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Transcriptional Repressor BCL6 Controls Th17 Responses by Controlling Gene Expression in Both T Cells and Macrophages

Arpita Mondal,1 Deepali Sawant,1 and Alexander L. Dent

The transcriptional repressor protein BCL6 regulates T cell differentiation by repressing Th2 responses and promoting follicular Th cell responses. However, little is known about the role of BCL6 in Th17 responses. We found that memory T cells from BCL6-deficient mice had increased IL-17 production. Additionally, BCL6 expression is upregulated in CD4 T cells cultured under Th17 conditions. T cells from BCL6-deficient mice showed defective Th17 differentiation and enhanced IL-4 production in vitro; however, normal Th17 differentiation was obtained with BCL6-deficient T cells under culture conditions when highly pure naive CD4 T cells were used, when IL-4 production was inhibited, or when TGF-β levels were increased. Retrovirus-mediated expression of BCL6 in CD4 T cells repressed IL-4 and augmented basal IL-17 mRNA expression. These data support the idea that BCL6 promotes Th17 differentiation through suppression of Th2 differentiation. BCL6-deficient T cells transplanted into Rag1−/− mice produced wild-type levels of IL-17, indicating that, in vivo, BCL6-deficient T cells develop relatively normal Th17 responses. Macrophages from BCL6-deficient mice showed strikingly increased expression of the Th17-promoting cytokines IL-6, IL-23, and TGF-β, and conditioned media from BCL6-deficient macrophages promoted augmented IL-17 expression by T cells. We propose that the increased Th17 activity in BCL6-deficient mice is due, in part, to BCL6-deficient macrophages promoting increased Th17 differentiation in vivo. T cells may require BCL6 for optimal Th17 differentiation; however, BCL6 function in macrophages critically regulates Th17 differentiation in vivo. We hypothesize that increased Th17 differentiation aggravates the severe Th2-type inflammatory disease in BCL6-deficient mice. The Journal of Immunology, 2010, 184:4123–4132.

T helper 17 cells are a subset of Th cells characterized by the production of the proinflammatory cytokines IL-17 (IL-17A) and IL-17F, as well as the multifunctional cytokines IL-21 and IL-22 (reviewed in Refs. 1–3). Th17 cells are associated with pathogenic autoimmune-type inflammation, such as inflammatory bowel disease, arthritis, encephalitis, and myocarditis (1–3). Th17 cells are also critical for effective antimicrobial immune responses, as mice deficient in Th17 responses are more prone to lethal bacterial infections (4, 5). In mice, Th17 cells can be generated from naive CD4 precursors by activation and differentiation in the presence of the cytokines IL-6 plus TGF-β (1–3). In humans, TGF-β is not required, but IL-6 can still drive Th17 differentiation (3). The cytokine IL-23 promotes Th17 activity, but IL-23 may be more important for stabilization or maintenance of the Th17 phenotype rather than initial Th17 differentiation (6). However, the precise role of IL-23 is still controversial (7, 8). The transcription factors STAT3, STAT4, and retinoid-related orphan receptor (ROR)-γT are critical for IL-17 expression and Th17 differentiation, and high expression of ROR-γT seems to define the Th17 lineage (3, 9, 10). The cytokine IL-21, which, like IL-6, activates STAT3, has been implicated as an important intermediate involved in Th17 differentiation (11–13). A number of factors have been shown to potently inhibit Th17 development in mice: IL-2/STAT5 (14), T-bet (15), and IL-4 and IFN-γ (1, 2).

