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ARTICLE

Bcl11b, a novel GATA3-interacting protein, suppresses Th1 while limiting Th2 cell differentiation

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GATA-binding protein 3 (GATA3) acts as the master transcription factor for type 2 T helper (Th2) cell differentiation and function. However, it is still elusive how GATA3 function is precisely regulated in Th2 cells. Here, we show that the transcription factor B cell lymphoma 11b (Bcl11b), a previously unknown component of GATA3 transcriptional complex, is involved in GATA3-mediated gene regulation. Bcl11b binds to GATA3 through protein–protein interaction, and they colocalize at many important cis-regulatory elements in Th2 cells. The expression of type 2 cytokines, including IL-4, IL-5, and IL-13, is up-regulated in *Bcl11b*-deficient Th2 cells both in vitro and in vivo; such up-regulation is completely GATA3 dependent. Genome-wide analyses of Bcl11b- and GATA3-regulated genes (from RNA sequencing), cobinding patterns (from chromatin immunoprecipitation sequencing), and Bcl11b-modulated epigenetic modification and gene accessibility suggest that GATA3/Bcl11b complex is involved in limiting Th2 gene expression, as well as in inhibiting non-Th2 gene expression. Thus, Bcl11b controls both GATA3-mediated gene activation and repression in Th2 cells.

Introduction

CD4 T helper (Th) cells play a central role during a variety of adaptive immune responses (Zhu et al., 2010). Upon activation through TCR, naive CD4 T cells can differentiate into distinct Th effector lineages, including type 1 Th (Th1), Th2, and IL-17–expressing Th (Th17) cells. Through signature cytokine production by these Th effectors (i.e., IFN-γ by Th1, IL-4 by Th2, and IL-17 by Th17), they play indispensable roles in protective immunity to various microorganisms. On the other hand, inappropriate activation of Th cells by self-antigens or harmless environmental agents may cause organ-specific autoimmune or allergic inflammatory diseases. In particular, Th2 cells play a major role in asthma, a common allergic disease (Lambrecht and Hammad, 2012). Th2 cells are capable of producing hallmark type 2 cytokines, including IL-4, IL-5, and IL-13. IL-5, together with eotaxin, induces the release of eosinophils from the bone marrow and recruits them into the lung tissue (Wills-Karp, 1999; Paul and Zhu, 2010). IL-4 can induce immunoglobulin class-switching to IgE in B cells, whereas IL-13 causes airway hypersensitivity (Wynn, 2015).

Lineage-specific transcription factors are responsible for the differentiation of specific Th effector cells (Zhu and Paul, 2010). The master transcription factors for each lineage are T-bet (Th1), GATA3 (Th2), RORγt (Th17), and Foxp3 (regulatory T [T reg] cell). During Th2 cell differentiation, GATA3 expression is up-regulated by TCR stimulation and IL-4-STAT6 signaling (Paul and Zhu, 2010). Continuous GATA3 expression is critical for naive CD4 T cells to differentiate into Th2 cells with the acquisition of Th2 cytokine-producing capacity. However, GATA3 is no longer required for IL-4 expression in differentiated Th2 cells, but is still indispensable for IL-5 and IL-13 production (Pai et al., 2004; Zhu et al., 2004; Yagi et al., 2011).

GATA3 protein level must be tightly regulated to an appropriate level; too much or too little GATA3 expression could suppress T cell survival and/or proliferation (Yui and Rothenberg, 2014). Furthermore, precise control of GATA3 transcription activity is critical for effectively expelling extracellular parasites and preventing allergic disorders. During T cell differentiation, the master transcription factor of one lineage also restricts the

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functions or expression of other lineage-specific genes. For example, T-bet represses GATA3 expression as well as GATA3 binding to the *Il5* promoter in Th1 cells (Hwang et al., 2005; Wei et al., 2011; Kanhere et al., 2012; Lazarevic et al., 2013). However, how lineage-nonspecific transcription factors regulate the expression and functions of the Th2 master regulator GATA3 is not clear.

Bcl11b, a zinc finger transcription factor expressed by all T cells starting from CD4/CD8 double-negative stage 2, is essential for T cell development (Li et al., 2010a,b). Although GATA3 also plays a critical role at multiple stages during T cell development, its expression is restrained by Bcl11b at the double-negative stage (Yui and Rothenberg, 2014). In mature T cells, Bcl11b further regulates T cell differentiation. CD4-Cre–mediated *Bcl11b* deletion leads to spontaneous inflammatory bowel disease (IBD) with increased number of IFN-γ and IL-17–producing cells, which is possibly caused by T reg cell dysfunction and/or a development-related defect (Vanvalkenburgh et al., 2011). In a mouse experimental autoimmune encephalomyelitis (EAE) model, dLck-Cre *Bcl11b*-deficient Th17 cells up-regulate GATA3 protein, and Bcl11b binds to the *Gata3* promoter (Califano et al., 2014). Recent studies suggest that Bcl11b also regulates the development of type 2 innate lymphoid cells (ILC2s) and is essential for maintaining ILC2 functions (Califano et al., 2015; Walker et al., 2015; Yu et al., 2015).

Both GATA3 and Bcl11b play critical roles in the development of T cells and ILC2s. Previous studies indicate that Bcl11b may regulate GATA3 expression either positively or negatively (Califano et al., 2014, 2015; Yu et al., 2015). In addition, it has been shown that GATA3 and Bcl11b are in the Foxp3-containing complex in T reg cells (Rudra et al., 2012). Our unpublished data also indicate that Bcl11b is part of the GATA3-containing complex in T reg cells. Despite their shared functions in many cell types at various developmental stages, the physical and functional relationship between GATA3 and Bcl11b is elusive. Furthermore, it is not known whether Bcl11b modulates GATA3 expression and functions in Th2 cells and whether Bcl11b is involved in Th2 cell differentiation and maintenance.

Here, we show that Bcl11b interacts with GATA3, and they cobind to common cis-regulatory elements of several important lineage-specific genes in Th2 cells. RNA-sequencing (RNA-seq) data suggest that Bcl11b and GATA3 cooperatively regulate the expression of both Th2-specific and Th1-associated genes. Bcl11b limits GATA3-mediated Th2 cytokine IL-4, IL-5, and IL-13 production both in vitro and in vivo. Both GATA3 and Bcl11b are required for suppressing many Th1-associated genes. Genome-wide changes in acetylation of histone H3 at Lys27 (H3K27ac) and DNase I hypersensitive sites (DHSs) upon *Bcl11b* removal suggest a critical role of this transcription factor in regulating epigenetic modifications. Strikingly, genes with epigenetic changes upon *Bcl11b* deletion are enriched for the genes to which Bcl11b and GATA3 cobind and with overlap peaks. Genome-wide analyses of gene regulation, epigenetic regulation, and DNA binding by these two transcription factors reveal an important relationship between Bcl11b and GATA3 in limiting Th2 responses while suppressing genes associated with alternative cell fates.

Results

Colocalization of Bcl11b and GATA3 binding in the genome

To better understand the Bcl11b- and GATA3-mediated transcriptional regulatory network, we applied chromatin immunoprecipitation (ChIP; with anti-Bcl11b and anti-GATA3 antibodies) followed by high-throughput sequencing (ChIP-seq) to genome-wide map Bcl11b and GATA3 binding to cis-regulatory elements of their direct target genes in Th2 cells. Interestingly, the genomic binding pattern of Bcl11b and GATA3 indicated a substantial overlap of their binding peaks (Fig. 1 a). For example, multiple GATA3-binding peaks that were identified within the Th2 cytokine gene loci containing *Il4* and *Il13* genes overlapped with Bcl11b-binding peaks (Fig. 1 b). At the genome level, we found 14,306 Bcl11b-binding peaks, among which 17% (2,434 peaks) or 32.8% (4,698) also contained GATA3-binding peaks in the vicinity of 30 or 150 bp, respectively (Fig. 1 c, left). Similarly, among the 25,704 GATA3-binding peaks, 10.5% (2,435 peaks) or 19.3% (4,963 peaks) contained Bcl11b-binding peaks in the vicinity of 30 or 150 bp, respectively (Fig. 1 c, right). After assigning all the GATA3- and/or Bcl11b-binding peaks to genes, we found that majority of the genes that were bound by GATA3 were also bound by Bcl11b (5,166 of 9,081). Similarly, GATA3 bound to most of the genes that Bcl11b bound (5,166 of 7,776). Strikingly, 25.2% of all the genes to which either GATA3 or Bcl11b could bind (2,950 of 11,691) displayed at least one cobinding of GATA3 and Bcl11b peak within 150 bp (Fig. 1 d). Frequent cobinding of Bcl11b and GATA3 at the whole genome indicates that these two transcription factors may function together to regulate gene expression. Consistent with the ChIP-seq data, GATA3 was detected in the Bcl11b immune-precipitation complex (Fig. 1 e). All these results raised the possibility that Bcl11b, as a component of the GATA3-containing transcriptional complex, could be involved in GATA3-mediated gene regulation in Th2 cells.

