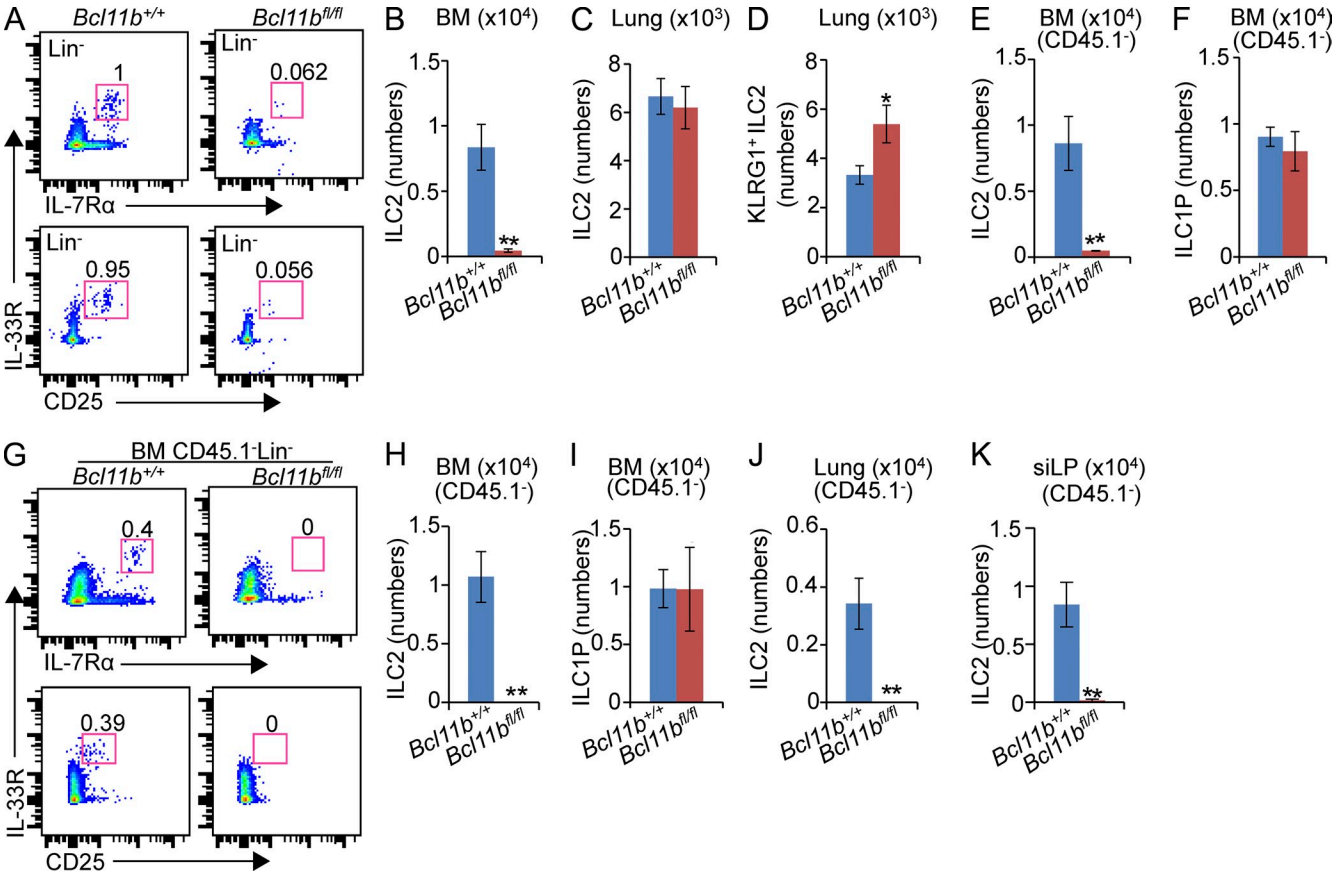

Figure 4. Bcl11b has critical and cell-autonomous functions in early ILC2 development. (A–D) ILC2s in BM (A and B) and lung (C and D) of *Bcl11b*$^{fl/fl}$ or control mice were analyzed by flow cytometry. (A) BM ILC2s were detected by either Lin$^-$IL-7Rα$^+$IL-33R$^+$ or Lin$^-$IL-33R$^+$CD25$^+$. (B) BM ILC2 numbers in *Bcl11b*$^{fl/fl}$ or control mice were analyzed as shown. (C and D) The numbers of lung ILC2s and KLRG1$^+$ILC2s in the *Bcl11b*$^{fl/fl}$ or the control mice were enumerated as shown. (E and F) Donor-derived BM ILC2s and ILC1Ps in chimeras engrafted with *Bcl11b*$^{fl/fl}$ BM cells 3–4 wk after Tam treatment. (G–K) LSKs from the *Bcl11b*$^{fl/fl}$ or control mice were transplanted into the *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ recipients (CD45.1$^+$). 8 wk after engraftment, the donor cells (CD45.1$^-$) were analyzed by flow cytometry. (G) Donor LSK-derived ILC2s in the BM were analyzed by flow cytometry. (H–J) ILC2s or ILC1s were enumerated as shown. (H) BM ILC2 numbers. (I) BM ILC1P numbers. (J) Lung ILC2 numbers. (K) siLP ILC2 numbers. Error bars indicate the SD. Numbers in flow cytometry plots denote percentages of cells in the indicated areas. All the experiments were independently repeated at least three times. *n* = 4 mice per genotype. Statistical significance is indicated as follows: *, P < 0.05; **, P < 0.01. Additional gating strategies are noted in Figs. S4 and S5.

Bcl11b deletion in these mice was achieved by administration of Tamoxifen (Tam; the treated mice are referred to as *Bcl11b*$^{fl/fl}$). We chose *Bcl11b*$^{+/+}$*Rosa26*$^{CreERT2/CreERT2}$ mice as the control (the treated mice are referred to as *Bcl11b*$^{+/+}$) to help exclude the potential defects from Cre toxicity. We analyzed the mice 2–3 wk after Tam administration. *Bcl11b* deletion