The BCL6 gene, originally identified as an oncogene for B cell lymphoma, encodes a transcriptional repressor protein (16–22). BCL6 is expressed in almost all cell types, but it is most highly expressed in B and T cells of the germinal center reaction (23, 24). BCL6-deficient (knockout [KO]) mice are significantly growth retarded and invariably die by 10 wk of age (25, 26). BCL6 KO mice have multiple immunological defects, including lack of germinal center formation and spontaneous development of severe Th2-type inflammatory disease, particularly affecting the heart and lung (25–27). We showed previously that BCL6 is a potent inhibitor of Th2 cell differentiation and that it regulates Th2 responses downstream of IL-4 and Stat6 (28, 29). The repression of Th2 responses by BCL6 can be explained, in part, by a posttranscriptional effect of BCL6 on the expression of the master Th2 transcription factor GATA3 (28; A.L. Dent, unpublished data). Recently, BCL6 was shown to be a master transcriptional regulator of follicular Th cells (Tfh) (30–32); thus, it has emerged as a prime regulator of T cell differentiation. Nonetheless, the mechanism by which BCL6 controls inflammatory disease is not well understood. The inflammatory disease in BCL6 KO mice is dependent upon T cells and involves defects in nonlymphoid cells (33; A.L. Dent, unpublished data). Macrophages from BCL6 KO mice have increased expression of IL-6 and several chemokines (33, 34) and likely are critical for the development of inflammatory disease in the mice.
Because Th17 cells are proinflammatory, we wondered whether the inflammatory disease in BCL6 KO mice might be associated with increased Th17 cell activity. Thus, we set out to examine the regulation of Th17 cells by BCL6. We found that IL-17 expression was strongly upregulated in BCL6 KO memory T cells and that BCL6 expression was upregulated by Th17 signals. Strikingly, we found that BCL6-deficient T cells have a defect in Th17 differentiation associated with increased IL-4 production and TH2 differentiation. We also found that macrophages from BCL6 KO overproduce the key cytokines that drive Th17 differentiation. Thus, BCL6 is a novel regulator of the Th17 cell fate, controlling Th17 responses via T cells and macrophages. These data have significant implications for understanding the development of Th17 cells. In particular, our results imply that Th17-promoting cytokines produced by APCs can dominantly control Th17 differentiation in vivo.

Recently, papers from two groups reported that BCL6 represses Th17 cell differentiation in the context of promoting Th1f differentiation (30, 31). One group reported that BCL6 represses ROR-γt transcriptional activity but not expression in CD4 T cells (30). The other group reported that BCL6 could directly repress ROR-γt expression in CD4 T cells (31). Our findings demonstrate a more complicated role for BCL6 in Th17 differentiation and contrast with these other data in ways that will be addressed in the Discussion. Taken together, the three papers support the idea that BCL6 is a critical regulator of Th17 differentiation, by mechanisms intrinsic and extrinsic to T cells.

Materials and Methods

Mice

BCL6-deficient mice on a mixed C57BL/6-129Sv background have been described (29). Rag1−/− mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were bred under specific pathogen-free conditions at the laboratory animal facility at Indiana University School of Medicine (IUSM) and were handled according to protocols approved by the IUSM Animal Use and Care Committee. Wild-type (WT), BCL6 heterozygote-deficient (Het), and BCL6 homozygote-deficient (KO) mice were genotyped by PCR, as previously described (29). BCL6−/− IL4−/− mice were described previously (20).

Cell preparation and stimulation

CD4+CD62L+ (naive) and CD4+CD62L− (memory) T cells were purified from lymph node (LN) and spleen using magnetic beads (MACS reagents; Miltenyi Biotec, Auburn, CA). Pure naive CD4+CD62L+ CD44− T cells were obtained by FACS following the staining of LN T cells with PerCp–anti-CD4, PE–anti-CD25, FITC–anti-CD62L, and APC–anti-CD44 Abs. All Abs were obtained from BD Biosciences/Miltenyi Biotec, Auburn, CA). Pure naive CD4+CD62L+CD44low T cells were obtained by FACS following the staining of LN T cells with PerCp–anti-CD4, PE–anti-CD25, FITC–anti-CD62L, and APC–anti-CD44 Abs. All Abs were obtained from BD Biosciences/Miltenyi Biotec, Auburn, CA). Pure naive CD4+CD62L+CD44low T cells were stimulated with 10 ng/ml IL-2 plus LN cells were stimulated at a concentration of 107 cells/ml.

Cytokines were measured from 24-h cell culture supernatants by ELISA. Cytokine-secretion analysis

Macrophage culture and stimulation

Macrophages were grown from bone marrow of WT and KO mice with M-CSF and stimulated with LPS, as previously described (33). Macrophage supernatants were obtained after 24 h of stimulation with LPS and used at a 50% final concentration in T cell differentiation cultures, without any added cytokines.

Statistical analysis

Experiments for which statistical analysis is indicated were performed in triplicate. Differences were calculated using the Student’s t test. A p value < 0.05 was considered significant.
**Results**

**BCL6 KO mice have significantly increased Th2 and Th17, but not Th1, responses**

Initially, to test the role of BCL6 in Th17 cell function, we tested the ability of total T cells from WT, heterozygote (BCL6+/−), and KO (BCL6−/−) mice to produce IL-17 following stimulation with anti-CD3 Ab. As shown in Fig. 1A, IL-17 was strongly increased in supernatants from KO cells compared with WT cells. IL-17 production from BCL6 heterozygous mice was comparable to the level produced by WT cells. As a comparison, we tested IL-4 and IFN-γ production; KO mice, consistent with earlier results (29), produced greatly elevated levels of IL-4 but only mildly increased amounts of IFN-γ. To further define the ability of BCL6 KO T cells to produce IL-17, we purified CD4+CD62L− memory T cells from WT and KO mice and tested IL-17 secretion following stimulation with anti-CD3 Ab. The memory population from KO mice produced ∼5-fold more IL-17 than did the memory population from WT mice (Fig. 1B). The KO memory population also produced much more IL-4 than did WT memory cells. These data are consistent with the idea that Th2 and Th17 differentiation is increased in BCL6 KO mice and that BCL6 is a repressor of Th2 and Th17 differentiation.