Bcl11b represses GATA3-mediated expression of Th2 cytokines in vitro

Because Bcl11b is involved in T cell development at multiple stages, to assess the function of Bcl11b in Th2 cell differentiation, we need to have a tool to delete *Bcl11b* in mature CD4 T cells to avoid any developmental defect. To this end, we crossed *Bcl11b* floxed mice (Li et al., 2010b) with CreER^{T2} mice to obtain *Bcl11b*$^{fl/fl}$-CreER^{T2} conditional KO mice so that *Bcl11b* can be deleted only after tamoxifen (TMX) treatment. Purified naive CD4 T cells from *Bcl11b*$^{fl/fl}$-CreER^{T2} mouse were cultured under Th1, Th2, or Th17 conditions. 4-Hydroxytamoxifen (4-OHT) was added at the beginning of the culture to delete *Bcl11b* during T cell differentiation. Bcl11b was highly expressed by Th1, Th2, and Th17 cells (Fig. S1 a). Deletion efficiency in Th2 cells was confirmed by flow cytometry and Western blot (Fig. S1, b and c). Unlike the essential role of Bcl11b in ILC2 development (Walker et al., 2015; Yu et al., 2015), *Bcl11b*-deficient Th2 cells produced more Th2 cytokines, including IL-4 and IL-5, compared with untreated WT Th2 cells (Fig. 2 a). However, *Bcl11b*-deficient Th1 and Th17 cells had reduced IFN-γ and IL-17A production, respectively (Fig. 2 a). These results suggested that Bcl11b is a negative regulator for Th2 cell differentiation. To further investigate Bcl11b function in mature Th2 cells, *Bcl11b*$^{fl/fl}$-CreER^{T2} Th2 cells that had not been

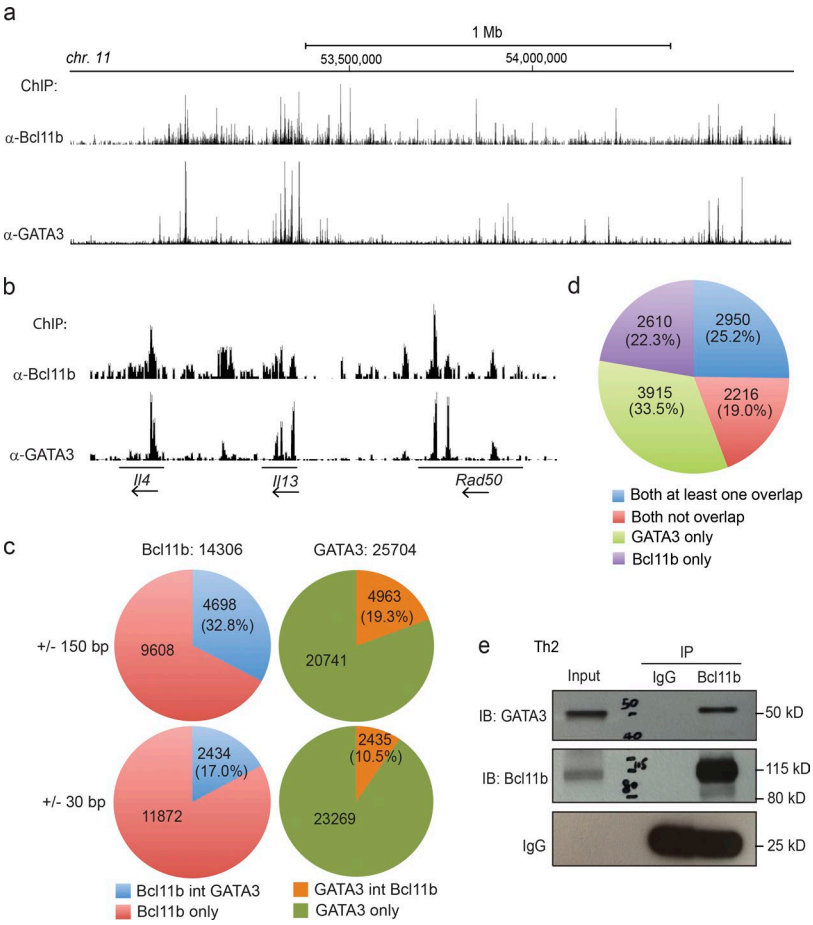

Figure 1. **Bcl11b and GATA3 interact with each other and colocalize to common cis-regulatory elements in Th2 cells. (a–e)** Naive CD4 T cells were purified and cultured under Th2 conditions in vitro. **(a–d)** ChIP-seq analysis was performed using anti-Bcl11b and anti-GATA3 antibodies. **(a)** UCSC genome browser view of Bcl11b and GATA3 binding to a segment of chromosome 11 is shown. **(b)** UCSC genome browser view of Bcl11b and GATA3 binding to the Th2 cytokine loci including genes *Il4*, *Il13*, and *Rad50* is shown. **(c)** Overlap of GATA3-binding peaks within Bcl11b-binding peaks, and of Bcl11b-binding peaks within GATA3-binding peaks that were defined as 150 or 30 bp upstream/downstream. **(d)** Pie graph of the distribution of the Bcl11b- and GATA3-binding genes in Th2 cells. Four populations of the genes, bound by both Bcl11b and GATA3 with at least one overlap peak (blue), bound by both Bcl11b and GATA3 without overlap peaks (red), bound by GATA3 only (green), and bound by Bcl11b only (purple), were identified. **(e)** Th2 cell lysate was immune-precipitated with anti-Bcl11b antibody, and coprecipitated proteins were analyzed by immune blotting (IB) with indicated antibodies. Data are representative of one (a–d) and two (e) independent experiments.

treated with 4-OHT during the initial culture were further stimulated under Th2 conditions (late Th2) with or without 4-OHT for another 4 d. As shown in Fig. 2 b, the percentages of IL-4, IL-5, and IL-13 single-positive cells, as well as IL-4/IL-5, IL-4/IL-13, and IL-5/IL-13 double-positive cells, were all increased when *Bcl11b* was deleted in already differentiated Th2 cells. Thus, the transcription factor Bcl11b is a negative regulator for Th2 cytokine production in vitro.

Bcl11b represses GATA3 expression and function during Th2 cell differentiation

Because GATA3 is the key transcription factor for Th2 cell differentiation, we assessed the impact of *Bcl11b* deletion on GATA3 expression. Deleting *Bcl11b* from the beginning of Th2 cell differentiation (early Th2) resulted in increased GATA3 expression, whereas Bcl11b did not affect GATA3 expression in Th1 or Th17 cells (Fig. 3 a and Fig. S1 d). Because GATA3 regulates its own expression during Th2 cell differentiation (Ouyang et al., 2000), increased GATA3 expression can be explained by enhanced GATA3 autoactivation in the absence of Bcl11b. Consistent with this notion, ChIP-seq data showed that both GATA3 and Bcl11b bound to the *Gata3* gene locus, including the NK and T cell enhancer, 280 kb downstream of the *Gata3* gene, which is important for *Gata3* gene expression in lymphocytes (Hosoya-Ohmura et al., 2011; Fig. 3 b). However, GATA3 expression was similar between WT and *Bcl11b* KO Th2 cells when *Bcl11b* was deleted in mature Th2 cells (Fig. 3 c). These data suggest that although Bcl11b can regulate GATA3 expression, Bcl11b also regulates the function of GATA3. To test whether Bcl11b's function in Th2 cells is GATA3 dependent, we generated *Bcl11b*$^{fl/fl}$-*Gata3*$^{fl/fl}$-CreER^{T2} mice to delete both *Bcl11b* and *Gata3*. *Bcl11b/Gata3* double-KO Th17 and Th1 cells had reduction in IL-17 and IFN-γ expression similar to that of *Bcl11b* KO cells (Fig. S1, e–g). However, we found that the expression of IL-4, IL-5, and IL-13 was no longer increased in *Bcl11b/Gata3* double-KO cells in both early and late Th2 cells (Fig. 3, d and e; and Fig. S1, h and i), suggesting that Bcl11b represses Th2 cytokine production through limiting GATA3 function.

Bcl11b promotes CD4 T effector cell proliferation

Th cell differentiation and effector cytokine production are regulated by epigenetic changes associated with cell division (Bird et al., 1998). CFSE dilution was impaired when *Bcl11b* was deleted under all culture conditions (Fig. S1 j). Ki-67 intracellular staining results also suggested that *Bcl11b* KO Th2 cells had a modest defect in cell proliferation (Fig. 4 a and Fig. S1 k). To better understand the relationship between changes in Th2 cytokine production and cell proliferation caused by *Bcl11b* deficiency, we measured IL-4 production by cells with different division history. In WT Th2 cells, the frequency of IL-4 production increased with cell division, and there was very little IL-4 production in cells that had divided fewer than three times (Fig. 4, b and c). However, *Bcl11b* deficiency resulted in enhanced IL-4 production compared with WT cells in each cell division, although the proliferation

