Development of chronic allergic responses by dampening Bcl6-mediated suppressor activity in memory T helper 2 cells

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Mice deficient in the transcriptional repressor B-cell CLL/lymphoma 6 (Bcl6) exhibit similar T helper 2 (T_H2) immune responses as patients with allergic diseases. However, the molecular mechanisms underlying Bcl6-directed regulation of T_H2 cytokine genes remain unclear. We identified multiple Bcl6/STAT binding sites (BSs) in T_H2 cytokine gene loci. We found that Bcl6 is modestly associated with the BSs, and it had no significant effect on cytokine production in newly differentiated T_H2 cells. Contrarily, in memory T_H2 (mT_H2) cells derived from adaptively transferred T_H2 effectors, Bcl6 outcompeted STAT5 for binding to T_H2 cytokine gene loci, particularly *Interleukin4* (*Il4*) loci, and attenuated GATA binding protein 3 (GATA3) binding to highly conserved intron enhancer regions in mT_H2 cells. Bcl6 suppressed cytokine production epigenetically in mT_H2 cells to negatively tune histone acetylation at T_H2 cytokine gene loci, including *Il4* loci. In addition, IL-33, a pro-T_H2 cytokine, diminished Bcl6's association with loci to which GATA3 recruitment was inversely augmented, resulting in altered IL-4, but not IL-5 and IL-13, production in mT_H2 cells but no altered production in newly differentiated T_H2 cells. Use of a murine asthma model that generates high levels of pro-T_H2 cytokines, such as IL-33, suggested that the suppressive function of Bcl6 in mT_H2 cells is abolished in severe asthma. These findings indicate a role of the interaction between T_H2-promoting factors and Bcl6 in promoting appropriate IL-4 production in mT_H2 cells and suggest that chronic allergic diseases involve the T_H2-promoting factor-mediated functional breakdown of Bcl6, resulting in allergy exacerbation.

Bcl6 | memory T_H2 cells | asthma

T helper 2 (T_H2) cells produce various effector cytokines [Interleukin (IL)-4, IL-5, and IL-13] (1, 2). GATA binding protein 3 (GATA3), a key regulator of T_H2 cell differentiation, subsequently facilitates T_H2 cytokine gene transcription in T_H2 cells (3, 4). In mice and humans, IL-4 is a key cytokine in T_H2 response initiation and IgE isotype class switching (5), whereas IL-5 and IL-13 are important in focal inflammation in allergic settings (5). The generation of lineage-committed effector T_H cells peaks within approximately 1 wk. Some of the effectors will survive and become long-lived memory cells. T_H2 effector cells can become memory T_H2 (mT_H2) cells (6), which are likely to be involved in maintaining allergic pathogenesis, although the regulatory mechanisms in these cells remain unclear.

The protooncogene B-cell CLL/lymphoma 6 (Bcl6) is a sequence-specific transcriptional repressor (7, 8). Increased T_H2 cytokine production has been observed after ex vivo T-cell stimulation in *Bcl6*-KO mice. We previously reported that Bcl6 repressed *Il5* expression (9). However, the molecular mechanisms underlying Bcl6-directed regulation of T_H2 cytokine genes remain unclear. Bcl6-binding DNA sequences resemble the IFN-γ–activated sequence motif bound by STAT proteins (10), suggesting that Bcl6 represses T_H2 cytokine gene expression via competitive binding against STAT factors in T_H2 cytokine gene loci (7). However, T_H2 cell differentiation was not influenced by the absence of Bcl6 under T_H2-skewing conditions (11). Additionally, T_H1 cell differentiation was similar between WT and Bcl6-KO cells under T_H1-skewing conditions (11). Conversely, the differentiation of T-follicular helper (T_{FH}) cells is believed to result from Bcl6-mediated suppression of differentiation to other T_H cell lineages (12–14). Conversely, we showed that excess exogenous Bcl6 in T cells suppressed T_H2 cytokine production in a murine model of chronic pulmonary inflammation (15). Therefore, considerable uncertainty surrounds the molecular mechanisms by which Bcl6 regulates T_H2 cell differentiation and cytokine production.

Recent studies recognized nonlymphoid-derived cytokines [thymic stromal lymphopoietin (TSLP), IL-25, and IL-33] as integral factors in promoting T_H2-type responses; however, their pathophysiological

Significance

It has been suggested that the transcriptional repressor Bcl6 suppresses T helper 2 (T_H2) immune responses underlying allergic diseases. However, the molecular role of B-cell CLL/lymphoma 6 (Bcl6) in T_H2 cells is incompletely understood in pathophysiological settings. We found that Bcl6 suppressed cytokine production in memory T_H2 cells through binding to intron 2 of the *Interkeukin 4* (*Il4*) locus using murine models. Furthermore, IL-33 controlled Bcl6 function at the chromatin level and consequently, augmented cytokine production in memory T_H2 cells. Therefore, pro-T_H2 cytokines, such as IL-33, play a role in chronic allergic diseases via the functional breakdown of Bcl6. This study identifies a relationship between T_H2-promoting factors and Bcl6 in T_H2 cells, which may lead to therapeutic strategies against chronic allergic diseases.

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The authors declare no conflict of interest.

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roles in mT_H2 cells are incompletely understood. The IL-33 receptor is expressed on T_H2 and innate immune cells, including basophils, mast cells, eosinophils, and type 2 innate lymphoid cells (16–18). In vitro-differentiated T_H2 cells are also activated to produce IL-5 and IL-13 but not IL-4 in response to IL-33, regardless of T-cell receptor (TCR) engagement (19, 20). Accordingly, IL-33 may regulate cellular functions in allergic diseases by cross-linking innate and adaptive immune responses. For example, IL-33 administration to WT mice induces T_H2 cytokines in the lungs. This pro-T_H2 inflammatory effect appears independently of the adaptive immune response because mice deficient in the recombinase-activating gene 2 (RAG2) develop a comparable response to IL-33 (21). Exogenous IL-33 can enhance allergen-nonspecific IgE Ab production in naïve WT mice by inducing IL-4 production mainly in innate cells (22). However, treatment with an Ab against ST2, an IL-33 receptor subunit (23), largely abrogated allergic airway inflammation and reduced antigen-specific IgE Ab and T_H2 cytokine production in a murine ovalbumin (OVA)-immunized allergy model. IL-33 does not induce IL-4 production in newly differentiated T_H2 cells (19, 20), and whether it induces the same in mT_H2 cells is uncertain.

In this study, we found that Bcl6 down-regulates T_H2 cytokine gene expression in mT_H2 cells. Furthermore, the findings of this study indicate that T_H2 cytokine gene regulation mediated by T_H2-promoting factors, such as IL-33, is associated with modulated Bcl6 function in mT_H2 cells, resulting in allergic exacerbation via enhanced T_H2 cytokine production.

Results

Role of Bcl6 in Cytokine Production. To investigate the role of Bcl6 in T_H2 cell differentiation and in vitro and in vivo maintenance, cultured naïve $CD4^+$ T cells were stimulated with antigen under T_H2-skewing conditions and expanded with IL-2 until day 7 or sequentially maintained with IL-7 for 28 d to yield T_H2 cells of in vitro early-phase (EP) or late-phase (LP) postdifferentiation types [T_H2 early-phase cells (T_H2EPs) and T_H2 late-phase cells (T_H2LPs), respectively]. T_H2EPs were also adoptively transferred into BALB/c WT mice to generate mT_H2 cells in vivo. T cell-intrinsic Bcl6 expression did not affect T_H2EP differentiation (Fig. 1*A*). However, the frequency of $IL\text{-}4^+$ WT T_H2LPs was decreased compared with that of *Bcl6*-KO T_H2LPs (Fig. 1*A*). *Il4*, *Il5*, and *Il13* mRNA expression levels were similar between *Bcl6*-KO and WT T_H2EPs, whereas their levels, particularly *Il4* and *Il5*, in *Bcl6*-KO T_H2LPs were significantly higher than those in WT cells (Fig. 1*B*). *Bcl6*-KO mT_H2 cells also exhibited augmented T_H2 cytokine production at the mRNA and protein level compared with WT mT_H2 cells (Fig. 1). However, the mRNA expression levels of *Gata3* in T_H2EPs, T_H2LPs, and mT_H2 cells were similar between *Bcl6*-KO and the WT, whereas their levels were decreased over time after T_H differentiation (Fig. S1*A*). According to T_H1 function, IFN-γ production was not significantly affected by Bcl6 at any T_H2-cell phase (Fig. 1*A*), and the mRNA levels of the T_H1 transcription factor *Tbox protein expressed in T cells* (*T-bet*) in both T_H1 and T_H2 cells were constant between *Bcl6*-KO and the WT (Fig. S1*B*).