caused severe phenotypes in the BM ILC2 compartment, with only ∼6% left compared with the control (Fig. 4, A and B). In contrast, lung ILC2s were not obviously reduced (Fig. 4 C and Fig. S4 A). Rather, more of them expressed KLRG1 (Fig. 4 D and Fig. S4 A), which is consistent with the result that the *Bcl11b* heterozygous mutants have increased mature ILC2s (Fig. 3 B and Fig. S3 A). This result shows that *Bcl11b* inactivation causes severe defects in early development of ILC2s in the BM.

To address whether the defects in ILC2s caused by *Bcl11b* deficiency were cell autonomous or intrinsic to hematopoietic cells, we intravenously injected *Bcl11b*$^{flox/flox}$*Rosa26*$^{CreERT2/CreERT2}$ or *Bcl11b*$^{+/+}$*Rosa26*$^{CreERT2/CreERT2}$ BM cells into lethally irradiated CD45.1$^+$ C57B6 recipients. The recipients were allowed 6–8 wk for donor cell reconstitution before Tam was administered to delete *Bcl11b*. BM cells from these mice were subsequently harvested 3–4 wk after Tam treatment. Again, only ∼6% of donor ILC2s was left in the BM after *Bcl11b* deletion (Fig. 4 E and Fig. S4 B). In contrast, no obvious changes were found in BM ILC1Ps (Lin$^-$NK1.1$^+$NKp46$^+$IL7Rα$^+$CD49a$^+$; Fig. 4 F and Fig. S4 C; Klose et al., 2014).

We next purified BM hematopoietic progenitors (LSKs) from either Tam-treated *Bcl11b*$^{flox/flox}$*Rosa26*$^{CreERT2/CreERT2}$ (*Bcl11b*$^{fl/fl}$) or control mice and intravenously injected these cells into lethally irradiated alymphoid *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ recipients (CD45.1$^+$; Serafini et al., 2014) with helper BM cells (CD45.1$^+$). After 8 wk of engraftment, the recipients were analyzed for ILC engraftment. Control LSKs (Bcl11b$^{+/+}$) efficiently generated ILC2s in the BM, the lung, and the siLP, as well as ILC1Ps in the BM (Fig. 4, G–K, and Fig. S5, A–C).