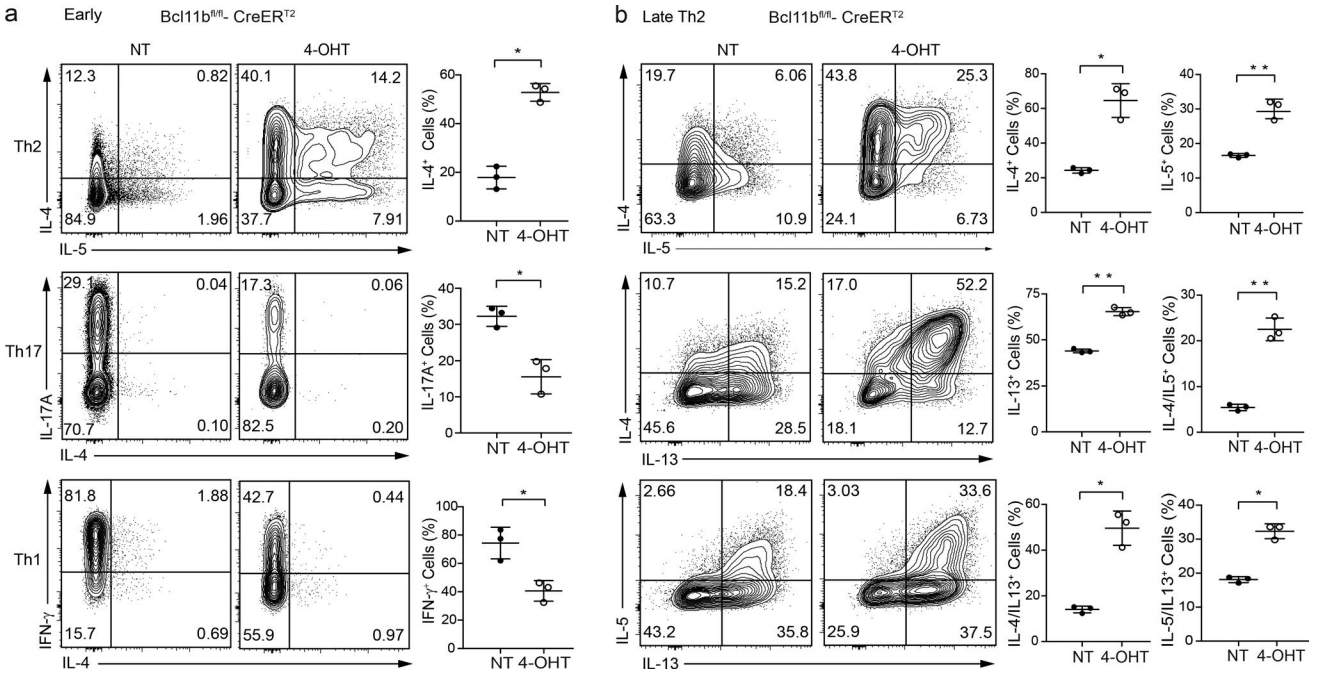

Figure 2. **Bcl11b negatively regulates the production of Th2 cytokines in vitro. (a)** Naive CD4 T cells were isolated from *Bcl11b*$^{fl/fl}$-CreER^{T2} mice and cultured under Th1, Th2, or Th17 conditions with or without 4-OHT for 4 d (early). Primed cells were restimulated with PMA and ionomycin for 4 h. Cytokine production was assessed by intracellular staining. **(b)** Naive CD4 T cells were isolated from *Bcl11b*$^{fl/fl}$-CreER^{T2} mice and cultured under Th2 conditions for 4 d. Then, after resting in IL-2–containing medium for 1 d, the primed cells were further cultured under Th2 conditions (late Th2) with or without 4-OHT for 4 d. Cytokine production was assessed by intracellular staining after restimulation with PMA and ionomycin. Data are representative of three independent experiments. *, P < 0.05; **, P < 0.01. Error bars represent mean ± SD.

index was reduced for the *Bcl11b* KO group (Fig. 4, b and c). Strikingly, even the *Bcl11b*-deficient cells that had divided fewer than three times were able to produce more IL-4 than the WT cells that had divided six times (Fig. 4 c).

To address the molecular mechanism of cell cycle–related defects in *Bcl11b* KO cells, we compared the expression of cell cycle–related genes between WT and *Bcl11b* KO Th2 cells using RNA-seq. We found that 26 of 309 cell cycle–related genes were dysregulated in both early and late *Bcl11b*-deficient Th2 cells (Fig. 4 d and Table S1). Based on the notion that Bcl11b mainly serves as a transcriptional repressor, many cell cycle–related genes that are increased in *Bcl11b* KO cells may be direct targets of Bcl11b, whereas reduced expression of genes in *Bcl11b* KO cells may be an indirect effect caused by cell cycle arrest. Indeed, all four genes that were negatively regulated by Bcl11b were bound by Bcl11b; however, among the 22 genes that appeared to be positively regulated by Bcl11b, only four were bound by Bcl11b (Fig. 4 d and Table S1). Cell cycle inhibitor gene *Cdkn1a*, which codes cyclin-dependent kinase inhibitor p21, was up-regulated in *Bcl11b* KO cells (Fig. 4 d). Aberrant p21 expression at the protein level in *Bcl11b* KO Th2 cells was confirmed by intracellular staining (Fig. 4 e). Strikingly, even among the cells with the same number of divisions, the whole population of *Bcl11b* KO Th2 cells expressed higher levels of p21 compared with WT Th2 cells (Fig. 4 e, bottom). Thus, transcription factor Bcl11b may positively regulate Th cell proliferation by directly repressing cell cycle inhibitor genes and indirectly affecting some cell cycle–promoting genes. However, the repression of Th2 cytokine production by Bcl11b seems to be independent of its function in regulating cell proliferation.

Genome-wide analysis of Bcl11b- and GATA3-mediated gene regulation

The data above suggest that Bcl11b inhibits Th2 cytokine expression and promotes effector T cell proliferation. To systematically investigate Bcl11b- and GATA3-mediated transcriptional gene regulation, we applied RNA-seq analysis with early and late Th2 cells derived from either *Bcl11b*$^{fl/fl}$-CreER^{T2} or *Gata3*$^{fl/fl}$-CreER^{T2} mice (Table S1). Overall, *Bcl11b* deficiency in early Th2 cells resulted in expression changes in more genes than *Bcl11b* deficiency in late Th2 cells (Fig. S2, a and b). The result suggests that Bcl11b may regulate many aspects of Th2 cell differentiation. We then focused on the genes that were regulated by Bcl11b in both early and late Th2 cells (73 genes positively regulated, and 145 genes negatively regulated, by Bcl11b). Gene ontology (GO) cellular component and biological process analysis with PANTHER overrepresentation test indicated that the 73 genes positively regulated by Bcl11b were highly enriched for the genes involved in the cell cycle process (Fig. S2 c). On the other hand, genes negatively regulated by Bcl11b contained many genes encoding extracellular components and proteins involved in the T cell secretion process (Fig. S2 d).

Because enhanced Th2 cytokine production resulting from *Bcl11b* deficiency depends on GATA3 (Fig. 3, d and e; and Fig. S1, e and f), to understand the Bcl11b- and GATA3-mediated transcriptional regulatory network at the global level, we further analyzed and compared the genes that were regulated by either Bcl11b or GATA3 in late Th2 cells. Comparison of four groups of genes—negatively regulated by Bcl11b (B-N), positively regulated by Bcl11b (B-P), negatively regulated by GATA3 (G-N),

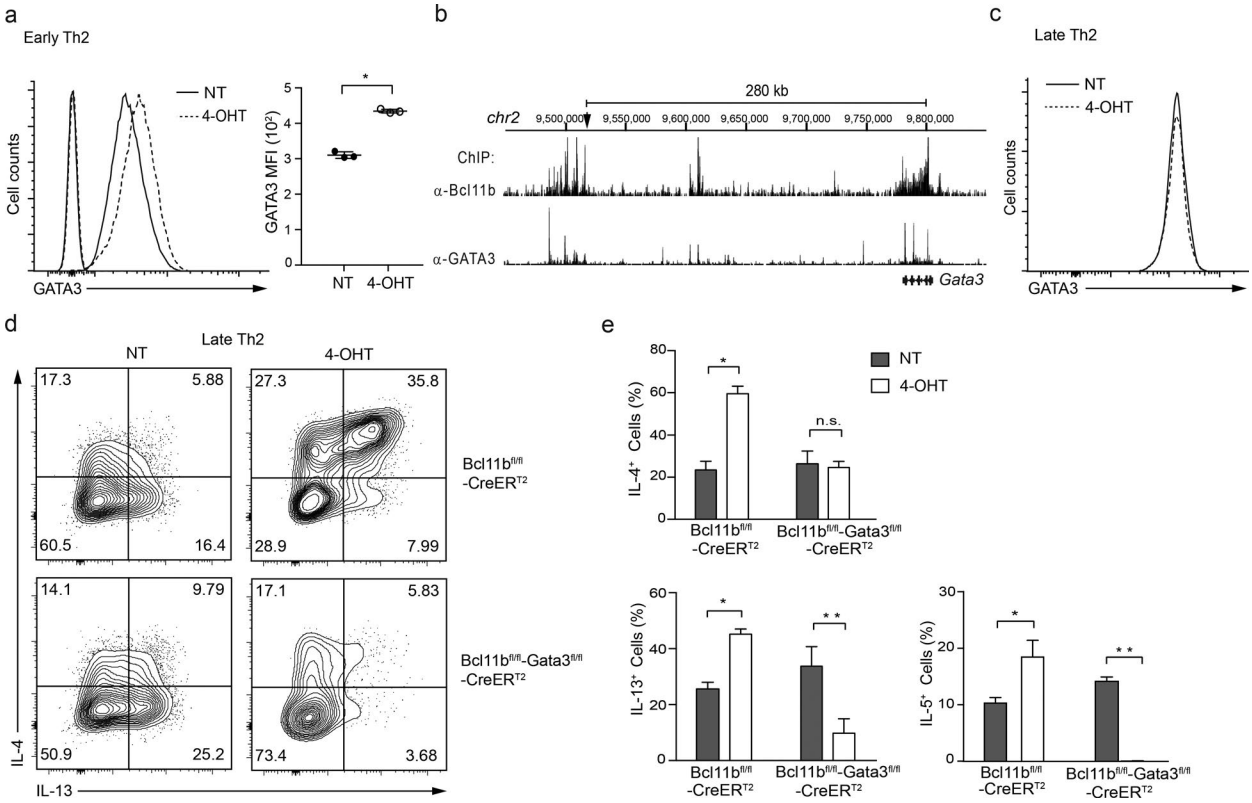

Figure 3. **Bcl11b inhibits in vitro Th2 response through modulating GATA3 expression and functions. (a)** Early Th2 cells prepared as described in Fig. 2 a. Cells were stained for intracellular GATA3 expression. Left peaks are fluorescence minus one (FMO) control. **(b)** UCSC Genome Browser view of Bcl11b and GATA3 binding to the *Gata3* gene locus including the NK and T cell enhancer (280 kb downstream of the *Gata3* gene body) in Th2 cells. **(c)** Late Th2 cells were prepared as in Fig. 2 b. Cells were stained for intracellular GATA3 expression. **(d and e)** Late Th2 cells from *Bcl11b*$^{fl/fl}$-CreER^{T2} or *Bcl11b*$^{fl/fl}$-*Gata3*$^{fl/fl}$-CreER^{T2} mice were restimulated with PMA and ionomycin for 4 h. IL-4, IL-5, and IL-13 expression were assessed by intracellular staining. Data are representative of one (b) and two (a and c–e) independent experiments. *, P < 0.05; **, P < 0.01; n.s., not significant. Error bars represent mean ± SD.

and positively regulated by GATA3 (G-P)—revealed 55 B-N/G-N genes and 25 B-N/G-P genes (Fig. 5 a). However, there was much less overlap between B-P and GATA3-regulated genes, suggesting that Bcl11b mainly plays an important role in negatively regulating GATA3-mediated gene regulation. This is consistent with the function of Bcl11b as a transcriptional repressor (Cismasiu et al., 2005). Furthermore, a substantial fraction of the genes that were coregulated by Bcl11b and GATA3 were cobound by these two factors with at least one overlapped peak (Fig. 5 b and Fig. S2 e). Interestingly, the genes that were cobound by Bcl11b and GATA3 but without overlap peaks or those that were bound by Bcl11b or GATA3 only did not seem to enrich for the coregulated genes (Fig. 5 b and Fig. S2 e). These results strongly suggest that GATA3 and Bcl11b cooperatively regulate gene expression at the genome level through formation of a transcriptional repressive complex and possibly an activation complex, with Bcl11b serving as a negative regulator of complex activity.