Bcl6-Mediated Histone Modification at T_H2 Cytokine Gene Loci. To investigate chromatin modification at Bcl6/STAT (BS) binding regions [IL5BS (9) and putative BSs] (Fig. S2*A*), we analyzed the acetylation levels of histone H3 (Ac-H3) at lysines (K9 and K14), which are known to play important roles in transcriptional activation and chromatin assembly at T_H2 cytokine gene loci in T_H2 cells at different phases using a ChIP assay (Fig. 2*A*). *Bcl6*-KO and WT T_H2EPs exhibited similar Ac-H3 levels, with particularly high levels at the *Il4* intron 2 region. Compared with *Bcl6*-KO cells, WT T_H2LPs displayed decreased Ac-H3 levels at each BS, excluding IL5BS, IL13BS, BS1, and BS2, and promoter regions of *Il4* (*Il4*p), *Il5*p, and *Il13*p. These decreased levels in WT T_H2LP cells were rescued after Bcl6 inhibition (Fig. 2*A*). A ChIP assay (Fig. 2*B*) revealed modest Bcl6 binding to each region in WT T_H2EPs;

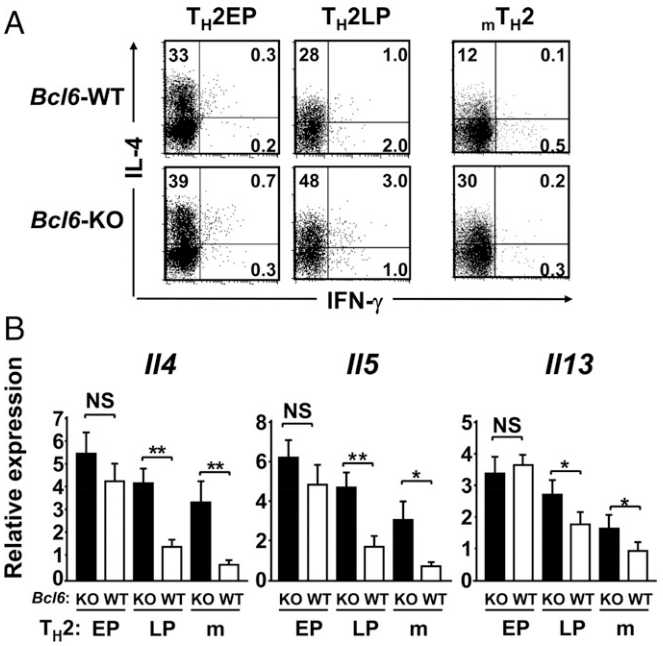

Fig. 1. Regulatory role of Bcl6 in the differentiation of T_H2 cells. T_H2 cells were differentiated from naïve T cells from *Bcl6*-KO and WT DO11.10 mice, which express the clonotypic KJ-1-26 TG TCR specific for the OVA peptide. (*A*) FACS analysis of IL-4 and IFN-γ–producing cells in KJ-1-26^+ CD4 T cells restimulated with anti-CD3 Abs. The figure shows representative data for T_H2EPs, T_H2LPs, or mT_H2 cells. T_H2EPs from WT or *Bcl6*-KO mice were adaptively transferred into BALB/c WT mice. Twenty-eight days later, mT_H2 cells were isolated as KJ-1^+CD4 T cells from the recipient spleens. All FACS data are representative of three independent experiments with similar results. The numbers in each corner represent the percentages of T cells in the column. (*B*) T_H2 cytokine mRNA expression in KJ-1^+ T_H2EPs, T_H2LPs, and mT_H2 cells from WT and *Bcl6*-KO was measured by qRT-PCR after restimulation for 24 h. Data are shown as means ± SDs ($n = 5$). All results are representative of five or six independent experiments with similar outcomes. NS, not significant. $^*P < 0.05$; $^{**}P < 0.01$.

conversely, binding of STAT5 rather than STAT6 was observed at each BS in both WT and *Bcl6*-KO cells. In all cultures, STAT5 associated with the T_H2 locus control region (LCR) and *Il4* intron 2 but not with BS1 and BS2. In WT T_H2LPs, broad Bcl6 binding was observed from IL5BS to BS7 (excluding IL13BS, BS1, and BS2), particularly at *Il4* intron 2 (BS3–S6), whereas STAT5 binding was lower in WT cells than in *Bcl6*-KO cells. GATA3 binding was observed at each major GATA (G) binding region [G1 in *Il5*p (24), G2 in Rad50 hypersensitive site 7 in T_H2LCR, CGRE upstream of the proximal *Il13*p (25), G3 in *Il4* intron 2 (26), G4 in HS5a (24), and G5 in conserved noncoding sequence 2 (CNS2) (Fig. 2*C*, *Upper* and Fig. S2*B*)], similar to WT and *Bcl6*-KO T_H2EPs. GATA3 binding was completely attenuated in both WT and *Bcl6*-KO T_H2LPs compared with in T_H2EPs (Fig. 2*C*). Whereas GATA3 predominantly associated with G3 among G sites in both WT and KO cells in all cultures, GATA3 binding was significantly greater in *Bcl6*-KO T_H2LPs than in WT cells. Regarding mT_H2 cells, Ac-H3 levels, and Bcl6, STAT5 and GATA3 binding to T_H2 cytokine gene loci resembled the findings in T_H2LPs (Fig. S2 *C–E*). Therefore, in T_H2LPs and mT_H2 cells, Bcl6 seems to directly outcompete STAT5 and indirectly attenuate GATA3 binding to T_H2 cytokine gene loci. To investigate the effect of Bcl6 on the level of corresponding transcriptional regulators for chromatin remodeling, we examined T_H2EPs and T_H2LPs from WT, *Bcl6*-KO, and *Bcl6*-transgenic (*Bcl6*-TG) mice. Intracellular staining (Fig. S3*A*) and Western blotting (Fig. S3*B*) revealed similar levels of critical activators, including GATA3 and phosphorylated STAT5 (pSTAT5), regardless of Bcl6 levels at each culture phase. Notably, the $pSTAT5^+$ population of