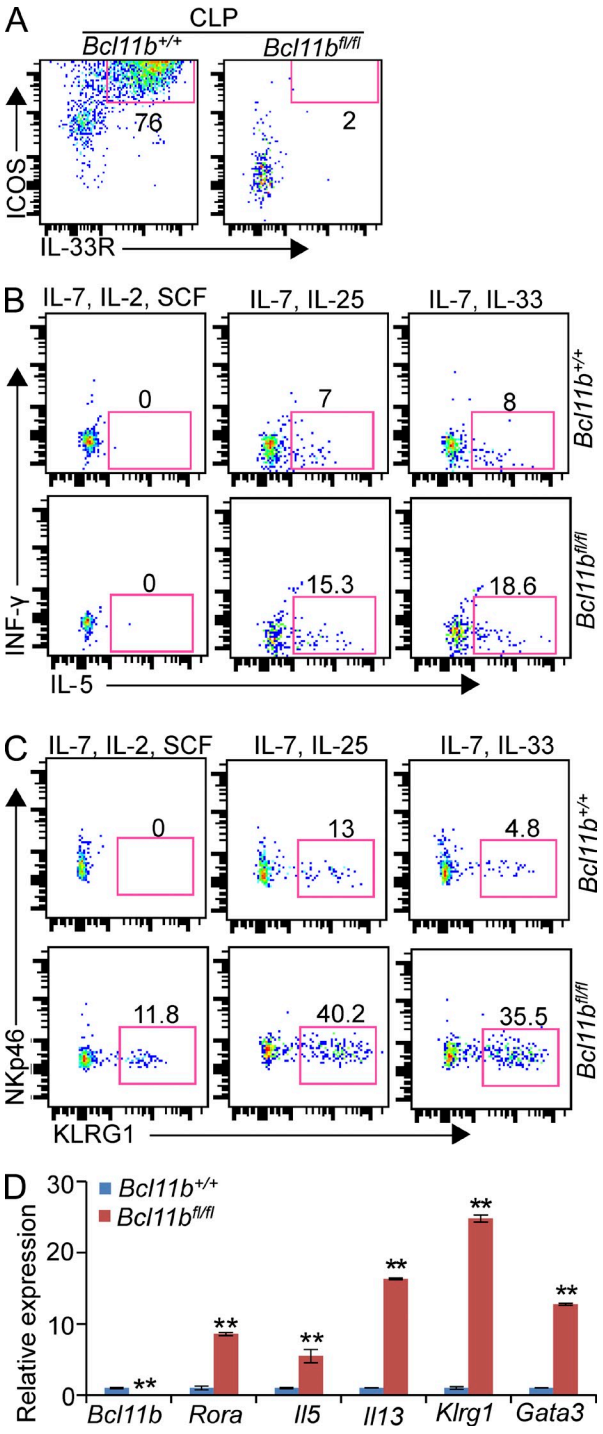

Figure 5. Bcl11b is required for ILC2 development from early progenitors in vitro but represses genes that are highly expressed in mature ILC2s. (A) In vitro differentiation potential of CLPs to ILC2s ($ICOS^+IL\text{-}33R^+$) was assessed by flow cytometry. *Bcl11b*$^{fl/fl}$ and control CLPs were cultured on OP9-DL1 cells in the presence of IL-7 and IL-33 for 22 d before flow cytometric analysis. In each experiment, CLPs from three to four mice of each genotype were pooled. (B and C) Purified BM ILC2s ($Lin^-Flt3^-IL\text{-}7R\alpha^+\alpha4\beta7^+CD27^+CD25^+CD244^-$) were cultured on OP9-DL1 cells in the indicated conditions, and expression of IL-5 and KLRG1 in these cells was assessed by flow cytometry 10–14 d after Tam treatment. (D) Expression of *Bcl11b*, *Rora*, *Il5*, *Il13*, *Klrg1*, and *Gata3* in cultured *Bcl11b*$^{fl/fl}$ ILC2s was assessed by qRT-PCR. Data represent mean values of three independent biological replicates, and all values were normalized to *Gapdh* expression. Expression of genes in the control cells was normalized as one. Error bars indicate the SD. Numbers in flow cytometry plots denote percentages of cells in the indicated areas. All the experiments were independently repeated at least three times. Statistical significance is indicated as follows: **, $P < 0.01$.

In contrast, *Bcl11b*$^{fl/fl}$ LSKs generated no ILC2s in the BM of chimeras (Fig. 4, G and H), and there were barely any detectable ILC2s in the lung or in the siLP (Fig. 4, J and K, and Fig. S5, B and C). On the other hand, ILC1Ps in the BM or LPL $CD4^+$ or $CD4^-$ LTi cells were not affected by *Bcl11b* deletion (Fig. 4 I and Fig. S5, A and C).