Bcl11b and GATA3 coinhibit Th1 gene expression

GATA3 not only promotes Th2 cell differentiation but also inhibits the expression of many Th1-associated genes (Yagi et al., 2010, 2011; Wei et al., 2011). Because Bcl11b also negatively regulates the genes that are negatively regulated by GATA3, we assessed the cooperative effect of Bcl11b and GATA3 on inhibiting the expression of Th1-associated genes. We compared the 55 genes that were negatively regulated by GATA3 and Bcl11b with the 91 Th1-associated genes previously reported (Wei et al., 2011). Strikingly, 10 of these 55 genes turned out to be Th1 associated (Fig. 5 c). We confirmed the expression change for 8 out of these 10 genes by quantitative RT-PCR (qRT-PCR; Fig. 5 d). Among these 8 confirmed genes, *Havcr2*, also known as *Tim3*, was identified as specifically induced during naive CD4 T cells differentiating into Th1 cells (Monney et al., 2002); *Ccl5* plays an important role in the recruitment of Th1 cells (Luther and Cyster, 2001); and *Anxa1* was reported to promote the differentiation of CD4 T cells into Th1 cells and negatively regulate Th2 cell differentiation (Kurata et al., 1999; D'Acquisto et al., 2007). Up-regulation of Th1-associated gene *Havcr2* and *Ccl5* in *Bcl11b* KO Th2 cells was confirmed at the protein level through either cell surface staining (for Tim3, Fig. 5 e) or ELISA (for CCL5, Fig. 5 f). Thus, despite an increased production of Th2 cytokines, *Bcl11b*-deficient Th2 cells also aberrantly express some Th1-associated genes.

Bcl11b negatively regulates CD69 expression

Among the 25 genes that were positively regulated by GATA3 but negatively regulated by Bcl11b, we identified another interesting gene, *Cd69*, in addition to *Il5* and *Il13* (Fig. 5 a and Table S1). CD69 is regarded as an early T cell activation maker and is associated with lymphocyte tissue residency (Dudakov et al., 2014). Furthermore, CD69 plays an important role in Th2 cell differentiation

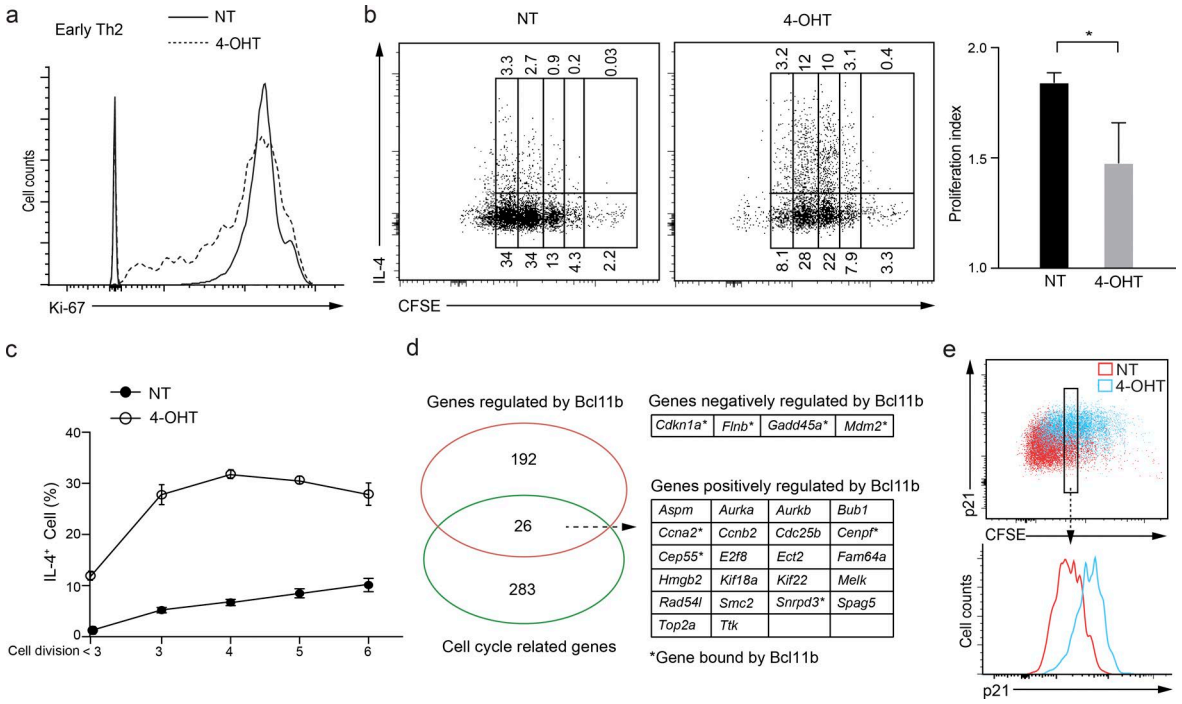

Figure 4. **Bcl11b is important for optimal Th cell proliferation. (a)** The expression of Ki-67 in early Th2 cells was measured by intracellular staining. Left peaks are the FMO controls. **(b and c)** Naive CD4 T cells purified from *Bcl11b*$^{fl/fl}$-CreER^{T2} mice were labeled with CFSE and cultured under Th2 conditions in vitro with or without 4-OHT. After 3 d, cells were restimulated with PMA and ionomycin. IL-4 expression was assessed by intracellular staining. Proliferation index was calculated using FlowJo software. The percentage of IL-4–expressing cells was calculated in cell populations that underwent different cycles of division based on CFSE dilution. **(d)** RNA-seq analysis was performed with the WT and *Bcl11b* KO early and late Th2 cells. Cell cycle–related genes regulated by Bcl11b are shown. The total cell cycle gene list (Table S1) is from Qiagen. **(e)** Early Th2 cells were processed as in b. The expression of p21 and CFSE dilution were assessed. Red, WT Th2 cells; blue, *Bcl11b* KO Th2 cells. Data are representative of three (a) and two (b, c, and e) independent experiments. *, P < 0.05. Error bars represent mean ± SD.

and migration in airway inflammation (Miki-Hosokawa et al., 2009; Kimura et al., 2017). Cell surface staining confirmed that CD69 was significantly up-regulated in *Bcl11b*-deficient Th2 cells (Fig. S3 a). Such up-regulation is completely GATA3 dependent because CD69 was no longer up-regulated in *Bcl11b*/*Gata3* double-deficient Th2 cells (Fig. S3 b). We also treated *Bcl11b*$^{fl/fl}$, *Bcl11b*$^{fl/fl}$-CreER^{T2}, and *Bcl11b*$^{fl/fl}$-*Gata3*$^{fl/fl}$-CreER^{T2} mice with TMX in vivo. CD69 expression in splenic CD4^{+}CD44^{high} cells was dramatically induced upon *Bcl11b* deletion, whereas *Bcl11b*/*Gata3* double deletion did not result in such induction (Fig. S3 c). Consistently, *Cd69* gene was cobound by transcription factor Bcl11b and GATA3 (Table S1 and Fig. S3 d). Additionally, in vitro–generated *Bcl11b* KO Th2 cells were more likely to migrate/retain in lung than similarly cultured WT Th2 cells upon transfer, and this tendency could be reversed by knocking down *Cd69* (Fig. S3 e). Altogether, our data indicate that Bcl11b inhibits GATA3-mediated CD69 induction, an important process for Th2 responses in vivo, in Th2 cells.

Bcl11b regulates epigenetic modifications in Th2 cells

Histone H3K27ac modification is a good indication for active chromatins in the genome and predicts developmental state (Creyghton et al., 2010; Calo and Wysocka, 2013). Interestingly, Bcl11b-binding sites often colocalize with H3K27ac peaks in Th2 cells. To address whether Bcl11b regulates H3K27ac at its target gene loci, we performed anti-H3K27ac ChIP-seq analysis with *Bcl11b* WT and KO Th2 cells (Fig. 6, Fig. S4, and Table S1). H3K27ac modification at the Th1-associated genes (*Havcr2* and *Ccl5*) that are negatively regulated by Bcl11b was increased in both early and late *Bcl11b* KO Th2 cells (Fig. 6, a and b). More importantly, regions with increased H3K27ac modification colocalized with the Bcl11b and GATA3 cobinding sites (Fig. 6, a and b). For Th2-associated genes (*Il4/Il13*, *Il5*, and *Gata3*), a modest increase of H3K27ac modification was also observed in early *Bcl11b* KO Th2 cells, although intronic enhancer HS2 region in the *Il4* gene and CGRE in the *Il13* gene (Yamashita et al., 2002; Tanaka et al., 2011) were not affected (Fig. 6, c and d; and Fig. S4 a). However, no obvious increase in H3K27ac was observed when *Bcl11b* was deleted in late Th2 cells, presumably because H3H27ac at these loci had already reached a maximum level in WT Th2 cells (Fig. S4 b). We further analyzed the correlation between Bcl11b/GATA3 binding and alterations in H3K27ac and found that H3K27ac modifications at the Bcl11b and GATA3 cobinding genes were more likely to be affected by *Bcl11b* deletion in both early and late Th2 cells (Fig. 6 e and Fig. S4 c). Analysis of DHSs is also widely used to identify accessible chromatin regions, which helps understand transcription factor–mediated regulation of the expression of lineage-specific genes (Loots et al., 2000; Boyle et al., 2008; Thurman et al., 2012). Thus, we further performed DHS analysis on WT and *Bcl11b* KO Th2 cells. Globally, increased chromatin accessibility was well correlated with increased H3K27ac modification in *Bcl11b*-deficient Th2 cells (Fig. 6, f–h; and Fig. S4,