**IL-17 is upregulated along with IL-4 in the inflamed organs of BCL6 KO mice**

Because BCL6 KO mice spontaneously develop severe inflammation of the heart and lungs that is associated with Th2 responses, we tested whether the inflammation in the KO mice is also associated with increased IL-17. The inflammation that develops in the mice varies in severity; thus, mice with different stages of disease were analyzed for IL-4 and IL-17 expression. As shown in Fig. 2, a KO mouse with little to no inflammation (mouse 3), showed no detectable increase in IL-4 or -17 expression in the heart and lung compared with WT mice, whereas mouse 2, with moderate inflammation, showed a 2- to 2.5-fold increase in IL-4 and a smaller increase in IL-17. Mouse 1, which displayed severe inflammation, had greatly elevated levels of both IL-4 and IL-17, although the fold increase in IL-17 was greater than the fold increase in IL-4. All three mice had similar levels of IFN-γ expression (data not shown), showing that IL-4 and IL-17 correlate specifically with the degree of inflammation in KO mice. Additionally, serum from WT and KO mice was tested for IL-4 and IL-17 levels; however, there was no significant increase in IL-4 or IL-17 levels in KO serum compared with WT serum, nor was there a correlation with the degree of inflammation (data not shown).

**BCL6 is upregulated by Th17 differentiation conditions**

Little is known about the signals that control BCL6 expression in T cells, although BCL6 expression is regulated in different T cell subsets, and BCL6 is strongly upregulated in Th cells (23, 24, 35–37). To further define the role of BCL6 in Th17 differentiation, we analyzed the expression of BCL6 in WT T cells stimulated with cytokines that promote Th17 differentiation. We first analyzed BCL6 mRNA expression in WT naive T cells stimulated under different Th cell-differentiation conditions for 96 h (Fig. 3A). We found that CD4 T cells cultured under Th17-differentiation conditions had significantly upregulated BCL6 expression compared with all other conditions. We compared the pattern of BCL6 expression by examining the transcription factor ROR-γT (tocr), which is upregulated in Th17 cells and is a master regulator of Th17 cells. The pattern of expression for ROR-γT was similar to BCL6 expression. We next analyzed the expression of BCL6 protein in T cells stimulated under similar conditions as those analyzed in Fig. 3A. Because mouse BCL6 is difficult to detect in whole-cell lysates in immunoblots and available anti-BCL6 Abs, we first immunoprecipitated BCL6 from T cells and then analyzed the immunoprecipitates by immunoblot. This method gives better BCL6 signal as a result of the removal of most nonspecific proteins as well as the increased concentration of BCL6 that can be loaded per well. As shown in Fig. 3B, we were able to detect BCL6 protein in T cells activated without added cytokines, and we could detect increased BCL6 in T cells cultured under Th17 conditions.

Other investigators reported that stimulation of T cells with IL-6 and/or -21 could promote increased BCL6 expression along with a Th1 phenotype (30, 38). Under slightly different T cell-stimulation conditions, we observed BCL6 upregulation with IL-6 alone (data not shown). Thus, BCL6 expression in CD4 T cells is regulated by complex mechanisms, and more work is required to understand how signals during T cell activation regulate BCL6 expression.