Bcl11b deletion promotes expression of genes that are at high levels in mature ILC2s

It was reported that the OP9-DL1 culture system allows CLPs to differentiate to ILC2s (Wong et al., 2012). We purified *Bcl11b*$^{fl/fl}$ CLPs and co-cultured them with OP9-DL1 stromal cells. No ILC2s were produced from *Bcl11b*$^{fl/fl}$ CLPs (Fig. 5 A), which is consistent with the previous in vivo data. Our previous study demonstrates that Bcl11b is essential for T cell identity maintenance (Li et al., 2010b). To investigate the functions of Bcl11b in the committed ILC2s and to test whether Bcl11b is also required for ILC2 identity maintenance, we purified ILC2Ps from *Bcl11b*$^{flox/flox}$*Rosa26*$^{CreERT2/CreERT2}$ conditional knockout mice and cultured them on OP9-DL1 stromal cells to produce ILC2s. We subsequently treated the cultured cells with Tam to delete *Bcl11b*. After IL-25 or IL-33 stimulation, *Bcl11b*$^{fl/fl}$ ILC2s were still able to produce IL-5 but not IFN-γ (Fig. 5 B), demonstrating that committed ILC2s are able to produce type 2 cytokine in the absence of Bcl11b, in contrast to its essential role in the early development of ILC2s. However, deletion of *Bcl11b* led to substantially more ILC2s that expressed KLRG1 (Fig. 5 C), which is consistent with the in vivo data (Fig. 3 B, Fig. 4 D, Fig. S3 A, and Fig. S4 A). Moreover, *Bcl11b* deficiency led to higher percentages of ILC2s producing IL-5 (Fig. 5 B). Therefore, Bcl11b is essential for the differentiation of ILC2s from hematopoietic progenitors but may also suppress genes that are highly expressed in mature ILC2s. Indeed, quantitative RT-PCR (qRT-PCR) confirmed up-regulation of *Il5*, *Il13*, *Klrg1*, *Gata3*, and *Rora*, which are highly expressed in mature ILC2s (Hoyler et al., 2012) in *Bcl11b*$^{fl/fl}$ ILC2 cultures (Fig. 5 D).

Immune challenges confirm no functional ILC2s generated from *Bcl11b*-deficient hematopoietic progenitors

Recent studies suggest a critical role for ILC2s in mediating protease-induced airway inflammation (for example, Halim et al., 2012a). We wished to confirm the essential role of Bcl11b in ILC2 development upon immune challenges. We purified LSKs from *Bcl11b*$^{flox/flox}$*Rosa26*$^{CreERT2/CreERT2}$*Rag1*$^{-/-}$ mice