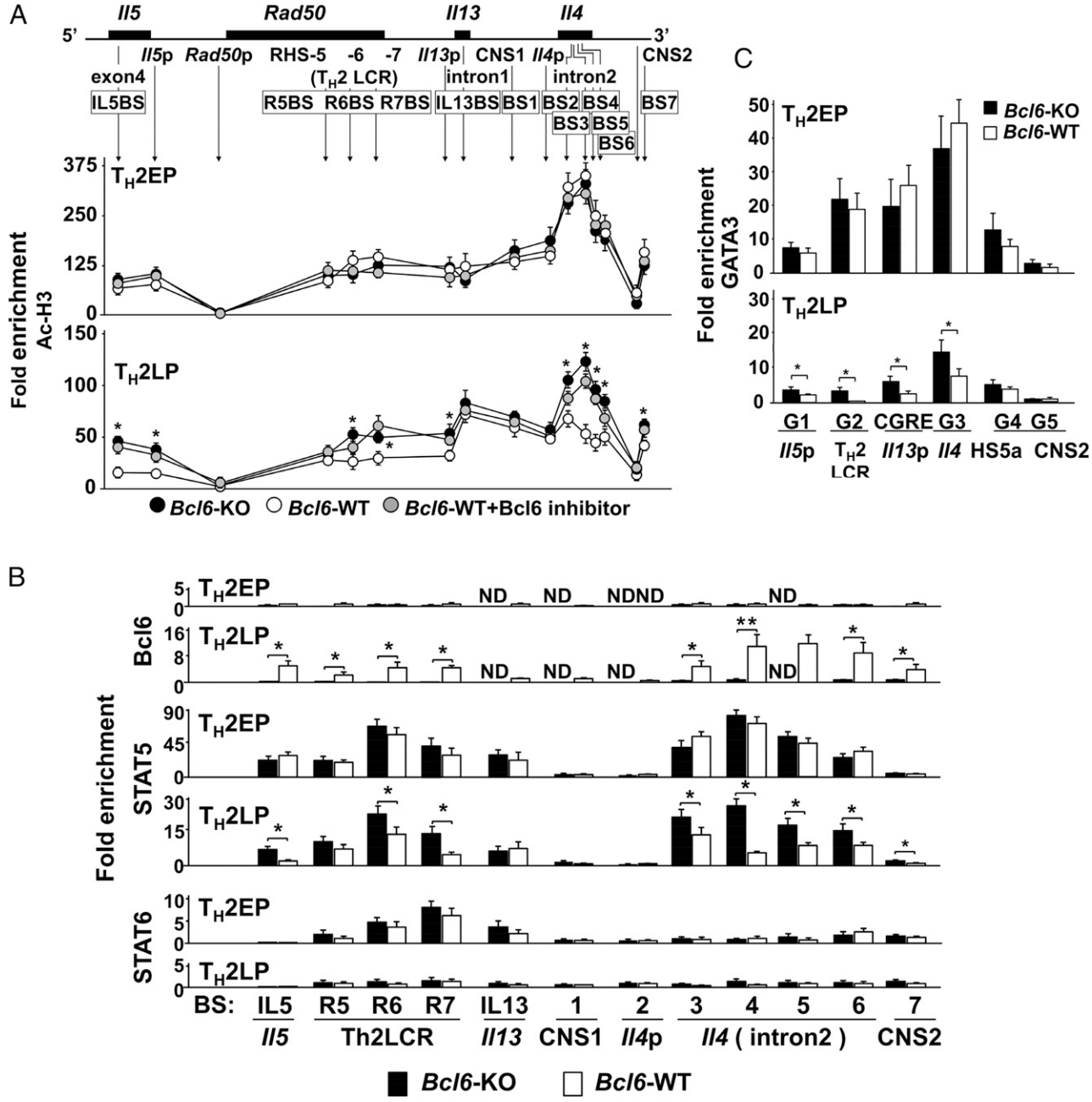

Fig. 2. Role of Bcl6 in chromatin remodeling of T_H2 cytokine gene loci. In vitro or transferred KJ-1-26$^+$ T_H2 cells [immunoprecipitation (IP) and LP] were analyzed by ChIP–quantitative PCR. The IP level at each site is presented as the relative fold enrichment compared with the minimum values. (*A*) Acetylation level of histone H3 at K9 and K14 in *Bcl6*-KO, WT, and Bcl6 inhibitor-pretreated WT T_H2 cells. (*B*) Binding of Bcl6, STAT5, and STAT6 to each BS in *Bcl6*-KO and WT T_H2 cells. (*A* and *B*) Data are pooled from five to seven independent experiments. (*C*) GATA3-binding G sites in *Bcl6*-KO and WT T_H2 cells. Data shown are means ± SDs ($n = 5$–6). All results are representative of (*A*) six or (*B* and *C*) seven independent experiments with similar outcomes. ND, not detected. $*P < 0.05$; $**P < 0.01$.

T_H2EPs is mainly included in the GATA3-expressing population and present at a similar ratio in WT and *Bcl6*-KO mice, regardless of IL-33 and/or TCR restimulation. T_H2LPs exhibited no significant GATA3$^+$ population but consistently expressed some pSTAT5, regardless of the *Bcl6* genotype. Notably, GATA3 protein expression levels were similar between WT and *Bcl6*-KO T_H2LPs after restimulation (Fig. S3*A*). Bcl6 protein levels had no apparent changes at each culture phase in WT and TG cells (Fig. S3*B*).

Dampening of Bcl6-Mediated Suppressor Activity by an Intron Enhancer in the *Il4* Locus. We focused on the *Il4* intron 2 region, wherein Bcl6/STAT5 strongly associates with multiple sites in mT_H2 cells. The DNA sequences of this 222-bp region, which included BS3 and G3, are highly conserved between mice and humans and designated as the highly conserved intron enhancer (hcIE) (Fig. 3*A*). The hcIE was included in DNase I hypersensitive sites (HS)2 (1.2 kbp), at which G3 was identified as a critical regulatory region for GATA3 binding-mediated *Il4* expression in a study of *HS2*-KO mice (26). To examine the role of hcIE, we generated hcIE-KO mice (Fig. S4) and observed markedly diminished IL-4 production in both hcIE-KO T_H2EPs and T_H2LPs compared with that in WT cells. Notably, hcIE-KO T_H2EPs and T_H2LPs also displayed reduced, albeit partially reduced, IL-5 and IL-13 production relative to WT cells, even in the presence of abundant exogenous

IL-4 (Fig. 3*B*). In T_H2EPs, increased Bcl6 binding and decreased STAT5 binding at *Il5*, *Rad50*, and *Il4* intron 2 (BS4–BS6) and decreased GATA3 binding at *Il5*, *Rad50*, *Il13*, and CNS2 were widely observed across the T_H2 cytokine gene loci when hcIE was abolished (Fig. 3 *C* and *D*). The differences in transcriptional factor binding dwindled between WT and hcIE-KO T_H2LPs.

Because Bcl6 binding was augmented in hcIE-KO T_H2 cells, hcIE may dampen this Bcl6-mediated suppressor activity. In fact,