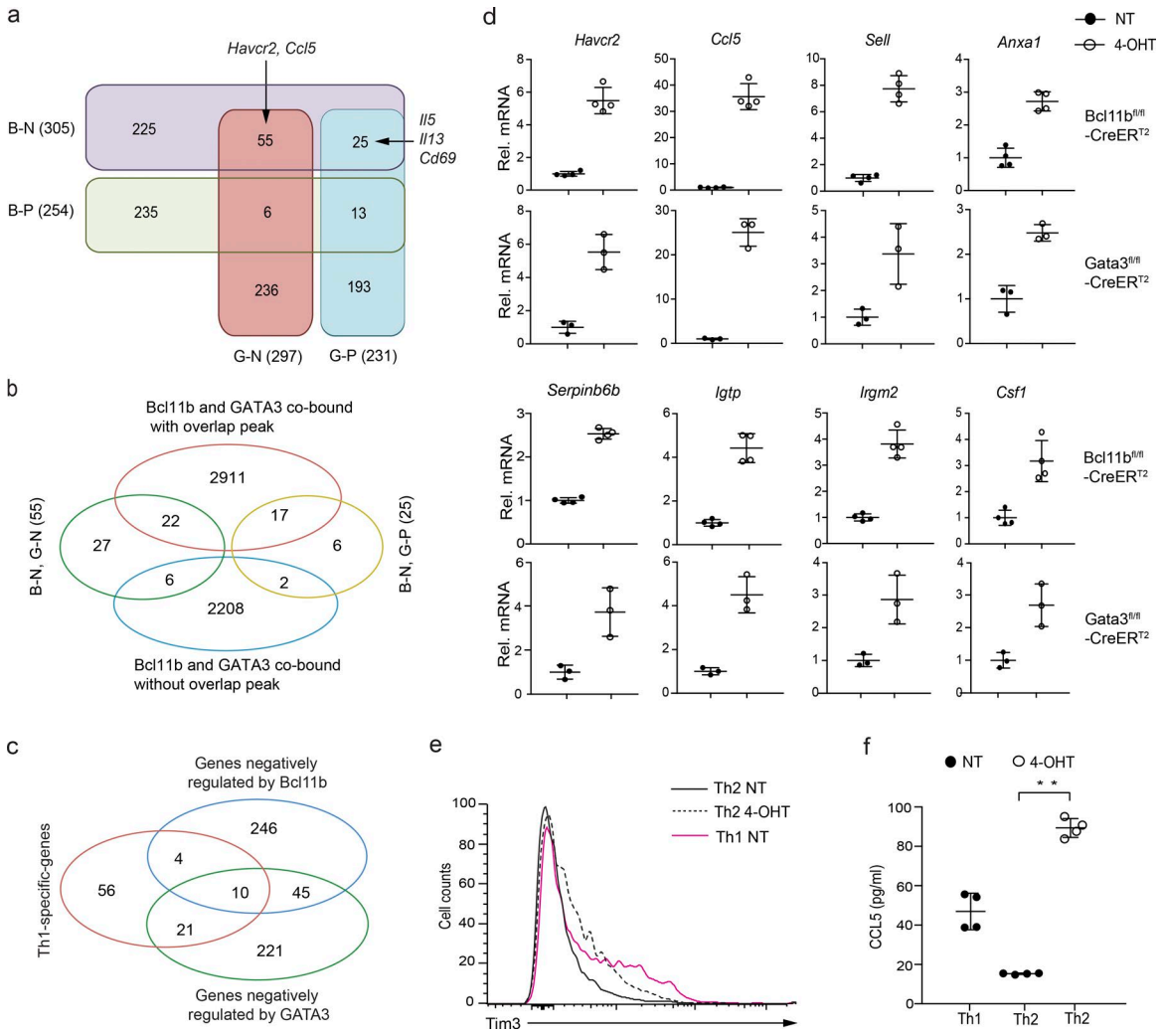

Figure 5. **Bcl11b/GATA3 complex inhibits the expression of Th1-associated genes. (a)** Overlap of genes that are negatively regulated by Bcl11b (B-N), positively regulated by Bcl11b (B-P), negatively regulated by GATA3 (G-N), or positively regulated by GATA3 (G-P) in late Th2 cells. **(b)** Overlap of genes that are negatively regulated by both Bcl11b and GATA3 (B-N, G-N), negatively regulated by Bcl11b but positively regulated by GATA3 (B-N, G-P), cobound by Bcl11b and GATA3 with at least one overlap peak, and cobound by Bcl11b and GATA3 without overlap peaks. **(c)** Overlap of Th1-associated genes and genes that are negatively regulated by Bcl11b and GATA3. **(d)** Total RNA was harvested from *Bcl11b*$^{fl/fl}$-CreER^{T2} and *Gata3*$^{fl/fl}$-CreER^{T2} late Th2 cells with or without 4-OHT. Relative (Rel.) mRNA expression of *Havcr2*, *Ccl5*, *Sell*, *Anxa1*, *Serpinb6b*, *Igtp*, *Irgm2*, and *Csf1* was measured by qRT-PCR. **(e and f)** Th1 and Th2 cells were prepared as Fig. 2 a. Tim3 (coded by *Havcr2* gene) expression was measured by cell surface staining (e). Th1 and Th2 cells were stimulated with CD3/CD28 beads for 24 h, and CCL5 amounts in supernatant were measured by ELISA (f). Data (d–f) are representative of two independent experiments. **, $P < 0.01$. Error bars represent means ± SD.

d–f). To further understand the relationship between epigenetic changes and gene regulation by Bcl11b, we compared these gene lists. Interestingly, we found that more than half (1,567 of 2,950) of the genes that Bcl11b and GATA3 cobind displayed altered H3K27ac upon *Bcl11b* deletion in either early or late Th2 cells; however, only 22% (352 of 1,567) of these genes also had altered gene expression (Fig. S4 g). Thus, Bcl11b can cause epigenetic changes without affecting gene expression, and Bcl11b-mediated epigenetic change is not the result of gene expression change. Some interesting genes that were cobound by Bcl11b and GATA3, including *Ccr9*, *Myb*, and *Batf*, displayed increased H3K27ac upon *Bcl11b* deletion; however, their gene expression was not altered (Table S1). Collectively, our data suggest that Bcl11b and GATA3 can cooperate to fine-tune the expression of Th2 genes and to inhibit the expression of Th1-associated genes through regulating epigenetic modifications and chromatin accessibility at their target loci.

Bcl11b deficiency promotes type 2 immune response in vivo

The above findings suggest that *Bcl11b* deletion in Th2 cells results in multiple changes in vitro, including enhanced Th2 cytokine expression and CD69 up-regulation, which may enhance the recruitment of Th2 cells into the lung, as well as reduced cell proliferation and aberrant expression of Th1-associated genes, which may dampen Th2 responses. To assess the net effect of *Bcl11b* deletion during type 2 immune response in vivo, we used an OVA/alum-mediated acute mouse asthma model. Mice with *Bcl11b* germline deletion die at birth (Wakabayashi et al., 2003). Surprisingly, even TMX-induced *Bcl11b* global deletion led to death of some *Bcl11b*$^{fl/fl}$-CreER^{T2} mice, particularly female mice.