**BCL6 impacts Th17 differentiation by modulating Th2 differentiation**

Next, we tested the ability of BCL6-deficient (KO) T cells to develop into IL-17–secreting Th17 cells in vitro. Naive CD4+CD62L+ T cells from WT and KO mice were activated for 5 d under standard Th17-differentiation conditions with 10 ng/ml IL-6 and 1 ng/ml TGF-β and then restimulated and analyzed for cytokine expression. As shown in Fig. 4A, WT cells produced high levels of IL-17 upon restimulation, whereas KO T cells produced substantially less IL-17 but concomitantly secreted a high level of IL-4. Previously, we found that KO T cells underwent greatly augmented Th2 differentiation in response to IL-6 (28). These data might indicate that the IL-6 in the Th17-differentiation culture is promoting abnormal Th2 differentiation in the absence of BCL6. To test this idea, we increased the dose of TGF-β in the Th17 culture to 5 ng/ml, on the assumption that increased TGF-β would inhibit Th2 differentiation (39, 40). As shown in Fig. 4A, increased TGF-β augmented the ability of WT and KO T cells to differentiate into IL-17–producing cells. At the same time, the increased TGF-β produced a relative advantage in the ability of KO T cells to produce IL-17, such that there was a statistically nonsignificant difference between IL-17 produced by WT and KO T cells. However, the increased TGF-β only slightly dampened the ability of the KO T cells to produce IL-4 (Fig. 4A). These data indicate that higher levels of TGF-β can overcome the inhibitory effects of IL-4 on Th17 differentiation.

Because CD4+CD62L+ T cells are not a perfectly pure population of naïve CD4 T cells, we wondered whether the Th17 defect we observed with KO CD4+CD62L+ T cells might have been due to contaminating CD4+CD62L+CD4+ T cells or contaminating cells that have not committed to a CD62L− memory phenotype. To test this idea, we sorted CD4+CD62L+CD4+ T cells from WT and KO mice and tested their ability to produce IL-17 following culture under Th17-differentiation conditions. As shown in Fig. 4B, more rigorously purified CD4+CD62L+CD4+ T cells from WT and KO mice and tested their ability to produce IL-17 following culture under Th17-differentiation conditions. As shown in Fig. 4C, KO T cells have a slight trend toward increased Th17 differentiation as assessed by the expression of IL-17, IL-17F, IL-21, IL-22, IL-23R, and ROR-γT (tocr), although none of the increases were statistically significant. However, IL-4 and IL-10 mRNA were significantly upregulated in the KO Th17 cells, indicating that BCL6 directly represses these genes in Th17 cells or that there is a low level of Th2 differentiation, even with pure naïve CD4 T cells from KO mice. These data indicate that pure naïve KO
CD4 T cells undergo relatively normal Th17 differentiation, with a trend toward enhanced Th17 differentiation. However, less pure naive KO CD4 T cells have a defect in Th17 differentiation that is associated with a bias toward Th2 differentiation.

To specifically examine the effect of increased IL-4 made by BCL6 KO T cells on Th17 differentiation, we used BCL6/IL-4 double KO mice (29). CD4+CD62L− memory T cells were stimulated overnight with plate-bound anti-CD3 Ab, and the supernatants were tested for cytokine expression by ELISA. Values represent mean cytokine levels ± SE from four WT, four BCL6 heterozygous-deficient, and six KO mice. When comparing WT and KO cytokine levels, p values were 0.006 for IL-17, 0.018 for IL-4, and 0.356 for IFN-γ. B, CD4+CD62L− memory T cells were stimulated overnight with plate-bound anti-CD3 Ab, and the supernatants were tested for cytokine expression by ELISA. Values represent averages ± SE from two WT and two KO mice. This experiment was repeated once with highly similar results. Het, BCL6 heterozygous deficient.

To specifically examine the effect of increased IL-4 made by BCL6 KO T cells on Th17 differentiation, we used BCL6/IL-4 double KO mice (29). CD4+CD62L− T cells were prepared from IL-4 KO and BCL6/IL-4 double-KO mice and cultured under standard Th17 differentiation conditions. As shown in Fig. 5A, T cells from BCL6/IL-4 double-KO mice showed no significant difference in IL-17 production compared with T cells from IL-4 KO mice. These data support the idea that increased IL-4 from BCL6 KO T cells can interfere with Th17 differentiation. Furthermore, like BCL6 KO mice, BCL6/IL-4 double-KO mice generate increased numbers of Th17 memory cells (Fig. 5B, 5C). Taken together, these data indicate that non-T cell factors are driving the increased Th17 cell differentiation occurring in BCL6 KO mice.