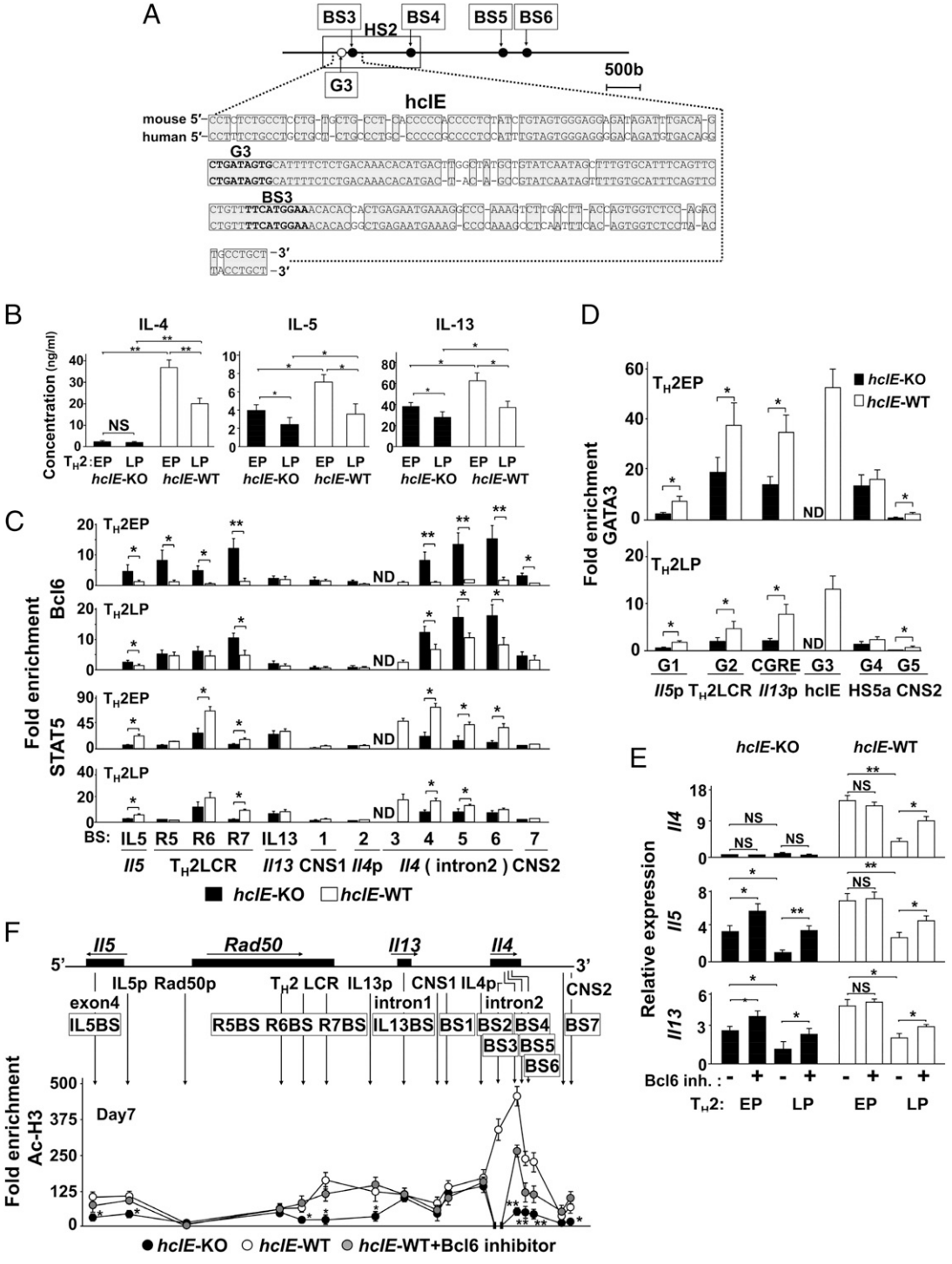

Fig. 3. Role of hcIE in the Bcl6 suppressive function for T_H2 cytokine gene loci. (*A*) A conserved sequence [positions +1,180 to +1,401 relative to the transcription start site; Mouse Genome Informatics (MGI) accession no. 96556] in the HS2 region of mouse hcIE is shown along with human hcIE. Conserved sequences between mice and humans are indicated by shaded boxes. (*B*) Cytokine production (EP and LP) by WT and hcIE-KO T_H2 cells estimated after restimulation with anti-CD3 Abs for 48 h. (*C* and *D*) Analysis of binding levels of (*C*, *Upper*) Bcl6, (*C*, *Lower*) STAT5, and (*D*) GATA3 by ChIP–quantitative PCR for hcIE-KO and WT T_H2 cells. (*E* and *F*) Effect of Bcl6 on cytokine gene expression estimated in EPs and LPs after restimulation for 24 h and acetylation of histone H3 at K9 and K14 in EPs at rest with/without Bcl6 inhibitor in hcIE-KO and WT T_H2 cells. Data are shown as means ± SDs. Data are representative of (*B*) four, (*C*) eight, (*D* and *E*) six, or (*F*) seven independent experiments with (*B*) $n = 4$–5, (*C*) $n = 7$–8, (*D*) $n = 5$–6, (*E*) $n = 6$–8, or (*F*) $n = 6$–7 samples in each experiment. ND, not detected; NS, not significant. $^*P < 0.05$; $^{**}P < 0.01$.

T_H2 cytokine gene expression, excluding *Il4*, was significantly restored in hcIE-KO T_H2EPs and T_H2LPs as well as in WT T_H2LPs after Bcl6 inhibitor treatment (Fig. 3*E*). In accordance with gene expression, Ac-H3 levels at *Il5*p and *Il13*p (except at *Il4*p) and those at various BSs (excluding *Il13*BS, BS1, and BS2) were distinctly reduced in hcIE-KO T_H2EPs compared with those in WT T_H2EPs (Fig. 3*F*). The reduced Ac-H3 levels were significantly restored in hcIE-KO T_H2EPs after Bcl6 inhibitor treatment, although this inhibitor had less effect on the residual intron 2 region relative to other sites. The Bcl6 mRNA expression level in WT mT_H2 cells but not in WT T_H2LPs cells was slightly elevated compared with that in WT T_H2EPs, whereas the level in each T_H2 cell type was constant, regardless of hcIE function (Fig. S5). Therefore, hcIE is critical for chromatin remodeling of T_H2 cytokine gene loci in T_H2 cells through the regulation of Bcl6-mediated suppressor activity.

Association Between IL-33 and Bcl6 in T_H2 Cells. The T_H2-promoting cytokine IL-33 is known to induce IL-5 and IL-13, but not IL-4, production in T_H2 cells (19, 20). We confirmed this effect of IL-33 on T_H2EPs (Fig. S6*A*). Increased expression of ST2, an IL-33 receptor subunit, has been shown in ex vivo WT mT_H2 cells (27), and we also confirmed augmented ST2 expression in WT T_H2LPs (Fig. S6*B*) as well as WT mT_H2 cells (Fig. S6*C*). The range of effective concentrations of IL-33 for inducing IL-4 in WT mT_H2 cells was wider than those of the other T_H2-promoting cytokines, namely TSLP and IL-25 (Fig. S6*D*). TCR-restimulated IL-4 production was significantly reduced in *Bcl6*-TG mT_H2 cells compared with that in WT cells. In contrast, there was no significant difference in IL-4 production between WT and *Bcl6*-KO mT_H2 cells in the presence of IL-33 (Fig. 4). To examine T_H2 phenotypes, T_H2 cells were simultaneously analyzed for ST2 and intracellular GATA3 expression levels. No significant differences were observed between the WT and *Bcl6*-KO for T_H2EPs and mT_H2 cells, regardless of IL-33 and/or TCR restimulation (Fig. S6*C*). Restimulation induced no apparent changes in ST2 expression or GATA3 protein levels in WT or *Bcl6*-KO T_H2EPs, whereas both ST2 expression and GATA3 expression levels were similarly increased in WT and *Bcl6*-KO mT_H2 cells after IL-33 and TCR restimulation. When we focused on the functional association between IL-33 and Bcl6 in mT_H2 cells, 30 ng/mL IL-33 augmented IL-4 production strongly in WT mT_H2 cells and modestly in *Bcl6*-KO mT_H2 cells (Fig. 4 *A*–*C*) and concomitantly, induced *Il4* expression after TCR stimulation (Fig. 4*D*). The effects of IL-33 on IL-5 (Fig. 4*C*) and IL-13 (Fig. 4 *A*–*C*) production and their gene expression (Fig. 4*D*) were apparently observed in WT mT_H2 and *Bcl6*-KO cells. These effects of IL-33 on each T_H2 cytokine were similar in the cell types, although cytokine production was dominated by *Bcl6*-KO cells rather than WT cells in the absence of IL-33.

Effects of IL-33 on Bcl6-Mediated Histone Modification. Because differences in TCR-stimulated cytokine production between WT and *Bcl6*-KO mT_H2 cells were diminished by IL-33 pretreatment in a concentration-dependent manner, we hypothesized that IL-33 may exert its effect by attenuating the function of Bcl6. In WT mT_H2 cells, Bcl6 binding to BSs was reduced by exogenous IL-33 partially at 10 ng/mL and fully at concentrations exceeding 30 ng/mL (Fig. 5*A*, *Upper*), at which histone acetylation within T_H2 cytokine gene loci resembled that in *Bcl6*-KO cells. *Bcl6* mRNA expression did not significantly change in mT_H2 cells after IL-33 treatment (Fig. S6*E*). Because GATA3 protein levels were minimal in T_H2LPs (Fig. S3) and mT_H2 cells (Fig. S6*C*) at rest in WT and *Bcl6*-KO mice, we examined GATA3 binding to these loci by IL-33, which induces *Gata3* expression and/or phosphorylation and subsequent IL-5 and/or IL-13 production (27).

Although the isolated WT mT_H2 cells displayed inferior GATA3 binding to the major sites compared with *Bcl6*-KO cells, IL-33 could significantly augment this binding (Fig. 5*A*, *Lower*) along with increases in histone acetylation at the responsible sites (Fig. 5*B*) to similar levels between *Bcl6*-KO and WT cells. The effects of IL-33 on histone acetylation within T_H2 cytokine gene loci occurred in a concentration-dependent manner. Bcl6 detaching and GATA3 binding were particularly associated with histone acetylation regarding T_H2 cytokine genes. Chromatin modification of *Il5* occurred in response to IL-33 at lower concentrations than those observed for *Il4* and *Il13* in WT mT_H2 cells, indicating that regulation of *Il5* is sensitive to IL-33 function among the gene foci. A maximum concentration of IL-33 broadly increased histone acetylation levels at BSs other than G sites to a similar extent in both WT and Bcl6-KO mT_H2 cells.