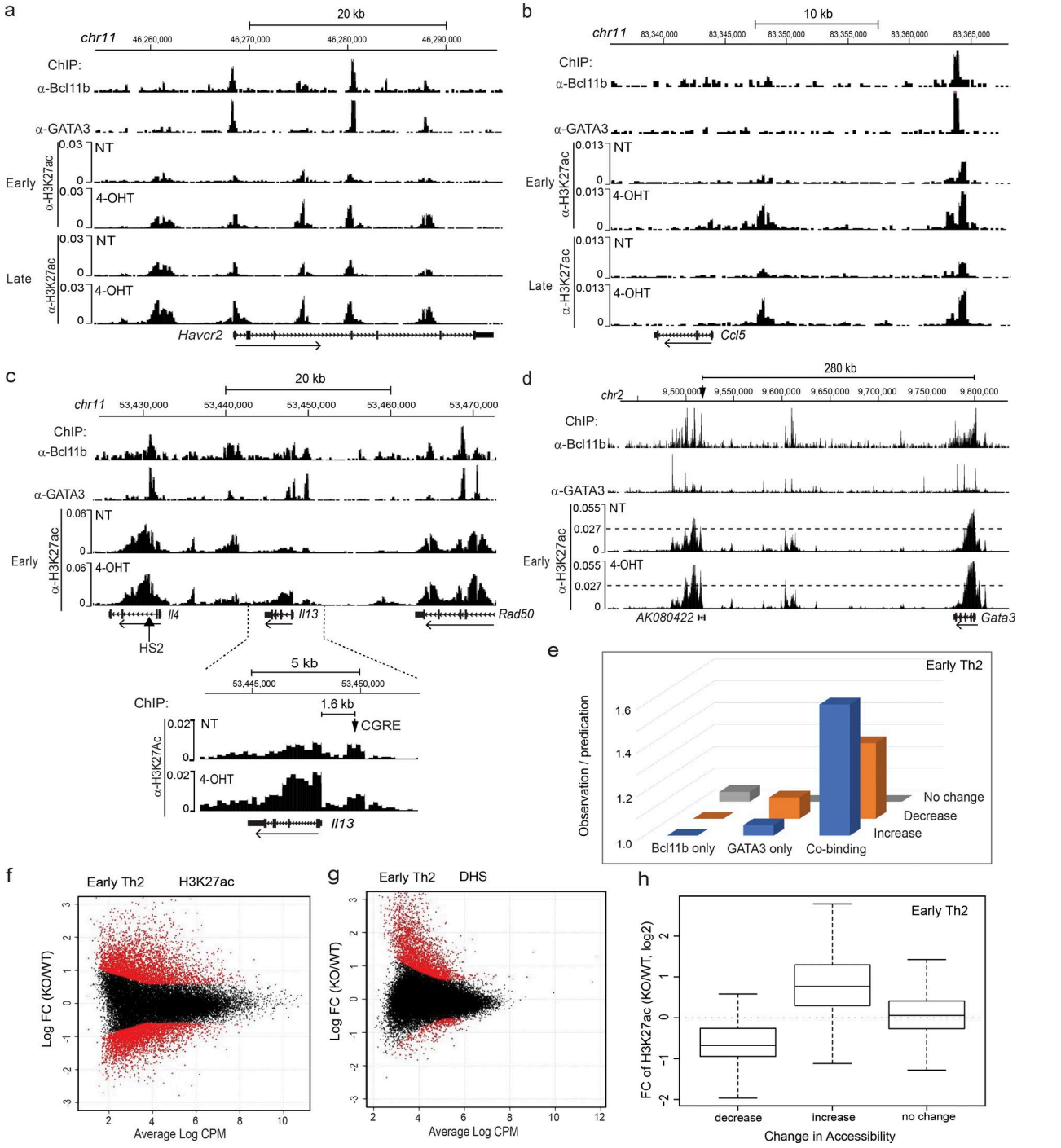

Figure 6. **Bcl11b regulates chromatin epigenetic modification.** Early and late Th2 cells were prepared from *Bcl11b*$^{fl/fl}$-CreER^{T2} mice with or without 4-OHT for DHSs and H3K27ac modification analyses. **(a and b)** UCSC Genome Browser view of Bcl11b and GATA3 binding and histone H3K27ac modification (in early and late Th2 cells) at the *Havcr2* and *Ccl5* gene loci. **(c and d)** UCSC Genome Browser view of Bcl11b and GATA3 binding and histone H3K27ac modification (in early Th2 cells) at the *Il4/Il13* and *Gata3* gene loci. **(e)** The ratio of observation and predication was calculated on genes with Bcl11b binding only, GATA3 binding only, and Bcl11b/GATA3 cobinding (with at least one overlapped peak) versus H3K27ac modification changed genes in *Bcl11b*-deficient early Th2 cells. **(f)** Scatter plot for the mean of H3K27ac level between *Bcl11b* KO cells and WT cells versus the fold-change in H3K27ac level for regions enriched with H3K27ac in early Th2 cells. **(g)** Scatter plot for the mean of accessibility between *Bcl11b* KO cells and control cells versus the fold-change in accessibility for DHSs in early Th2 cells. **(h)** Boxplot for the fold-change in H3K27ac level at nonpromoter DHSs sorted based on their change in accessibility induced by *Bcl11b* KO; similar results were obtained for promoter DHSs. H3K27ac data shown in the genome browser view (a–d) represent one of the two biological repeats.

To avoid the unwanted effect of *Bcl11b* deletion in non-T cells, we transplanted bone marrow cells from *Bcl11b*$^{fl/fl}$, *Bcl11b*$^{fl/fl}$-CreER^{T2}, or *Bcl11b*$^{fl/fl}$-*Gata3*$^{fl/fl}$-CreER^{T2} mice into sublethally irradiated TCR-α–deficient mice to generate bone marrow chimeras. ILC2 is another component of the type 2 immune response (Spits et al., 2013), and Bcl11b is essential for ILC2 maintenance (Califano

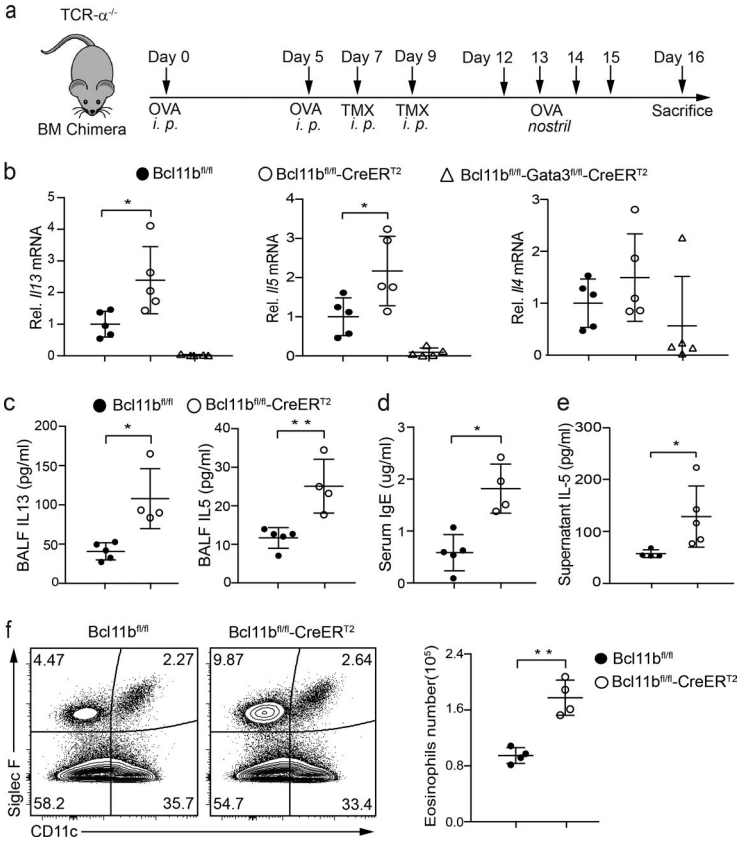

Figure 7. **Bcl11b inhibits Th2 responses in vivo. (a)** Diagram of the mouse acute asthma model. **(b)** *Bcl11b*$^{fl/fl}$, *Bcl11b*$^{fl/fl}$-CreER^{T2}, or *Bcl11b*$^{fl/fl}$-*Gata3*$^{fl/fl}$-CreER^{T2} bone marrow chimeric mice were treated as in panel a. CD4 T cells purified with CD4 (L3T4) microbeads were stimulated with plate-bound anti-CD3/CD28 antibodies for 2 h. Total RNAs were harvested, and *Il4*, *Il5*, and *Il13* mRNA levels were assessed by qRT-PCR. Rel., relative. **(c and d)** BAL fluids (c) and sera (d) were collected from *Bcl11b*$^{fl/fl}$ and *Bcl11b*$^{fl/fl}$-CreER^{T2} bone marrow chimeras treated as in panel a. IL-13 and IL-5 in BALF (c) and IgE (d) in serum were measured by ELISA. **(e)** CD4 T cells were purified from *Bcl11b*$^{fl/fl}$ and *Bcl11b*$^{fl/fl}$-CreER^{T2} bone marrow chimeras treated as in panel a. Cells were further cultured with OVA peptide in the presence of antigen-presenting cells for 24 h. IL-5 was measured by ELISA. **(f)** Late Th2/OT-II cells were transferred into CD45.1/CD45.2 F1 recipients. Mice were then challenged with OVA for 4 d. The percentage and cell number of SiglecF$^+$CD11c$^-$ eosinophils in the lung were measured through cell surface staining. Data are representative of two (b–f) independent experiments. *, P < 0.05; **, P < 0.01. Error bars represent means ± SD.

et al., 2015). Unlike conventional lymphoid cells, tissue-resident ILCs are irradiation resistant (Dudakov et al., 2014). Therefore, such chimeric mice also allow us to minimize the involvement of ILC2 defect caused by *Bcl11b* deletion.

Chimeric mice were immunized with OVA/alum as shown in the diagram (Fig. 7 a). To assess the expression of type 2 cytokine genes *Il4*, *Il5*, and *Il13*, we purified CD4 T cells from lungs of treated mice and stimulated them with plate-bound anti-CD3/CD28 antibodies for 2 h. *Il13* and *Il5* mRNA were significantly up-regulated in *Bcl11b*-deficient cells, although there was a big variation for *Il4* mRNA expression in the lung CD4 T cells (Fig. 7 b). Consistent with in vitro data, *Bcl11b*/*Gata3* double deletion abolished GATA3-dependent *Il5* and *Il13* expression (Fig. 7 b). Also, secreted IL-13 and IL-5 proteins in bronchoalveolar lavage fluid (BALF) and IgE in serum were significantly increased in *Bcl11b*-deficient chimeric mice (Fig. 7, c and d). Furthermore, IL-5 produced by antigen-specific lung CD4 T cells was also increased in the *Bcl11b*-deficient group (Fig. 7 e). The total number of *Bcl11b*-deficient CD4 T cells in the lung was significantly lower than that of WT counterparts (Fig. S5 a). On the other hand, GATA3 protein expression in the lung effector CD4 T cells (CD4$^+$CD44^{high}) was comparable between WT and *Bcl11b* KO chimera groups (Fig. S5 b). To further confirm whether the proliferation defect of *Bcl11b* KO T cells in vivo is cell intrinsic, we cotransferred CD45.1 (WT) and CD45.2 (*Bcl11b*$^{fl/fl}$ or *Bcl11b*$^{fl/fl}$-CreER^{T2}) bone marrow cells into irradiated TCR-α–deficient mice. After 8 wk, the mice were treated as shown in Fig. 7 a. The ratio of CD45.2/CD45.1 CD4 T cells in the lung was significantly reduced in the TMX-induced *Bcl11b*-deficient chimera group (Fig. S5 c).

Bcl11b was reported to regulate T reg cells in an inflammatory bowel disease model (Vanvalkenburgh et al., 2011). However, in our study, we failed to detect any notable difference in the percentage of Foxp3-expressing cells in the lung CD4 T cell population or in the mean fluorescence intensity of Foxp3 in T reg cells between WT and *Bcl11b* KO chimeras that were immunized with OVA/alum (Fig. S5, d and e). We also tested the ILC2 population in these chimeras. The percentage of Lin$^-$CD127$^+$KLRG1$^+$ ILC2 cells in the lung was comparable between WT and *Bcl11b* KO chimeras as expected because they should be largely host derived (Fig. S5 f).