We next wanted to test the effect of increased levels of BCL6 on IL-17 expression in T cells. Retroviruses were used to express BCL6 in activated WT CD4+CD62L− T cells. Following retrovirus infection, control and BCL6 retrovirus-positive cells were isolated by cell sorting and tested for gene expression either after culture for 24 h or after stimulation with anti-CD3 and CD28 Abs for 5 h. Intriguingly, BCL6-retrovirus–infected T cells cultured for 24 h without restimulation showed a >3-fold increase in steady-state IL-17 mRNA levels compared with control cells (Fig. 6A). However, CD3/CD28-activated BCL6-retrovirus–infected T cells did not show a significant change in IL-17 mRNA levels compared with control cells (Fig. 6B). BCL6 significantly repressed IL-4 expression under both conditions. BCL6-retrovirus infection did not significantly affect ROR-γT in naïve CD4 T cells (data not shown). Furthermore, BCL6-retrovirus

**FIGURE 1.** T cells from BCL6 KO mice produce greatly increased IL-17 and IL-4 levels. A, Total spleen and LN cells were stimulated overnight with plate-bound anti-CD3 Ab, and the supernatants were tested for cytokine expression by ELISA. Values represent mean cytokine levels ± SE from four WT, four BCL6 heterozygous-deficient, and six KO mice. When comparing WT and KO cytokine levels, p values were 0.006 for IL-17, 0.018 for IL-4, and 0.356 for IFN-γ. B, CD4+CD62L− memory T cells were stimulated overnight with plate-bound anti-CD3 Ab, and the supernatants were tested for cytokine expression by ELISA. Values represent averages ± SE from two WT and two KO mice. This experiment was repeated once with highly similar results. Het, BCL6 heterozygous deficient.

**FIGURE 2.** IL-4 and IL-17 are upregulated in the inflammatory disease of BCL6 KO mice. The bar graph shows the fold increase in IL-4 and IL-17 RNA levels of total heart (H) and lungs (L) from three individual KO mice compared with WT mice. The fold-increase is provided at the top of each bar. The three KO mice shown had different degrees of inflammatory disease as assessed by histological analysis of heart and lung sections.
T cells in the neutral condition. Ab used for immunoprecipitation. ThN, a gel and blotted to Nylon membrane. The BCL6 protein was immunoprecipitated, indicated conditions. T cells were lysed, BCL6 protein was immunoprecipitated, and then immunoprecipitates were run on a gel and blotted to Nylon membrane. The blot was probed with the same anti-BCL6 Ab used for immunoprecipitation. ThN, T cells in the neutral condition.

infection did not affect IL-17 expression in CD4+CD62L+ T cells activated under Th17 conditions, nor did BCL6 affect IL-17 expres-

The function of BCL6 in Th17 cells is subordinate to in vivo conditions

We next wondered whether KO T cells could differentiate normally into Th17 cells in vivo. To test this, we transferred CD4+CD62L+ T cells from WT and KO mice into Rag1−/− mice, which lack T and B cells. Mice that received T cells were then immunized with the protein Ag conalbumin in alum adjuvant to provoke an immune response. Seven days after immunization, LN cells were harvested and tested for cytokine secretion following stimulation with anti-CD3 Abs. As shown in Fig. 7, KO T cells produced comparable levels of IL-17 to WT cells when the T cells were primed in vivo and analyzed ex vivo. Consistent with the previously observed phenotype of KO T cells, the KO T cells also produced greatly increased IL-4 and similar levels of IFN-γ compared with WT T cells. These data suggest that other cell types, such as APCs, can provide signals to KO T cells that override the intrinsic Th17 defect of these cells.

Macrophages from BCL6 KO mice overproduce cytokines that promote Th17 differentiation

The above data pointed to a model in which APCs in BCL6 KO mice can promote the increased differentiation of BCL6 KO T cells into Th17 cells, despite the defect of BCL6 KO T cells to develop into Th17 cells. To test this model, we examined BCL6-deficient macrophages for their ability to produce cytokines that influence Th17 differentiation. Previously, we observed that macrophages from BCL6 KO mice produced increased levels of certain chemokines as well as increased IL-6 (33, 34). IL-18 has also been described as a BCL6 target gene in macrophages (41). However, other cytokines that promote Th17 differentiation (i.e., TGF-β and IL-23) have not been analyzed in BCL6 KO macrophages. Therefore, we grew macrophages from the bone marrow of WT and BCL6 KO mice using M-CSF and analyzed gene expression after 6 h of stimulation with LPS. As shown in Fig. 8, KO macrophages produce greatly increased levels of the previously characterized BCL6 target genes IL-6 and CCL2 compared with WT macrophages. Strikingly, TGF-β and IL-23 were also significantly increased in the KO macrophages, consistent with the idea that KO macrophages produce more pro-Th17 cytokines. The proinflammatory cytokines IL-18 and TNF-α were not significantly increased in the KO cells, showing that the KO macrophages do not have a general augmentation in cytokine gene expression. IL-12 p40 expression was significantly increased in the KO macrophages but at a lower level than the pro-Th17 cytokines. Thus, these data show that BCL6 represses the expression of the three major Th17-promoting cytokines in macrophages. We also examined whether KO dendritic cells (DCs) overexpressed pro-Th17 cytokines by growing DCs from WT and KO bone marrow with GM-CSF. Following LPS stimulation, KO DCs showed similar levels of IL-6 and -23 and TGF-β as WT DCs (data not shown), indicating a unique role for BCL6 in controlling pro-Th17 cytokines in macrophages.