Because ERK-1– and ERK-2–mediated phosphorylation induces Bcl6 degradation (28), we hypothesized that IL-33–induced MAPK activation (19) is important for Bcl6-mediated decreases in T_H2 cytokine production in mT_H2 cells. In cultured T_H2LPs, ERK phosphorylation levels increased within 30 min of stimulation with 10 ng/mL IL-33; conversely, Bcl6 protein levels decreased to 10% of baseline, but they were restored in the presence of FR180204, which selectively inhibits ERK-1/2 activity (29) (Fig. 5*C*).

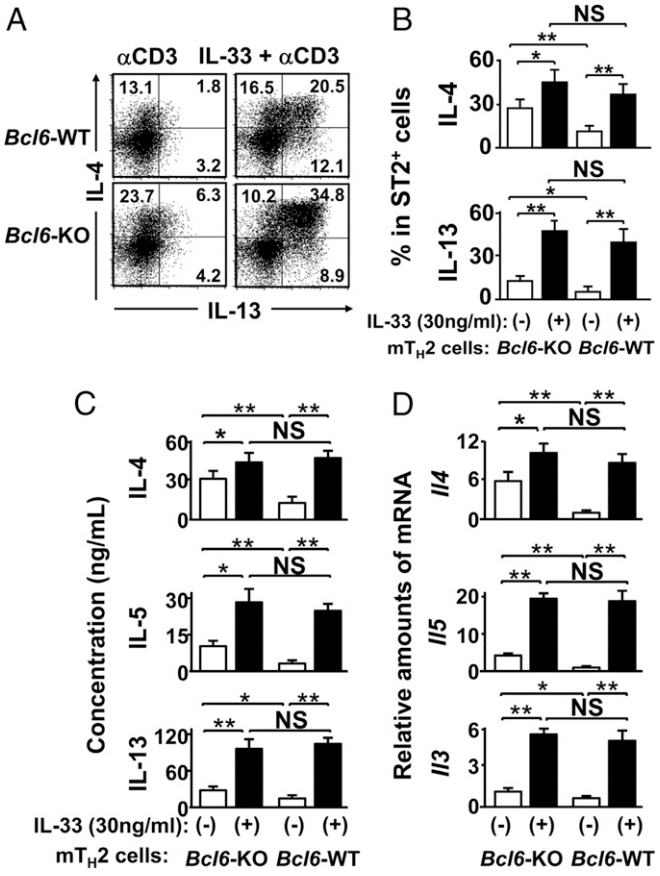

Fig. 4. Role of IL-33 in cytokine production by mT_H2 cells. KJ-1-26$^+$ T_H2EPs from WT or *Bcl6*-KO mice were adaptively transferred into naïve WT BALB/c mice. Twenty-eight days later, mT_H2 cells were isolated from the recipient spleens. These mT_H2 cells were incubated ex vivo with IL-33 or medium alone for 3 h before reactivation with anti-CD3 Abs. (*A*) FACS analysis for IL-4– and IL-13–producing cells among T_H2 cells (gate: ST2$^+$ populations) after restimulation. The figure shows representative FACS data. The numbers in each corner represent the percentages of T cells in the column. (*B*) Percentage of the indicated cytokine-positive population (gate: ST2$^+$ T_H2 cells). (*C*) Protein concentration in culture supernatants at 48 h and (*D*) mRNA levels at 24 h for IL-4, IL-5, and IL-13 produced by the sorted ST2$^+$ T_H2 cells after restimulation. (*B*–*D*) Data are shown as means ± SEMs (n = 7–8). All results are representative of eight independent experiments with similar outcomes. NS, not significant. *$P < 0.05$; **$P < 0.01$.

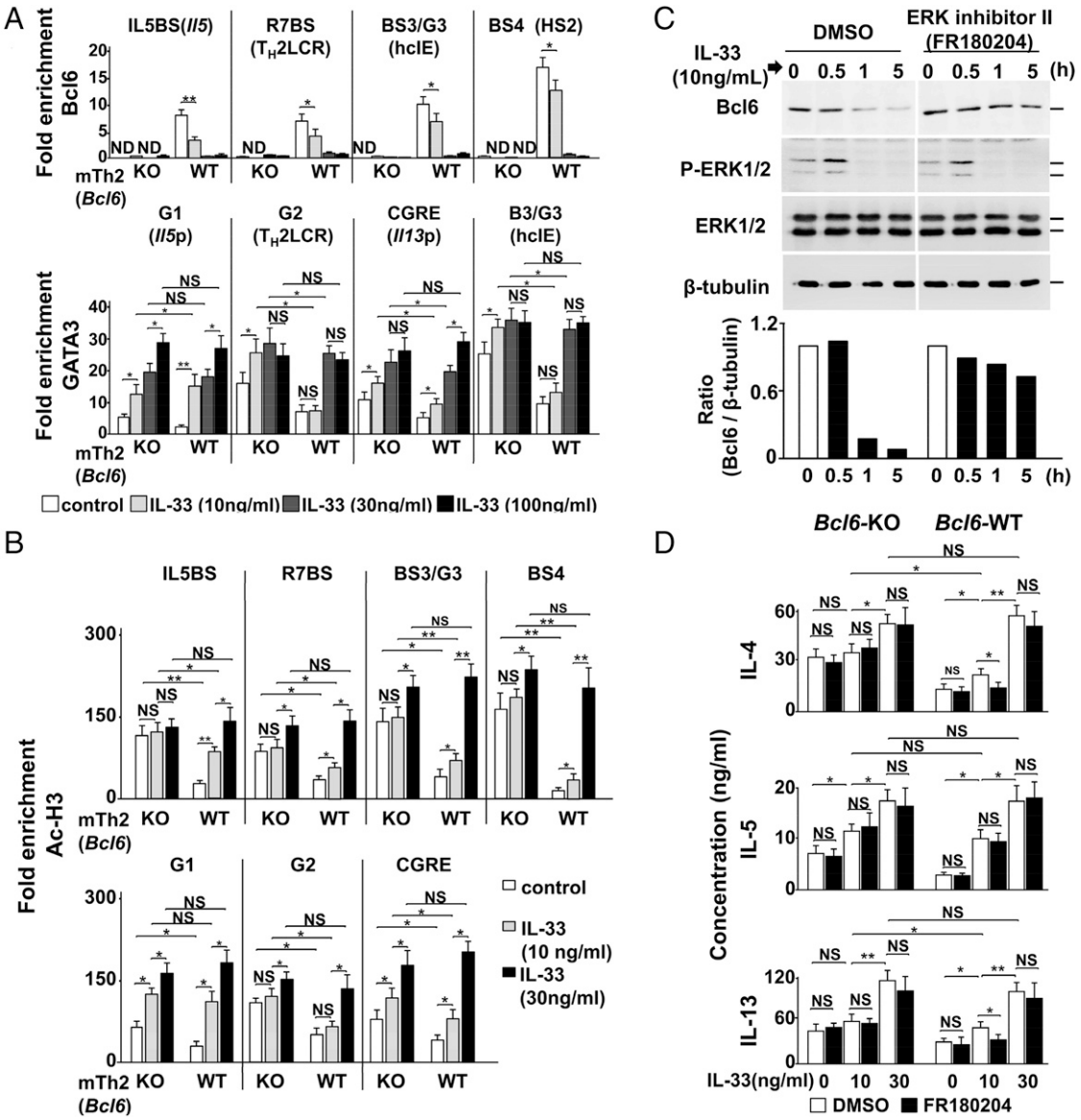

Fig. 5. Role of IL-33 in the functioning of Bcl6 at T_H2 cytokine gene loci. (*A* and *B*) ChIP analyses of (*A*, *Upper*) Bcl6 and (*A*, *Lower*) GATA3 binding and (*B*) Ac-H3 in the T_H2 cytokine gene loci of *Bcl6*-WT KJ-1-26$^+$ mT_H2 cells isolated from recipient spleens. Cells were incubated with IL-33 (10, 30, or 100 ng/mL) or medium alone as a control for 6 h before the analysis. (*C*) Bcl6 protein levels in T_H2LPs from immediately before (0 h) until 5 h after stimulation with IL-33 (30 ng/mL) in the presence of an ERK inhibitor (FR180204) or vehicle. Bcl6, pERK-1/2, and ERK-1/2 protein levels were analyzed. β-Tubulin was used as a loading control. The figure shows representative data from (*C*, *Upper*) a Western blot and (*C*, *Lower*) densitometric analysis. The quantified amount of each protein is indicated as the ratio of densitometric values of bands containing the protein between controls and experimental samples. (*D*) T_H2 cytokine production by *Bcl6*-WT mT_H2 cells. Concentrations of T_H2 cytokines in the culture supernatants of mT_H2 cells preincubated ex vivo with IL-33 for 3 h followed by activation with anti-CD3 Abs in the presence of IL-33 with/without FR180204 for 48 h. Data are shown as means ± SEMs (n = 5–12). All results are representative of (*A*–*C*) six or (*D*) seven independent experiments with similar outcomes. NS, not significant. *$P < 0.05$; **$P < 0.01$.