We noticed that TMX treatment alone inhibited eosinophil recruitment from bone marrow into the lung, which is consistent with another study (Cai et al., 2012). Therefore, we were not able to collect meaningful eosinophil data from the lung or BALF in these TMX-treated chimeras. To overcome the TMX effect, we crossed *Bcl11b*$^{fl/fl}$-CreER^{T2} with transgenic OT-II mouse strain. OT-II/*Bcl11b*$^{fl/fl}$-CreER^{T2} naive CD4 T (CD45.2) cells were sorted and cultured under Th2 conditions in the presence of OVA peptide in vitro. The primed Th2 cells were further cultured under Th2 conditions for 4 d with or without 4-OHT. Viable Th2 cells were transferred into CD45.1/CD45.2 recipients, which were subsequently challenged with OVA protein (i.n.) for the next 4 d (Fig. S5 g). Although *Bcl11b* deletion in these cells resulted in reduced cell numbers of donor CD45.2 CD4 T cells in the lung (Fig. S5 h), eosinophil recruitment was significantly increased in mice received *Bcl11b*-deficient Th2 cells compared with the mice received WT control Th2 cells (Fig. 7 f). Thus, despite multiple targets being regulated by Bcl11b in Th2 cells and its important

function in promoting Th2 cell proliferation, Bcl11b mainly serves as a negative regulator of type 2 immune response in vivo.

Discussion

In this study, we show that the transcription factor Bcl11b interacts with GATA3 at the protein level in Th2 cells, and that these two factors colocalize at the cis-regulatory elements of many important Th1- and Th2-related genes. *Bcl11b*-deficient Th2 cells produce higher amounts of type 2 cytokines than WT both in vitro and in vivo, although cell recovery is reduced when *Bcl11b* is deleted. Furthermore, our results indicate that several important gene regulatory functions of Bcl11b are GATA3 dependent. Thus, Bcl11b, an important component of GATA3-containing transcriptional complex, is a critical regulator of GATA3 in Th2 cells.

GATA3 protein levels are up-regulated in *Bcl11b*-deficient early Th2 cells. This is consistent with binding of Bcl11b to the *Gata3* gene locus and a modest increase in H3K27ac at the *Gata3* locus in *Bcl11b*-deficient early Th2 cells. Because GATA3 also binds to its own gene locus and is capable of self-regulating during Th2 differentiation, Bcl11b may limit GATA3 auto-regulation. Therefore, in addition to Bcl11b's role in regulating GATA3 expression in Th2 cells, Bcl11b may regulate GATA3 expression during T cell development in the thymus. Indeed, GATA3 expression is increased when *Bcl11b* is deleted in CD4/CD8 double-negative thymocytes (Li et al., 2010a). Interestingly, GATA3 protein level is equivalent between WT and *Bcl11b*-deficient late Th2 cells, yet *Bcl11b*-deficient late Th2 cells produce more Th2 cytokines. This finding strongly supports the idea that Bcl11b also limits the function of GATA3 in inducing the expression of Th2 cytokines. Similarly, although epigenetic status at the *Il4/Il13* Th2 cytokine loci is different between early WT and *Bcl11b* KO Th2 cells, consistent with the idea that the GATA3/Bcl11b complex regulates chromatin accessibility, enhanced cytokine production in late *Bcl11b* KO Th2 cells without H3K27ac increase suggests that either other GATA3/Bcl11b targets may indirect induce Th2 cytokine production in late Th2 cells or the GATA3/Bcl11b complex also affects other epigenetic modifications that may promote gene expression. Given critical functions of both GATA3 and Bcl11b during T cell development at multiple stages, Bcl11b may also limit the functions of GATA3 in thymocytes, a topic that requires further investigation.

Not only is GATA3 essential for Th2 cell differentiation, but it is also important for suppressing alternative lineage fate. Our RNA-seq data show that Bcl11b and GATA3 inhibit Th1-specific gene expression in Th2 cells. Interestingly, among the 31 Th1-specific genes that are negatively regulated by GATA3, 10 are also negatively regulated by Bcl11b. Therefore, the GATA3/Bcl11b complex is involved in repressing Th1 cell differentiation. Although it is possible that GATA3 interacts with another transcription repressor to inhibit the remaining 21 Th1 genes, GATA3 may directly suppress the expression of these genes by interfering with the functions of T-bet and/or Runx3 through protein–protein interaction.

GATA3 is essential for ILC2 development and maintenance (Hoyler et al., 2012; Mjösberg et al., 2012; Yagi et al., 2014). Interestingly, recent studies show that Bcl11b is also involved in ILC2 development and mature ILC2 maintenance (Califano et al., 2015; Walker et al., 2015; Yu et al., 2015; Zhong and Zhu, 2015). Califano et al. (2015) show that *Gata3* mRNA is down-regulated in TMX-induced *Bcl11b*-deficient ILC2s; deletion of Bcl11b from mature ILC2s results in aberrant lineage switching to ILC3-like cells. However, Yu et al. (2015) showed that *Gata3* mRNA is up-regulated in bone marrow–derived *Bcl11b*-deficient ILC2s in vitro, consistent with our observation in Th2 cells and an earlier study in thymocytes (Li et al., 2010a). It is possible that the regulatory relationship between Bcl11b and GATA3 is dependent on cell context and/or developmental stage. Therefore, whether Bcl11b regulates GATA3 expression and/or functions in ILC2s requires further investigation. Future mapping of the binding pattern of Bcl11b and GATA3 in different stages of ILC2s through ChIP-seq, in comparison to their binding patterns in Th2 cells, may yield new insights.

In summary, we have shown that Bcl11b is an important component of the GATA3-containing transcriptional regulatory complex in Th2 cells. The complex not only negatively regulates Th2-specific genes, thereby limiting the magnitude of Th2 cell differentiation, it also represses Th1-associated genes to silence an alternative cell fate. Both functionalities of Bcl11b depend on GATA3. Therefore, Bcl11b controls the differentiation of Th2 cells through modulating GATA3 expression and function. *Bcl11b* deletion results in changes in H3K27ac modification and chromatin accessibility, particularly at the regions where Bcl11b and GATA3 can cobind (more than half of these genes have altered H3K27ac status upon Bcl11b removal), whereas it affects the expression level of only a subset of these genes, indicating that the GATA3/Bcl11b complex suppresses gene expression through epigenetic modifications. The net outcome of *Bcl11b* deletion in vivo results in enhanced Th2 response. It is possible that polymorphisms and/or mutations at the *Bcl11b* gene may result in Th2-related immune diseases. On the other hand, enhancing the expression and/or function of Bcl11b may be considered a novel strategy to treat Th2 cell–mediated inflammation such as asthma.

Materials and methods

Mice

Bcl11b$^{fl/fl}$ mice (Li et al., 2010b) on C57BL/6 background were bred to CreER^{T2} (Taconic line 10471) to generate *Bcl11b*$^{fl/fl}$-CreER^{T2}. *Bcl11b*$^{fl/fl}$-CreER^{T2} mice were further crossed with OT-II (Taconic line 187) to obtain OT-II/*Bcl11b*$^{fl/fl}$-CreER^{T2} mice. *Gata3*$^{fl/fl}$-CreER^{T2} mice (Yagi et al., 2014), also available as Taconic line 8445, CD45.1 congenic mice (Taconic line 7), CD45.1/CD45.2 congenic mice (Taconic line 8422), and TCR-α$^{-/-}$ mice (Taconic line 98) were obtained from the National Institute of Allergy and Infectious Diseases (NIAID)-Taconic repository.

To prepare bone marrow chimeras, TCR-α$^{-/-}$ mice were sublethally irradiated (450 rad) and reconstituted (i.v.) with 5 million bone marrow cells. The chimeric mice were kept for 8 wk before being used for experiments. All mice were bred and/or maintained in the NIAID specific pathogen–free animal facilities, and the experiments were done when mice were 8–16 wk of age under a protocol approved by the NIAID Animal Care and Use Committee. Because of possible effects of TMX on female mice, all the immunized chimeric mice used in this study were males.

Experimental acute asthma induced by OVA/alum immunization

The experimental protocol for inducing acute airway hypersensitivity by OVA/alum sensitization is as follows. The chimeras were immunized with OVA protein and alum on days 0 and 5 through i.p. injection, treated (i.p.) with TMX (T5648; Sigma-Aldrich) on days 7 and 9 (3 mg TMX in 150 µl corn oil per mouse), and challenged i.n. with OVA daily from day 12 to day 15. The mice were analyzed on day 16 according to the protocol (Han and Ziegler, 2013). Late OT-II/Th2 cells were made in vitro. Two million cells were transferred into CD45.1/CD45.2 congenic mice. The recipient mice were challenged with OVA i.n. daily for 4 d. The mice were analyzed 24 h after the final OVA challenge.

Tissue cell preparation

Lung digestion was conducted according to the protocol previously described (Moro et al., 2015). In brief, mice were perfused with cold PBS right after euthanization. The lung was cut into small pieces and digested with DNase I (10104159001; Roche) and liberase TM (05401119001; Roche) for 30 min at 37°C. Cell suspensions were made with a Lung Dissociation kit (130-095-927; MACS Miltenyi Biotec) following the manufacturer's instructions. Eosinophils were stained and identified as MHC-II$^-$Ly6G$^-$Siglec-F$^+$CD11c$^-$. ILC2 were stained and identified as Lin$^-$CD127$^+$KLRG1$^+$. Mouse CD4 (L3T4) MicroBeads were used to purify CD4 T cells from lung cell suspensions. CD4 T cells were stimulated with plate-bound anti-CD3/CD28 antibodies for 2 h before total RNAs were prepared.