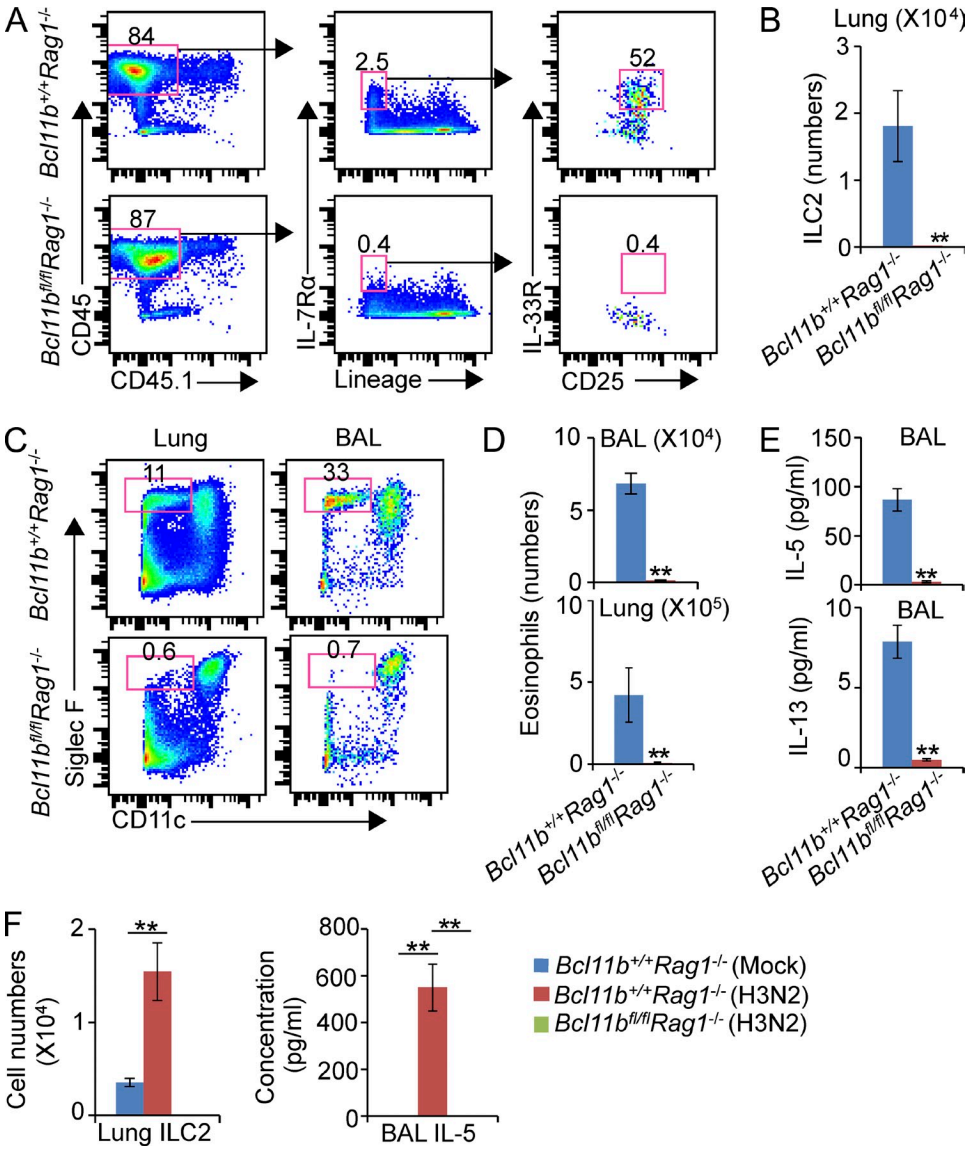

Figure 6. Immune challenges confirm no ILC2 development from *Bcl11b*-deficient hematopoietic progenitors. LSKs of *Bcl11b*$^{fl/fl}$*Rag1*$^{-/-}$ or the control (*Bcl11b*$^{+/+}$*Rag1*$^{-/-}$) were transplanted into *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice recipients (CD45.1^{+}) and subjected to papain treatment (intranasally) or influenza virus infection (inoculated intranasally). (A–E) ILC2-mediated lung immune responses after papain treatment were assessed by flow cytometry. (A and B) Donor ILC2s (CD45.1^{-}Lin^{-}IL-7Rα^{+}IL-33R^{+}CD25^{+}) in the lung were analyzed by flow cytometry and enumerated as shown. (C and D) Eosinophil (Siglec-F^{+}CD11c^{-}) infiltration in the lung and BAL of mouse recipients was enumerated as shown. (E) IL-5 and IL-13 production in the BAL fluid of recipient mice was assessed by flow cytometry. (F) Lung ILC2s or BAL fluid IL-5 in recipients after influenza virus infection (H3N2) or allantoic fluid diluted 1:500 in PBS as the mock was assessed by flow cytometry and was quantitated as shown. (A and C) Numbers in the flow cytometry plots denote percentages of cells in the indicated areas. (A–F) Error bars indicate the SD. All the experiments were independently repeated at least three times (n = 4 mice per genotype). Statistical significance is indicated as follows: **, $P < 0.01$. Additional gating strategies are noted in Fig. S6.

that were treated with Tam (*Bcl11b*$^{fl/fl}$*Rag1*$^{-/-}$) and transplanted them to sublethally irradiated alymphoid *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ recipients (CD45.1^{+}). 8–10 wk after engraftment, the recipients were intranasally challenged with papain, which induces rapid IL-5 production and eosinophil infiltration in the airway (Halim et al., 2012a). Consistent with our above results (Fig. 4 J and Fig. S5 B), no ILC2s were elicited in the lung after papain challenge in *Bcl11b*$^{fl/fl}$*Rag1*$^{-/-}$ chimeras at 84 h (Fig. 6, A and B). Papain induced eosinophil infiltration in the bronchoalveolar lavage (BAL) fluid and in the lung in control recipients, but not in the *Bcl11b*$^{fl/fl}$*Rag1*$^{-/-}$ ones (Fig. 6, C and D). Furthermore, the papain challenge also failed to induce IL-5 or IL-13 production in the BAL fluid in the recipients from *Bcl11b*$^{fl/fl}$*Rag1*$^{-/-}$ LSKs (Fig. 6 E).