We then wondered whether elevated levels of Th17-promoting cytokines made by KO macrophages could promote increased Th17 differentiation of CD4 T cells in vitro. Thus, CD4+CD62L+ T cells from WT mice were activated and cultured in the presence of conditioned media from either WT or KO LPS-stimulated macrophages and then tested for Th17 differentiation by restimulation and measurement of IL-17 secretion. As shown in Fig. 9, factors produced by KO macrophages promoted >3-fold more IL-17 secretion than did factors made from WT macrophages. These data support the model that KO macrophages promote the increased Th17 differentiation in BCL6 KO mice.

Discussion

In this study, we made several novel findings relating to the role of the BCL6 transcription factor and Th17 cells. First, we showed that T cells from BCL6 KO mice produce more IL-17 than do comparable T cells from WT mice. Second, we showed that BCL6 expression is upregulated in Th17 cells, suggesting that BCL6 plays a T cell-intrinsic role in Th17 cells. Third, BCL6 has a modest, but positive, role directly on IL-17 expression and Th17 differentiation, in part by inhibiting Th2 differentiation. Fourth, BCL6 represses three key Th17-promoting cytokines in macrophages: IL-6 and -23 and TGF-β. Together, these data indicate a model wherein BCL6 is required to suppress Th2 differentiation during Th17 priming conditions to allow optimal Th17 differentiation, BCL6 is required to repress
Th17-promoting cytokines in macrophages, and increased Th17 differentiation in BCL6 KO mice is due to increased pro-Th17 cytokine production by KO macrophages. Our data are also consistent with the idea that the increased Th17 differentiation enhances the overall inflammatory phenotype of BCL6 KO mice.

All BCL6 KO mice die at an early age, and the majority of mice die with severe inflammation of the heart and lungs (25–27). The etiology of the inflammation in KO mice is complex and seems to depend on defects in several cell types, including T cells, myeloid cells, and stromal cells (27, 33). Whether the inflammatory disease...
is autoimmune in nature or is hyperresponsiveness to endogenous bacteria is not known. The inflammation in KO mice is generally classified as Th2-type because it is associated with eosinophils, and this is consistent with the strong bias of BCL6 KO T cells to develop into Th2 cells (28, 29). The heart and lungs of BCL6 KO mice show elevated expression of Th2 cytokines and macrophage-derived chemokines (25, 33). We assessed IL-17 levels in heart and lungs from BCL6 KO mice with various degrees of inflammation and detected increased IL-17 that correlated with the severity of inflammation (Fig. 2). These data suggest that the increased Th17 differentiation in BCL6 KO mice may be a factor in the heart and lung inflammation observed in these mice and that the inflammation in BCL6 KO mice involves Th2 and Th17 cells. Whether Th17 cells are required for the inflammatory disease in KO mice is unclear and needs to be addressed in future studies. One possible complication to this issue is that there is redundancy in the role of T cells in the KO inflammatory disease, such that Th2 and Th17 cells need to be disabled to block inflammation. Although the precise etiology of the inflammatory disease in the BCL6 KO mouse is unknown, previous work from our laboratory showed that T cells are absolutely required for the heart and lung inflammation (A.L. Dent, unpublished data). Because nonhematopoietic cells are required for the heart and lung inflammation (27), our current hypothesis is that there are cellular defects in the myocardium and blood vessels of BCL6 KO mice that lead to tissue damage and inflammation, which is severely exacerbated by proinflammatory KO macrophages and T cells.

Some of the increased Th17 activity in the BCL6 KO mice may originate from heightened bacterial infections observed in the mice (26), wherein bacterial products stimulate macrophages to release pro-Th17 cytokines. Increased Th17 activity in the BCL6 KO mice may also be a novel feedback response to increased Th2 responses, wherein activated Th2 cells stimulate KO macrophages to secrete pro-Th17 cytokines. Although we observed high levels of IL-4 and IL-17 in the severely inflamed heart and lung of a KO animal (Fig. 2), comparison of IL-4 and IL-17 expression in the spleens of other individual BCL6 KO mice did not show a clear relationship between the two cytokines: some KO mice had high IL-4 and intermediate IL-17, whereas other mice had lower IL-4 and high IL-17 (data not shown). Thus, Th2 and Th17 responses in lymphoid tissues outside of inflamed organs are not necessarily linked in BCL6 KO mice. Different patterns of CD4 effector T cell activity in the KO mice either relate to specific infections that promote different T cell responses in different mice or are due to differences in the temporal interplay between Th2 and Th17 responses in the KO mice at the time of analysis.