IL-4, IL-5, and IL-13 production was increased by IL-33 in a concentration-dependent manner in WT and *Bcl6*-KO mT_H2 cells. In particular, IL-5 production levels in both cell types were remarkably similar in the presence of IL-33 at any concentration, and its production was not prevented by ERK inhibition. In WT mT_H2 cells, 10 ng/mL IL-33 slightly increased the production of IL-4 and IL-13, and their levels in WT cells approached those in KO cells. This effect of IL-33 was significantly suppressed by ERK inhibition in WT mT_H2 cells but not KO cells. A higher concentration of IL-33 (100 ng/mL) further augmented IL-4 and IL-13 production; meanwhile, their levels were not reduced by ERK inhibition, and they were similar between WT and *Bcl6*-KO mT_H2 cells (Fig. 5*D*), indicating that IL-33 could introduce permissive chromatin modification of T_H2 cytokine genes via some critical functions, such as GATA3 activation, in addition to Bcl6 degradation.

Role of Bcl6 in the Effects of IL-33 Regarding an mT_H2 Cell-Mediated Allergic Response. When *Bcl6*-KO or WT T_H2EPs on a DO11.10 background were adoptively transferred into naïve WT mice, we observed that *Bcl6*-KO mT_H2 cells exacerbated allergic airway inflammation with increases in T_H2 cytokine production and IgE Ab levels during the chronic phase (Fig. S7). Accordingly, to examine the association between Bcl6 and high IL-33 levels, we modified our OVA model by simultaneously administering bacteria-derived LPSs, which are known to induce IL-33 production (30) (OVA-LPS model). When *Il33*-WT recipients were challenged with OVA

or OVA plus LPS during the chronic phase, LPS augmented the OVA-induced recruitment of mT_H2 cells (Fig. S8*A*) and increase in IL-33 levels, which were much larger than those of IL-25 and TSLP (Fig. S8*B*) in the lungs in both *Bcl6*-KO and WT T_H2 cell recipients. In response to excess IL-33 production in the OVA-LPS model (Fig. 6*A*), T_H2 cytokine gene expression was augmented in mT_H2 cells isolated from mediastinal lymph nodes (MLNs) similarly among *Il33*-WT recipients of *Bcl6*-KO and WT T_H2 cells relative to cells challenged with OVA alone (less IL-33 production). In the OVA-LPS model, *Il33*-KO recipients displayed a significant attenuation of expression of each cytokine gene, regardless of the *Bcl6* genotype of T_H2 cells (Fig. 6*A*). Expression of *Il14*, *Il5*, and *Il13* was not different between WT mT_H2 and *Bcl6*-KO cells, although differences in *Il4* expression in *Bcl6*-KO cells between *Il33*-KO and *Il33*-WT recipients were modest compared with those in WT cells (Fig. 6*A*). Notably, in the presence of OVA alone, *Il4* and *Il13* expression was not affected by the presence of IL-33 (Fig. 6*A*, *Middle*). Conversely, T_H2 cytokine levels in bronchial alveolar lavage fluid (BALF) were apparently increased in the OVA-LPS model for both genotypes of T_H2 cells compared with those in the OVA model (Fig. S8*C*). However, contrary to the gene expression profile in MLNs, cytokine protein levels were not significantly affected by the absence of IL-33 in this model (Fig. S8*C*). Finally, serum IgE levels were markedly increased to a similar extent among all *Il33*-WT recipients with WT or *Bcl6*-KO mT_H2 cells in the OVA-LPS model compared with those in the OVA alone model (Fig. 6*B*). On the contrary, in the OVA alone model, IgE levels in *Il33*-WT recipients with *Bcl6*-KO mT_H2 cells were more strongly elevated than those in the presence of WT mT_H2 cells. However, IL-33 was not significantly involved in IgE Ab production, regardless of the *Bcl6* genotype of mT_H2 cells in not only the OVA model but also, the OVA-LPS model. The IgE production levels closely corresponded to gene expression levels in MLNs rather than to those in BALF.

Discussion

We showed that T_H2 cytokine genes are negatively regulated by Bcl6 through chromatin remodeling and that interactions between Bcl6 and STAT5 physiologically contribute to histone modulation and consequently, cytokine production in mT_H2 cells rather than to T_H2 cell differentiation. Although *Bcl6*-KO mice exhibit severe general tissue eosinophilia with striking cytokine production of the T_H2 type but not the T_H1 type, the intrinsic functions of Bcl6 for T_H cell differentiation, in particular T_H1 and T_H2, remain obscure. In earlier reports, we and other groups previously showed that Bcl6 has no significant intrinsic function for both T_H1 and T_H2 differentiation in the full commitment experiments in vitro. In later studies, which focused on T_{FH} cells, Bcl6 was shown to suppress effector T cells, including T_H1, T_H2, and T_H17 cells, resulting in the induction of T_{FH} cell differentiation. For T_{FH} differentiation, exogenous Bcl6 in T cells was found to suppress *T-bet* at the gene level. Conversely, Bcl6 suppressed GATA3 function by reducing protein stability or repressing gene expression via microRNA. By contrast, we observed in this study that the expression of *T-bet* as well as *Gata3* was not influenced by Bcl6 in T_H2EPs, T_H2LPs, or mT_H2 cells. *T-bet* expression in each type of T_H1 cells was also not reduced by intrinsic Bcl6. On the contrary, intrinsic Bcl6 attenuated T_H2 but not T_H1 cytokine production by T_H2EP-derived memory T cells in BALB/c-recipient mice. However, when T_H2EPs were adaptively transferred into nude mice, Bcl6 appeared to play an antagonistic role for both T_H1 and T_H2 cytokine production in the memory donor T cells (Fig. S9). There was a possibility that T_H2EPs transferred into nude mice possibly experience homeostatic proliferation. It was previously indicated that a memory phenotype, central memory (CM) T cells, was maintained by homeostatic proliferation induced under a lymphopenic condition. Because CM T cells display plasticity of helper function (31), T_H2EPs might proliferate to give rise to CM T_H1 cells in nude mice, especially under the *Bcl6*-KO condition, suggesting that Bcl6 could be involved in T_H cell functions other than T_H2 cells under