Influenza virus infection induces type 2 immune responses and production of prototypical cytokines such as IL-5 by ILC2s (Gorski et al., 2013), which is linked to asthma exacerbation. We thus examined immune responses of BM chimeras

reconstituted with LSKs upon influenza virus infection. Influenza virus infection induced expansion of ILC2s in the lung of the control recipient chimeras at day 5 after infection (Fig. 6 F and Fig. S6). In contrast, chimeras of *Bcl11b*$^{fl/fl}$*Rag1*$^{-/-}$ LSKs completely lacked ILC2s in the lung (Fig. 6 F and Fig. S6). The control recipients, but not *Bcl11b*$^{fl/fl}$*Rag1*$^{-/-}$ LSK chimeras, produced abundant IL-5 in the BAL fluid after influenza virus infection (Fig. 6 F). In summary, both immune challenges confirm the complete lack of ILC2 development potential in the *Bcl11b*-deficient hematopoietic progenitors.

We report here that Bcl11b is specifically expressed in ILCPs that are committed to ILC2s and is essential for ILC2 development. Several transcription factors have been shown to have important functions in ILC2 development. Gata3 is critical for controlling the cell fate of ILC2s (Hoyler et al., 2012; Mjösberg et al., 2012; Klein Wolterink et al., 2013) and also for all IL-7Rα–expressing ILC development (Yagi et al., 2014). Rora appears to be specifically required for inducing ILC2s (Halim et al., 2012b; Wong et al., 2012), but not RORγt-expressing ILCs (Halim et al., 2012b). However, Rora is also expressed in other ILC subsets (see microarray data in Hoyler et al., 2012). Nfil3 is shown to control type 2 Th cytokine expression (Kashiwada et al., 2011), but recent results demonstrate that it is a key regulator of the development of other ILC subsets essential for immune protection in the lung and gut (Geiger et al., 2014; Seillet et al., 2014). Bcl11b is different from these factors. It is the first transcription factor that is found specifically expressed in ILC2s and is required for development of ILC2s from hematopoietic progenitors, a function similar to its role in T cell development. Interestingly, deletion of *Bcl11b* in ILC2s led to higher expression of many ILC2 genes, including several transcription factors mentioned above (Fig. 5 D). This is also reminiscent of Bcl11b's role in T cells, where deleting *Bcl11b* results in up-regulated expression of genes of mature T cells (Kastner et al., 2010). Further dissection of the molecular and cellular mechanisms of Bcl11b in ILC2s and ILC-specific deletion of Bcl11b using the Cre system should facilitate better understanding of ILCs and of Bcl11b's potential role in human disease.

MATERIALS AND METHODS

Mice. The *Bcl11b*-Tdtomato reporter mice (*Bcl11b*$^{Td/Td}$) and *Bcl11b*$^{flox/flox}$ *Rosa26*$^{CreERT2/CreERT2}$ mice were generated on the C57BL/6 genetic background by backcrossing to C57BL/6 mice for 11 generations. The *Bcl11b*$^{flox/flox}$ *Rosa26*$^{CreERT2/CreERT2}$ mice were crossed to *Rag1*$^{-/-}$ mice (C57BL/6) to generate *Bcl11b*$^{flox/flox}$*Rosa26*$^{CreERT2/CreERT2}$*Rag1*$^{-/-}$ mice. *Bcl11b* germline heterozygous mutant (+/−) and the wild-type control mice (+/+) were on the 129S5 background. C57BL/6 CD45.1^{+} wild-type or *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice (C57BL/6 CD45.1; Serafini et al., 2014) were used as recipients for transplantation of BM cells or LSKs. All mice used were from colonies maintained at the research support facility of the Sanger Institute. Housing and breeding of mice and experimental procedures were done according to the UK 1986 Animals (Scientific Procedures) Act and the Animal Welfare and Ethical Review Body of the Wellcome Trust Sanger Institute.