An unexpected result of our study is that BCL6 is a positive and negative regulator of Th17 differentiation. Although BCL6 is required in T cells for optimal Th17 differentiation (see later discussion), BCL6 is also required to inhibit the expression of cytokines that promote Th17 differentiation from macrophages. Specifically, we found that BCL6 is a potent regulator of IL-6 and IL-23 and TGF-β in macrophages. Although IL-6 was shown to be a direct target of BCL6 in macrophages, whether BCL6 directly represses IL-23 and TGF-β is not known and requires further experimentation. BCL6 may repress other transcription factors that are critical for TGF-β and IL-23 expression in macrophages. Surprisingly, we found that the role of BCL6 in controlling cytokine expression by macrophages seems to dominate over the role of BCL6 in T cells in vivo. This finding has major implications for understanding the regulation of Th17 responses, and modulation of APC activity may be key to controlling Th17 responses therapeutically.

Ironically, the KO T cell defect in Th17 differentiation may be a benefit for the BCL6 KO mice, because pro-Th17 macrophages in the KO mice with WT T cells might result in even worse inflammatory pathology than the mice already have. Along the same lines, we cannot rule out that the increased Th2 differentiation in the BCL6 KO mice is also protective to the mice, in terms of downregulating Th17 responses and limiting tissue damage from Th17 activity. Important questions are how much the increased Th2 differentiation in the KO mice is due to overproduction of IL-6 by macrophages, because IL-6 promotes abnormal Th2 differentiation with KO T cells, and the degree to which IL-6 promotes Th2 and Th17 responses in the KO mice. We are currently producing BCL6/IL-6 double-KO mice to address this question.

Although the effect of BCL6 in T cell Th17 differentiation seems to be subordinate to the effect of BCL6 on macrophages or other
APC types in vivo, the question of how BCL6 regulates the ability of T cells to undergo Th17 differentiation is important. Our results suggest that the primary effect of BCL6 in T cells is to repress Th2 differentiation and IL-4 expression, to promote maximal Th17 differentiation. In contrast, two recent reports showed that BCL6 strongly represses Th17 differentiation, apparently by acting on ROR-γT (30, 31). However, Nurieva et al. (30) and Yu et al. (31) presented conflicting mechanisms for BCL6 regulating ROR-γT. Nurieva et al. (30) showed that BCL6 represses ROR-γT transcriptional activation but not its expression, whereas Yu et al. (31) reported that BCL6 binds to the ROR-γT promoter and inhibits its expression. In support of Nurieva et al. (30), we see no evidence that BCL6 represses ROR-γT (Fig. 4C, data not shown). Nonetheless, Nurieva et al. (30) and Yu et al. (31) showed that BCL6 overexpression with retrovirus significantly repressed IL-17 expression and Th17 differentiation. These data clearly conflict with our results (Fig. 6), although we performed the retrovirus expression experiment >10 times and are confident in our results. Interestingly, in some early experiments with less pure naive BCL6 KO T cells (CD4+CD62L+ T cells), we observed increased Th17 differentiation compared with WT T cells, indicating a repressive role for BCL6 in Th17 differentiation. However, we found that in these experiments, there was a high percentage of contaminating macrophages, apparently due to the fact that some KO mice develop abnormal accumulations of macrophages as a by-product of their inflammatory disease (A.L. Dent, unpublished data). When we used more highly purified CD4+CD62L+CD44low T cells for our Th17-differentiation experiments, we did not observe a significant difference between WT and KO T cells, as shown in Fig. 4.

The case for repression of Th17 differentiation by BCL6 presented by Yu et al. (31) was made primarily from experiments involving the overexpression of BCL6 by retrovirus in CD4 T cells, wherein they observed decreased Th17 differentiation over the course of several days at the same time that Tfh differentiation was being promoted by BCL6. Thus, these results may be explained by an increase in differentiation toward the Tfh lineage and/or an outgrowth of Tfh cells and may not be a direct effect of BCL6.
is strengthened by the fact that they observed the strongest Th17-repressive effects with BCL6 after 5 d of retrovirus-mediated BCL6 expression. Further, the chromatin-immunoprecipitation (Chip) data for ROR-γT shown by Yu et al. (31) was curiously performed with human cells instead of mouse T cells, whereas the rest of their Th17 experiments used mouse T cells. Further, the control for their ROR-γT Chip experiment was B cells, which may not be an ideal control for this experiment. A Chip reaction with isotype control Ab in T cells would better reflect the background level of ROR-γT promoter in a Chip assay in T cells.