Flow cytometry analysis and reagents

Cell surface staining and cytokine intracellular staining were performed as previously described (Zhu et al., 2004). In brief, cells were stimulated with 10 ng/ml PMA and 500 nM ionomycin for 4 h in the presence of monensin (00-4505-41; eBioscience). Cells were fixed in 4% PFA for 15 min at room temperature. A Foxp3 Staining Buffer Set (00-5523-00, eBioscience) was used to stain transcription factors according to the manufacturer's instructions. Flow cytometry data were collected through the LSR II or FORTESSA (BD Biosciences), and the results were analyzed with FlowJo software (Tree Star). Antibodies were purchased from sources indicated below. Antibodies against CD4 (RM4-5), CD8 (53-6.7), CD25 (PC61.5), CD44 (IM7), CD45.1 (A20), CD45.2 (104), CD62L (MEL-14), F(ab')2 anti–rabbit IgG FITC (11-4839-81), B220 (RA3-6B2), IFN-γ (XMG1.2), Ki-67 (SoIA15), MHC-II (M5/114.15.2), Foxp3 (FJK-16s), IL-5 (TRFK5), IL-13 (eBio13A), IL-17A (eBio17B7), and Ly-6G (RB6-8C5) were from eBioscience; antibodies against CD45RB (16A), CD11b (M1/70), CD11c (HL3), F4/80 (T45-2342), Siglec-F (E50-2440), CD127 (SB/199), KLRG1 (2F1), and GATA3 (L50-823) from BD Biosciences; anti-p21 (F-5) from Santa Cruz Biotechnology; anti–IL-4 (11B11) from BioLegend; 2.4G2 (PUR001) from Harlan; anti-Bcl11b (A300-385A-2) from Bethyl Laboratories; and α-rabbit IgG light chain specific-HRP (211-032-171) and α-rabbit IgG light chain specific-HRP (115-035-174) from Jackson ImmunoResearch. For ILC2 staining, lineage antibodies were used as previously described (Moro et al., 2015).

Cell culture

Naive CD4 T cells (CD4$^+$CD44^{low}CD62L^{high}CD25$^-$) were purified from lymph nodes. Cells were cultured in RPMI 1640 (Invitrogen) supplemented with 10% FBS (HyClone), 200 mM glutamine, 100 mM sodium pyruvate (Gibco), 50 µM β-mercaptoethanol (Sigma-Aldrich), 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified incubator supplied with 5% CO_2. Th1 conditions: α-CD3 1 µg/ml, α-CD28 3 µg/ml, α-IL-4 10 µg/ml, IL-12 10 ng/ml, and IL-2 100 U/ml; Th2 conditions: α-CD3 1 µg/ml, α-CD28 3 µg/ml, α-IFN-γ 10 µg/ml, α-IL-12 10 µg/ml, and IL-4 5000U/ml; Th17 conditions: α-CD3 1 µg/ml, α-CD28 3 µg/ml, α-IL-4 10 µg/ml, α-IFN-γ 10 µg/ml, α-IL-12 10 µg/ml, TGFβ1 1 ng/ml, IL-6 10 ng/ml, and IL-1β 10 ng/ml; Thneu conditions: α-CD3 1 µg/ml, α-CD28 3 µg/ml, and IL-2 100 U/ml. T cell–depleted splenocytes were used as antigen-presenting cells in in vitro T cell culture. For RNA-seq, Dynabeads Mouse T-Activator CD3/CD28 (11453D) was used for T cell activation. Purified naive CD4 T cells or activated CD4 T cells were labeled with CFSE (C34554; Thermo Fisher Scientific) following the manufacturer's instructions.

ELISA

Serum IgE concentrations were measured with ELISA (Mouse IgE Ready-SET-Go!, 88-50460; eBioscience). Approximately 2 ml BALF was collected from each mouse, and IL-5 and IL-13 were measured with ELISA (Mouse IL-5 ELISA MAX Standard Set, 431202 from BioLegend; Mouse IL-13 DuoSet ELISA, DY413-05 from R&D Systems) according to the manufacturers' instructions. The supernatants from in vitro cultured Th1 and Th2 cells were harvested for testing CCL5 by ELISA (DY478-05; R&D Systems). To measure IL-5 produced by antigen-specific CD4 T cells, purified lung CD4 T cells were further cultured with CD11b$^+$ cells and OVA peptide for 24 h before cell supernatant was harvested.

qRT-PCR

Total RNA was extracted with RNAeasy Mini kit (74104; Qiagen). The diluted RNA was reverse transcribed and analyzed with a 7900HT Fast Real-Time PCR system (Applied Biosystems) using FastStart Universal SYBR Green Master (Rox; 04913850001; Roche). The primers listed in Table S2 were used to amplify the specific fragments of the gene transcripts. *Hprt* was used for normalization.

Coimmunoprecipitation

The total cell lysates were prepared in RIPA buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 1% (vol/vol) Triton X-100, 1% deoxycholate, and protease inhibitor cocktail (04-693-132-001; Roche). The whole-cell homogenates were centrifuged twice at 12,000 rpm, and the supernatants were incubated with antibodies and rotated at 4°C overnight, then incubated with protein A sepharose (17-5138-01; GE Healthcare Life Sciences) for an additional 2 h. Immunoprecipitates were washed five times, boiled in loading buffer, and subjected to SDS-PAGE.

RNA-seq, ChIP-seq, DHSs, and data analysis

RNA was extracted with the RNAeasy Mini kit (74104; Qiagen). 10 ng RNA was amplified with the Ovation RNA-seq System V2 (7102-08; NuGEN) according to the manufacturer's

instructions. 220 ng double-stranded DNA was sonicated into 200–400 bp in size using the Bioruptor Pico (Diagenode). ChIP-seq experiments were performed with an iDeal ChIP-Seq kit for Transcription Factors (C01010055; Diagenode) using anti-Bcl11b (A300-385A-2; Bethyl Laboratories) and anti-GATA3 (L50-823; BD Biosciences), and iDeal ChIP-seq kit for Histones (C01010051; Diagenode) using anti-H3K27ac (C15410196; Diagenode). Both cDNA and ChIP DNA were blunt-ended, ligated to the Solexa adaptors, amplified, and sequenced with an Illumina HiSeq system, and 50-bp reads were generated by the National Heart, Lung, and Blood Institute DNA Sequencing and Computational Biology Core. Both RNA-seq and ChIP-seq reads were mapped to the mm9 genome (UCSC Genome Browser). Gene expression levels were measured by reads per kilobase of exon per million reads (RPKM). Differentially expressed genes were identified by edgeR (Robinson et al., 2010) with the following criteria: false discovery rate (FDR) <0.001, fold change $\log_2$ ≥1, and RPKM ≥5 in *Gata3*-sufficient, *Bcl11b*-sufficient, *Gata3*-deficient, or *Bcl11b*-deficient samples. ChIP-seq reads were also mapped to the mm10 genome using Novoalign (Novocraft). To identify significant GATA3- or Bcl11b-binding peaks genome-wide from the ChIP-seq experiments, we used a cutoff with P value of 10^{-6} for peak identification through MACS v.1.4.2 (Zhang et al., 2008) with setting keep-dup = 1 to call peaks, followed by PeakSplitter using the default height cutoff of 5. Peaks overlapping satellite or ribosomal RNA repeats were removed from the analysis. We scored subpeaks identified by PeakSplitter using DefinedRegionScanSeqs from USeq v.8.8.8 (Nix et al., 2008) and retained significant peaks with $\log_2$ ratio ≥1 in ChIP versus input control and *q*-value FDR <0.1% or <0.003% for Bcl11b and GATA3, respectively. GATA3/Bcl11b cobound regions were obtained by taking the peak summit ±30 or ±150 bp for both Bcl11b and GATA3 and intersecting peaks between datasets using BEDTools (Quinlan and Hall, 2010). Hypersensitive sites based on DNase-seq reads were called by SICER with a window of 200 bp, a gap of 400 bp, and an *E*-value of 1 (Zang et al., 2009). For each condition, namely the treatment or the control group, only peaks predicted by both duplicates were kept. To call peaks with differential accessibility between conditions, the numbers of DNase-seq reads for each DHS were extracted from each duplicate and supplied to EdgeR 3 to make the prediction (fold change >1.5; FDR <0.01; Robinson et al., 2010). Peaks were assigned to genes using FindNeighboringGenes (USeq).

Statistics

Differences between groups were determined by two-tailed Student's *t* test with Prism 7 software (GraphPad). P < 0.05 was considered significant.

Accession numbers

RNA-seq, ChIP-seq, and DNase-seq data are available in the Gene Expression Omnibus (GEO) database under accession no. GSE109109.

Online supplemental material

Fig. S1 shows Bcl11b expression in Th1, Th2, and Th17; deletion efficiency of Bcl11b by TMX; GATA3 expression and its function in *Bcl11b*-deficient Th1 and Th17 cells; the effect of *Bcl11b* deletion on cytokine production in early Th2 cells; CFSE dilution in Th cells; and Ki-67 staining in late Th2 cells. Fig. S2 shows genes regulated by Bcl11b and/or GATA3 and the overlap of Bcl11b and GATA3 binding and regulated genes. Fig. S3 shows the effect of Bcl11b on *Cd69* expression. Fig. S4 shows H3K27ac modification and DHSs regulated by Bcl11b. Fig. S5 shows the effect of Bcl11b on T cell recovery, T reg cells, and ILC2s in vivo. Table S1 is an Excel file of six worksheets. Worksheet 1, genes regulated by Bcl11b in early and late Th2 cells; worksheet 2, genes regulated by GATA3 in early and late Th2 cells; worksheet 3, genes bound by Bcl11b and/or GATA3 in Th2 cells; worksheet 4, cell cycle–related genes regulated by Bcl11b; worksheet 5, genes with altered DHSs upon *Bcl11b* deletion in early and late Th2 cells; and worksheet 6, genes with altered H3K27ac upon *Bcl11b* deletion in early and late Th2 cells. Table S2 is an Excel file of primer sequences that were used to amplify specific fragments of gene transcripts.

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Author contributions: D. Fang, R. Yagi, and J. Zhu conceived the project. D. Fang performed the majority of the experiments. D. Fang and J. Zhu wrote the manuscript. K. Cui contributed to RNA-seq, ChIP-seq, and DNase-seq experiments. G. Hu and A.J. Oler performed bioinformatic analysis. R.M. Gurram and S. Sharma performed some asthma-related experiments. C. Zhong contributed to RNA-seq experiments. R. Yagi, M. Zhao, P. Liu, B. Sun, and K. Zhao provided critical advice to the project and edited the manuscript. P. Liu provided the *Bcl11b* floxed mouse line. J. Zhu supervised the project.

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