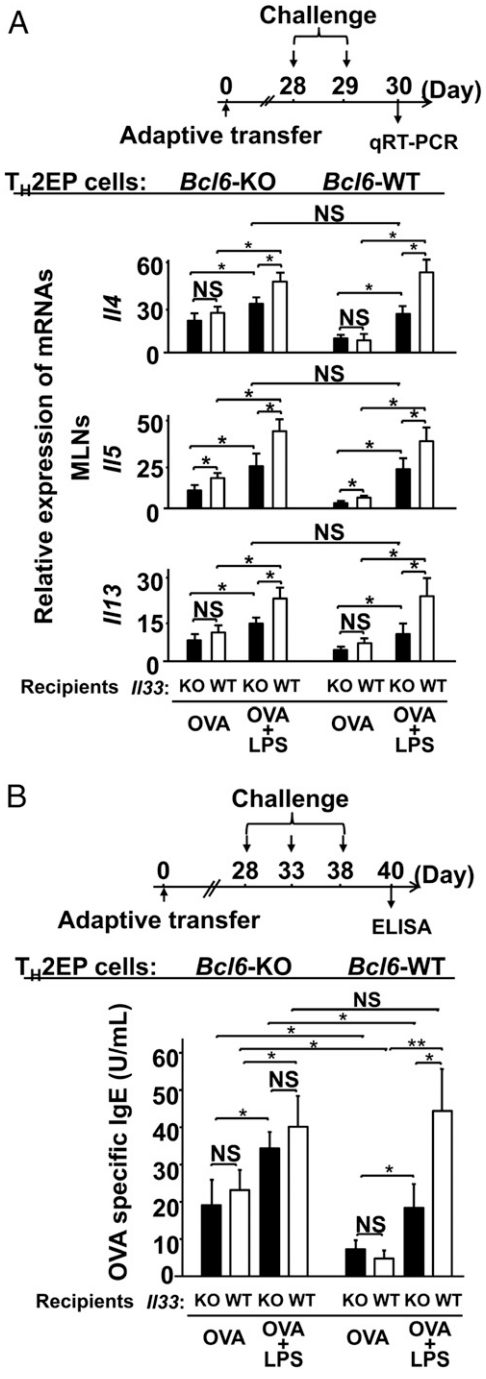

Fig. 6. Role of Bcl6 on the effects of IL-33 regarding mT_H2 cell-mediated allergic responses. WT and Bcl6-KO KJ-1$^+$ T_H2EPs (3×10^7) were adoptively transferred into IL-33–WT or –KO BALB/c background recipient mice. (*A*) The recipient mice were given two intratracheal administrations of OVA or LPS plus OVA at a 12-h interval on day 28 posttransfer. Levels of *Il4*, *Il5*, and *Il13* mRNA in mT_H2 cells isolated from MLNs 36 h after the final challenge (day 30) are shown. (*B*) Recipients were challenged with three instillations of either OVA or saline at 5-d intervals beginning on day 1 or 28 posttransfer. Serum from each recipient was subjected to analysis to determine the titer of OVA-specific IgE Abs at 48 h (day 40) after the final challenge. Data are shown as means ± SEMs with (*A*) n = 7–10 or (*B*) n = 10–13. (*A*) Results are representative of three independent experiments with similar outcomes. (*B*) Data are pooled from five independent experiments. NS, not significant. *$P < 0.05$; **$P < 0.01$.

certain settings. In this study, to focus on authentic mT_H2 cells, we analyzed WT BALB/c mice as a more representative physiological condition as opposed to nude mice (32).

For STAT5, GATA3 is required for rapid histone acetylation at HS2 during early T_H2 cell differentiation (33). Zhu et al. (33) also showed that STAT5-induced chromatin accessibility at the T_H2 cytokine loci of T_H2 cells was augmented by GATA3 overexpression, indicating that GATA3 binding to G3 may support STAT5 binding to BSs in T_H2 cytokine loci and maintain the permissive chromatin remodeling of T_H2 cells. Therefore, STAT5 and GATA3 cooperate in permissive histone modification of the *Il4* locus by binding to hcIE during T_H2 cell differentiation and memory development. GATA3 directly activates *Il5*p and *Il13*p but not *Il4*p (34, 35). IL-5 and IL-13 production is completely lost after the conditional deletion of *Gata3* after T_H2 cell lineage commitment (35). However, the IL-4 production level is apparently reduced in these *Gata3*-deleted T_H2 cells, despite no change in IL-4^+ cell frequency. Thus, hcIE may induce and maintain active chromatin structures of the *Il4* locus to control the efficiency and rate of transcription by protecting them against epigenetic silencing, similar to a globin gene enhancer (36). Accordingly, to induce chromatin activation effectively in the *Il4* locus, STAT5- and GATA3-mediated epigenetic activity of hcIE may be induced in differentiating T_H2 cells by directly and/or indirectly protecting the Bcl6-mediated silencing, because Bcl6 binding was augmented in the absence of hcIE. Regarding the relationship with STAT5, Bcl6-binding DNA sequences resemble the IFN-γ–activated sequence motif bound by STAT proteins (10), suggesting that Bcl6 represses T_H2 cytokine expression via competitive binding against STAT factors in T_H2 cytokine gene loci (7), which results in a shift toward histone deacetylation. On the contrary, it is certain that Bcl6 reduces GATA3 binding based on data from the *Bcl6*-KO study. In T_H2LPs, GATA3 and pSTAT5 levels decreased, whereas Bcl6 protein levels remained nearly unchanged throughout the long-term culture period. Accordingly, during the memory phase, hcIE activity may be suppressed by Bcl6. However, uncertainty largely remains regarding the Bcl6-mediated machinery for GATA3 binding without changes in its gene expression. Therefore, additional studies are required in the future. Bcl6 might function in a concentration-dependent manner; however, we believe that the suppressor activity does not simply depend on its levels, because we observed that cytokine production by T_H2EPs derived from Bcl6-TG mice was not reduced compared with that in Bcl6-KO T_H2EPs (Fig. S10). Dent et al. (37) also showed that *Bcl6*-KO T_H1 cells have no ability to produce T_H2 cytokines. Accordingly, we think that some activators (GATA3 and STATs) are essential for transcriptional processes in which the repressor activity of Bcl6 can be determined in functional balance with transcriptional activators, such as GATA3 and STATs. Therefore, in mT_H2 cells, Bcl6 properly tunes *Il4* chromatin modification by preventing exaggerated activation of the gene, because it is strongly suggested that hcIE regulates chromatin remodeling at the *Il4* locus by Bcl6 through direct competition with STAT5 and/or through indirect functional competition with GATA3. We previously reported that Bcl6 binds to *Il5*BS (9), also revealing that *Il13* was regulated by Bcl6, despite the apparent lack of Bcl6 binding to IL13BS. However, histone acetylation levels at *Il13*p and GATA3 binding to CGRE were influenced by Bcl6 in mT_H2 cells. Regarding this result, Bcl6 might be involved in chromatin remodeling at the T_H2LCR, which positively affects the coordinated expression (38) of *Il4* and *Il13*. Accordingly, Bcl6 seems to negatively regulate these genes by directly regulating the function of T_H2LCR, raising a query about the effects of hcIE on *Il5* and *Il13*, because Bcl6 inhibitor treatment restored their expression levels in hcIE-KO T_H2 cells. Although there is a possibility of cooperative repression by Bcl6 via binding to hcIE for T_H2 cytokine gene foci, additional studies are required to investigate this obscure mechanism.

We investigated whether the function of Bcl6 is physiologically modified at the protein level in some pathological settings without gene manipulation, resulting in regulatory perturbation of T_H2 cytokine genes. Because ERK-phosphorylated Bcl6 is degraded by the ubiquitin–proteasome system (28), we observed a decrement of Bcl6 protein expression in mT_H2 cells in an IL-33–induced ERK-1/2 activation-dependent manner in vitro. As was observed in T_H2 cytokine production by mT_H2 cells, in which Bcl6 dissociated from target chromatin in the presence of IL-33, we hypothesized that the ERK signaling-mediated Bcl6 decrement might have a prominent role in cytokine gene regulation. Unfortunately, ERK inhibition did not reduce the IL-33–induced hyperproduction of T_H2 cytokines, such as IL-4, indicating that mT_H2 cell exposure to excess IL-33 reinforces permissive T_H2 cytokine gene modification by both decreasing Bcl6 protein levels and inducing GATA3, a representative IL-33–induced activator. In contrast, IL-33 augments the production of IL-5 and IL-13, but not IL-4, in T_H2EPs after TCR restimulation (19), supporting the hypothesis that, in highly polarized T_H2 cells, intron 2 of the *Il4* locus is completely opened by activated GATA3 before IL-33 exposure, which activates *Il5* and *Il13* but not *Il4* by inducing GATA3 binding to the respective promoters. In addition to IL-33, IL-25 and TSLP are also known to induce T_H2 cytokine production via the expression of some critical transcriptional activators, such as *Gata3*, in T_H2 cells. Among the T_H2-promoting cytokines, we observed a wider range of effective concentrations of IL-33 than IL-25 and TSLP for inducing IL-4 in ex vivo WT mT_H2 cells. Because we observed that IL-33 exhibited a greater increase than IL-25 and TSLP in the OVA-LPS model, we expected IL-33 to play an important role in not only antagonizing Bcl6 function via protein degradation but also, inducing GATA3 for the activation of T_H2 cytokine genes, particularly *Il4*, in mT_H2 cells. Consistent with this finding, higher concentrations of IL-33 enhance the production of T_H2 cytokines, such as IL-4, in both WT and *Bcl6*-KO mT_H2 cells. These data imply that IL-33 at lower levels can function as an inducer by mainly suppressing Bcl6 via histone deacetylation; however, IL-33 at higher levels can function as a strong inducer via an additional function of activating GATA3. Conversely, in the presence of large amounts of Bcl6 (i.e., *Bcl6*-TG mT_H2 cells), TCR restimulation-induced IL-4 production was lower in *Bcl6*-TG mT_H2 cells; however, the inhibitory effect became moderate with increased IL-33 levels. Thus, Bcl6 could play an important role as an attenuator of hyperimmune response and inflammation against allergic pathology, whereas IL-33–induced functions could overcome Bcl6 activity in the highly severe pathology of asthma. In this pathology, IL-33 could induce robust inflammation with increases in IL-4 production and IgE serum levels, regardless of the presence of Bcl6 in the OVA-LPS model. These results indicate that Bcl6 functions as a repressor for the T_H2 cytokine production in the OVA model, whereas in the OVA-LPS model, IL-33 did more for the cytokine production in mT_H2 cells than alleviate BCL6-mediated suppression in a more severe allergy pathology. However, the possibility of there being other significant activating factors other than GATA3 for T_H2 response induction remains.