The data from Nurieva et al. (30) on the repression of Th17 differentiation and ROR-γT regulation by BCL6 are somewhat harder to reconcile with our data. Nonetheless, there are critical variations between their system and our system. They used a different BCL6 KO mouse strain from ours, which may have a stronger Th17 phenotype and, unlike us, they also used an OT-II TCR transgenic system for their Th17-differentiation assays. Thus, the form of T cell stimulation was different between our studies. Because BCL6 KO mice are sickly and develop spontaneous inflammatory disease, there are also likely variables related to the commensal organisms in the mouse colony used by Nurieva et al. (30) compared with our mouse colony. These differences could impact the Th17 phenotype of KO mice. There are also critical differences in the experimental protocols of the retrovirus infection and cell-stimulation conditions between our work and Nurieva et al. (30). In contrast to our retrovirus experiments, Nurieva et al. used transgenic T cells and APCs for their retrovirus experiments, including a longer incubation period with the BCL6 retrovirus. As with Yu et al. (31), a repressive effect of BCL6 on Th17 cells may be more apparent after a longer expression phase, but again this leads to the issue of whether there is truly a direct repressive effect of BCL6 on Th17 differentiation or whether there is an outgrowth of Tf α cells. In contrast, we performed two retrovirus infection experiments in which the T cells were restimulated at low density because of poor cell sort yields. In these instances, we observed that BCL6 strongly repressed IL-17 expression. In other experiments with cells at a higher standard stimulation density, BCL6 augmented IL-17 expression or had a neutral effect following stimulation. Thus, there may be specific T cell stimulation conditions under which BCL6 represses IL-17 and ROR-γT expression. The repressive effect of BCL6 on ROR-γT and Th17 differentiation may dominate under lower-density culture conditions. This issue requires further experimentation to resolve. Nonetheless, we should emphasize that we consistently observed more potent effects of BCL6 on Th2 differentiation than on Th17 differentiation, using BCL6 KO T cell and BCL6 retrovirus approaches. We conclude that although BCL6 strongly and directly represses Th2 differentiation, the effect of BCL6 on Th17 differentiation is subtle and involves indirect effects from APCs, such as macrophages. Consistent with our results, Nurieva et al. (30) and Yu et al. (31) found that IL-17 is increased in the context of a BCL6 KO immune system, either in BCL6 KO mice or in mice reconstituted with BCL6 KO bone marrow. However, neither Nurieva et al. (30) nor Yu et al. (31) examined the role of macrophages in the BCL6 KO Th17 response. Our results with TGF-β and Th17 cell differentiation may be instructive as to the role of BCL6 in CD4 T cells. TGF-β was shown to repress Th2 differentiation by downregulating the activity of the master Th2 factor GATA3 (39, 40). We found previously that BCL6 modulates GATA3 expression (28); more recently, we found that BCL6 is an extremely potent repressor of GATA3 transcriptional activity (A. Mondal, D. Sawant, S. Kusam, and A.L. Dent, manuscript in preparation). Our finding that increased TGF-β can restore the ability of BCL6 KO T cells to undergo Th17 differentiation likely relates to the ability of TGF-β to inhibit GATA3 and block Th2 differentiation. BCL6 KO T cells may be less sensitive to the effects of TGF-β during Th17 differentiation because of increased GATA3 activity in the absence of BCL6. One key effect of BCL6 on Th17 differentiation may be to inhibit GATA3 activity and subsequent Th2 differentiation, thus allowing for full Th17 differentiation. The lack of sensitivity of BCL6 KO T cells to TGF-β suggests that there is a previously unappreciated role for BCL6 in regulatory T cell responses. Indeed, a regulatory T cell defect due to BCL6 deficiency may be an important component of the inflammatory disease in BCL6 KO mice. Ongoing studies in our laboratory indicate that BCL6 regulates FoxP3 expression as well as the suppressive activity of regulatory T cells (manuscript in preparation).

In summary, we found a novel role for BCL6 in controlling Th17 responses. Although BCL6 is critical for optimal Th17 differentiation, BCL6 also potently regulates the expression of Th17-promoting cytokines in macrophages. These findings have important implications for understanding the molecular regulation of Th17 differentiation, as well as understanding the regulation of Th17 differentiation in vivo.

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Disclosures
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References