Reagents. Fluorochrome- or biotin-labeled monoclonal antibodies (clones denoted in parentheses) against B220 (RA3-6B2), CD19 (6D5), CD3ε (145-2C11), CD8α (53–6.7), TCRβ (B20.6), CD49b (DX5), TCRγδ (GL3), NK1.1 (PK136), Nkp46 (29A1.4), CD11b (M1/70), CD11c (N418), Gr1 (RB6-8C5), Ter119 (TER-119), c-kit (2B8), Flt3 (A2F10), Sca1 (D7), IL-7Rα (SB/199), CD25 (PC61), CD45.1 (A20), CD45 (30-F11), Siglec-F (E50-2440), CD27 (LG.3A10), CD244 (2B4), α4β7 (DATK32), IL-33R (RWST2-2), CCR6 (29-2L 17), IL-5 (TRFK5), IL-13 (eBio13a), IFN-γ (XMG1.2), Gata3 (L50-823), and RORγt (Q31-378) were purchased from BD, BioLegend, or eBioscience. LEGENDplex Mouse Th2 Panel (6-plex) (BioLegend) was used for BAL cytokine detection.

Flow cytometry and cell sorting. RBCs were removed using ACK (Ammonium-Chloride-Potassium) Lysing Buffer (Lonza). Cells were suspended in a solution of 2% (vol/vol) FBS in PBS. Fc receptors were blocked with anti-CD16 (2.4G2) before antibody labeling. Cells were stained with antibodies on ice for 20 min before washing. Intracellular staining was performed according to the instructions of the FOXP3 Fix/Perm Buffer Set (BioLegend). Cells were analyzed on a Fortessa (BD) or sorted on a MoFlo XDP (Beckman Coulter) according to the manufacturers' standard operating procedures. Data were analyzed with FlowJo software, version X.0.7 (Tree Star).

Preparation of cell suspensions. BM cells were isolated by gently crushing femurs and tibias before filtration (70-µm filter). Cells from lung and siLP were prepared according to the instructions of the Lung Dissociation kit (Miltenyi Biotec) and Lamina Propria Dissociation kit (Miltenyi Biotec), respectively. The tissues were digested in a shaking water bath at 37°C for 30 min. After dissociation, leukocytes were enriched by percoll gradient centrifugation.

Ex vivo production of cytokines from ILC2 cells. ILC2s were stimulated ex vivo with PMA and Ionomycin (Cell Stimulation Cocktail; eBioscience) for 4 h for IL-5 production, in the presence of the protein transport inhibitor cocktail (eBioscience). The cells were subsequently stained intracellularly for IL-5.

Cytokine administration in vivo. Mice were given intraperitoneal injections of 500 ng IL-25 or IL-33 (BioLegend) on days 0–3. On day 4, tissues were collected for analysis.

Adoptive transfers in vivo. For adoptive transfer experiments, cell populations highly purified by flow cytometry were injected intravenously into sublethally irradiated (1 × 450 rad) *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ recipient mice (CD45.1^{+}) via the tail vein. The drinking water was supplemented with antibiotics for 2 wk after irradiation.

BM chimeras. Single-cell suspensions of BM cells from the *Bcl11b*$^{flox/flox}$ *Rosa26*$^{CreERT2/CreERT2}$ and control *Bcl11b*$^{+/+}$*Rosa26*$^{CreERT2/CreERT2}$ mice were injected intravenously into lethally irradiated (2 × 500 rad) C57B6 recipient mice (CD45.1^{+}) for reconstitution for 6–8 wk. The drinking water was supplemented with antibiotics for 2 wk after irradiation.

For hematopoietic progenitor reconstitution in vivo, the LSKs were purified from the BM of *Bcl11b*$^{fl/fl}$, *Bcl11b*$^{fl/fl}$*Rag1*$^{-/-}$, or the *Bcl11b*$^{+/+}$ or *Bcl11b*$^{+/+}$*Rag1*$^{-/-}$ control mice. The sorted LSKs (10,000 cells) were injected into sublethally irradiated (1 × 450 rad) *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ recipient mice (CD45.1^{+}) or with helper CD45.1^{+} BM cells (2 × 10^{5} cells) into lethally irradiated (2 × 500 rad) *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ recipient mice (CD45.1^{+}) via the tail vein.

Papain administration. The mice were anesthetized with 3% isoflurane and then were intranasally administered with papain (10 µg in 40 µl PBS) every 24 h on days 0–2. 12 h after the last challenge, lungs and BAL fluid were collected and analyzed.