IL-4 should critically contribute to the development of allergic diseases, including asthma, because STAT6 phosphorylation in B cells is required for IgE class switching, depending on IL-4 in mice and either IL-4 or IL-13 in humans. In addition, *Il4*/*Il13*-KO mice exhibited completely abolished IgE production (39). Recently, IL-4–producing T_{FH} cells with low-level *Gata3* expression were shown to induce IgE class switch recombination (CSR) by direct Ig class switching in the germinal center (GC), resulting in short-lived plasma cells producing low-affinity IgE Ab (40). Thus, transfer T_H2 cell-derived T_{FH} cells might be involved in CSR to IgE. On the contrary, it has been reported that high-affinity memory IgG^+ B cells derived from GC migrate to the outer follicle and differentiate into high-affinity IgE^+ B cells via sequential Ig class switching with IL-4 (41). Furthermore, it has been suggested that IL-4 derived from T_H2 cells, rather than T_{FH} cells, is critical at the outer follicle for sequential class switching for memory allergic responses (39).

When we examined OVA-specific Ab production in an allergic model (Fig. S11), hcIE-KO mice, in which T_{FH} cells could produce sufficient IL-4 similarly to WT mice (Fig. S11 *A* and *B*), exhibited a normal memory response for IgG1 production after antigen challenge (Fig. S11*C*). By contrast, IgE production, the primary response of which was not detected, was strikingly impaired in hcIE-KO mice after challenge (Fig. S11*C*), supporting that IgE production is mainly conducted as a memory response depending on mT_H2 cells but not T_{FH} cells. In addition, because Bcl6 is a critical master regulator for T_{FH} cell differentiation, it is unlikely that *Bcl6*-KO T_H2 cells give rise to T_{FH} cells that augment IgE production because of the impairment of T_{FH} cell differentiation caused by deficiency of T cell-intrinsic Bcl6 (12–14).

It is known that IL-33 induces innate cells to produce IL-4, leading to allergen-nonspecific IgE production and T_H2 cell differentiation (22). However, the molecular mechanism by which IL-33 regulates cytokine production, in particular IL-4 production, by mT_H2 cells remains unclear. Our T_H2 cell transfer murine models showed that IL-33 is involved in the augmentation of IL-4 and antigen-specific IgE Ab levels by mT_H2 cells. However, the use of *Il33*-KO recipients in the OVA-LPS model illustrated a partial effect of IL-33 on both *Bcl6*-KO and WT mT_H2 cells in this severe asthma model. This result suggests that some unidentified mechanisms/factors, including IL-25 and TSLP, are also involved in the T_H2-promoting function in vivo. However, we propose that functional competition for epigenetic regulation via a balance between activation induced by T_H2-promoting factors, such as IL-33, and Bcl6-mediated suppression has enormous significance in the development of allergic diseases. Because some pathogenic bacteria contribute to the severity of asthma (42), the OVA-LPS model implies that the pathogenesis of chronic allergic diseases, particularly asthma and its exacerbation, is caused by the elevation of numerous risk factors, including IL-33 and IL-25. These factors may play an important role in conquering the protective function of Bcl6 against T_H2-type allergic immune responses.

Concerning the role of Bcl6 in the regulation of cytokine gene expression, we discovered a function of Bcl6 that directly regulates *Il4* and *Il5* and indirectly regulates *Il13* at the chromatin level. We considered that Bcl6 physiologically promotes appropriate T_H2 cytokine production in mT_H2 cells and that functional balance between Bcl6 and activators may critically direct *Il4* activation in particular. Such a function of Bcl6 could be involved in protection against the development of allergic disease. Because the repressor activity of Bcl6 must be influenced by activators, including GATA3 and STAT5, even in the presence of excess Bcl6, activators overcoming the repressor can induce *Il4* expression. Therefore, we propose a mechanism whereby the interaction between T_H2-promoting factors and Bcl6 tunes T_H2 cytokine gene activation and contributes to the pathology of severe allergic diseases, and therefore, dampening of Bcl6 may be involved in the maintenance and exacerbation of chronic allergic pathogenesis. Thus, T_H2-promoting factors that suppress Bcl6 function may represent therapeutic targets for T_H2 cell-mediated disease.

Materials and Methods

Abs and Reagents. The Abs and reagents are described in *SI Materials and Methods*.

Mice. *Bcl6*-KO (43) and Bcl6-TG mice with exogenous *Bcl6* under the control of the Lck proximal promoter (44) have been described previously. These mice were backcrossed with BALB/c mice more than 10 times. DO11.10-TG mice were purchased from The Jackson Laboratory. *Il33*-KO mice (45) have been described previously. hcIE-KO mice were generated at the Biomedical Research Center, Chiba University (*SI Materials and Methods*). All mice were used at 8–12 wk of age. All procedures conformed to the Chiba University Resolution on the Use of Animals in Research, and they were approved by the Institutional Animal Care and Use Committee at Chiba University School of Medicine. Mice were maintained under specific pathogen-free conditions in the animal center of Chiba University Graduate School of Medicine.

Purification of CD4 T Cells and Induction of T_H Cells. Splenic naïve CD4 T cells and transferred T cells were isolated by a cell sorter (FACSVantage; BD Biosciences). Naïve T cells were stimulated for differentiation of T_H1 and T_H2 cells as previously described (11). Details of the experimental methods are described in *SI Materials and Methods*.

FACS Analysis of Intracellular Cytokines. As previously described (11), T_H cells were restimulated for 6 h and treated with monensin (2 μM) for the last 3 h. After intracellular cytokine staining, cells were subjected to flow cytometric analysis. Details of the experimental procedure are described in *SI Materials and Methods*.

ChIP Assay. ChIP was performed as described previously (9). Details of the experimental procedure are described in *SI Materials and Methods*.

mRNA Measurements. cDNA was synthesized from total RNA with the SuperScript III First-Strand Synthesis as described previously (9). System (Invitrogen) was used for quantitative real-time RT-PCR (qRT-PCR) analysis. A list of primer sets used is included in *SI Materials and Methods*.

ELISA. The levels of IL-4 and IL-5 in culture supernatants or BALF were determined using a BD Cytometric Bead Array Kit (BD Biosciences). The levels of IL-13 and IL-33 in the samples were measured using ELISA kits (R&D Systems). Mouse IgE and IgG1 anti-OVA Abs were measured using assay kits (Chondrex). Specific absorbance was measured, and OD was quantified at 410 nM using a Multiskan JX Plate Reader (Thermo Lab System).

Western Blot Analysis. In vitro-differentiated T_H2 cells were subjected to the analysis. Details of the experimental procedure are described in *SI Materials and Methods*.

Analysis of Antigen-Induced Airway Inflammation. Details of the examination are described in *SI Materials and Methods*.

Statistical Analysis. Statistical significance was determined using Student's *t* tests (two-tailed) for two groups or one-way ANOVA (with Tukey's multiple comparisons tests) for three or more groups. P values <0.05 were considered significant.

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