Influenza virus infection. The BM chimeras were anesthetized with 3% isoflurane and were inoculated intranasally with influenza A virus X31 (H3N2) in 50 µl PBS of 10^{4} PFU. The virus was grown and collected from embryonated chicken eggs (48–72 h). The control (mock infected) mice were treated intranasally with allantoic fluid diluted 1:500 in PBS. On day 5 after infection, lung and BAL fluid (lavaged with 0.4 ml PBS) were collected.

In vitro culture assays. The ILC2 differentiation potential of hematopoietic progenitors was performed as previously reported (Wong et al., 2012). In brief, freshly sorted CLPs from the *Bcl11b*$^{flox/flox}$*Rosa26*$^{CreERT2/CreERT2}$ and control *Bcl11b*$^{+/+}$ *Rosa26*$^{CreERT2/CreERT2}$ mice that were treated with Tam 4 d earlier (4.0 mg Tam by intraperitoneal injection over three consecutive days) were cultured on OP9-DL1 monolayers in the presence of 10 ng/ml IL-7 (PeproTech) and 10 ng/ml IL-33 (PeproTech) for 22 d.

For the short-term fate assay, purified Bcl11b^{-}ChILPs and Bcl11b^{+}ChILPs were cultured on OP9 monolayers in the presence of 25 ng/ml IL-7 (PeproTech) and 25 ng/ml Stem Cell Factor (SCF; PeproTech) for 6 d as previously described (Constantinides et al., 2014).

For deleting *Bcl11b* in ILC2s, sorted ILC2Ps from the BM of *Bcl11b*^{floxflox}*Rosa26*$^{CreERT2/CreERT2}$ and control mice were cultured on OP9-DL1 monolayers in the presence of 20 ng/ml IL-7, 20 ng/ml SCF, and 10 ng/ml IL-2 or 20 ng/ml IL-7 plus 20 ng/ml IL-25 or plus 20 ng/ml IL-33. After 3–5 d, Tam was added in the medium to induce *Bcl11b* deletion in vitro. Cells were collected and analyzed 10–14 d after Tam treatment.

qRT-PCR. RNA was extracted from in vitro cultured ILC2 and reverse transcribed. TaqMan primer and probe sets (Applied Biosystems) were used for quantification of the expression of *Bcl11b*, *Rora*, *Tcf7*, *Gata3*, *Il-5*, *Il-13*, and *Klrg1*. Expression was quantified relative to that of *Gapdh*.

Statistical analysis. Data were statistically analyzed and figures were prepared using Microsoft Excel. A two-tailed Student's *t* test was used throughout this work to evaluate statistical significance. Significance is indicated as follows: *, $P < 0.05$; **, $P < 0.01$.

Online supplemental material. Fig. S1 contains gating strategies of ILCs. Fig. S2 shows the gating strategies of ILCPs. Fig. S3 presents the gating strategies of ILC2s in the BM and lung of wild-type and Bcl11b heterozygous mutant mice. Fig. S4 contains gating strategies of ILCs in the *Bcl11b*$^{flox/flox}$*Rosa26*$^{CreERT2/CreERT2}$ mice, control mice, or in BM chimeras. Fig. S5 shows gating strategies of donor LSK-derived ILCs in chimeras. Fig. S6 presents gating strategies of ILC2s in the lung of recipients engrafted with the control or *Bcl11b*$^{fl/fl}$*Rag1*$^{-/-}$ LSKs after influenza virus (H3N2) infection. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20142318/DC1.

We thank Paul Kellam's group at the Sanger Institute for producing virus X31 (H3N2). We would also like to thank the Sanger Institute research support facility (Andrea Kirton, Georgina Owen, Paul Green, Claire Rogerson, Matthew Brealey, George Notley, and Scott Kemp) and the flow cytometry core facility (Bee-Ling Ng and Jennifer Graham) for technical assistance.

This work is supported by a grant from the Wellcome Trust (098051) to P. Liu and by grants from the National Natural Science Foundation of China (grants 31370904 and 30972691), Shanghai Science and Technology Development Fund (grant 10QA1405900), Training Program of the Excellent Young Talents of the Shanghai Municipal Health System (grant XYQ20110115), and Shanghai Municipal Science and Technology Foundation (grant 11JC1410802) to L. Lu.

The authors declare no competing financial interests.

Submitted: 12 December 2014
Accepted: 31 March 2015

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