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Kristin Hollister, Saritha Kusam, Hao Wu, Ninah Clegg, Arpita Mondal, Deepali V. Sawant and Alexander L. Dent

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Insights into the Role of Bcl6 in Follicular Th Cells Using a New Conditional Mutant Mouse Model

Kristin Hollister, Saritha Kusam, Hao Wu, Ninah Clegg, Arpita Mondal, Deepali V. Sawant, and Alexander L. Dent

The transcriptional repressor Bcl6 controls development of the follicular Th cell (T_{FH}) lineage, but the precise mechanisms by which Bcl6 regulates this process are unclear. A model has been proposed whereby Bcl6 represses the differentiation of T cells into alternatively activated effector lineages, thus favoring T_{FH} cell differentiation. Analysis of T cell differentiation using Bcl6-deficient mice has been complicated by the strong proinflammatory phenotype of Bcl6-deficient myeloid cells. In this study, we report data from a novel mouse model where Bcl6 is conditionally deleted in T cells (Bcl6^{fl/fl}Cre^{CD4} mice). After immunization, programmed death-1 (PD-1)\textsuperscript{high} T_{FH} cells in Bcl6^{fl/fl}Cre^{CD4} mice are decreased >90% compared with control mice, and Ag-specific IgG is sharply reduced. Residual PD-1\textsuperscript{high}CXCR5\textsuperscript{+} T_{FH} cells in Bcl6^{fl/fl}Cre^{CD4} mice show a significantly higher rate of apoptosis than do PD-1\textsuperscript{high} CXCR5\textsuperscript{+} T_{FH} cells in control mice. Immunization of Bcl6^{fl/fl}Cre^{CD4} mice did not reveal enhanced differentiation into Th1, Th2, or Th17 lineages, although IL-10 expression by CD4 T cells was markedly elevated. Thus, T cell–extrinsic factors appear to promote the increased Th1, Th2, and Th17 responses in germline Bcl6-deficient mice. Furthermore, IL-10 may be a key target gene for Bcl6 in CD4 T cells, which enables Bcl6 to promote the T_{FH} cell phenotype. Finally, our data reveal a novel mechanism for the role of Bcl6 in promoting T_{FH} cell survival. The Journal of Immunology, 2013, 191: 3705–3711.

During an immune response, CD4 Th cells can differentiate into several unique effector lineages that promote different immune responses via the secretion of distinct types of cytokines. Follicular Th cells (T_{FH}) are a recently characterized CD4 lineage whose major function is to help B cells form germinal centers (GCs) and produce high-affinity Abs (reviewed in Refs. 1–5). T_{FH} cells are characterized by a high level of expression of the chemokine receptor CXCR5, which binds the chemokine CXCL13 expressed in B cell follicles. CXCL13, acting on CXCR5, promotes migration of T_{FH} cells to the B cell follicle. T_{FH} cells have an activated effector T cell phenotype and express elevated ICOS and programmed death-1 (PD-1). T_{FH} cells control both the initiation as well as the outcome of the GC B cell response. Thus, T_{FH} cells are critical for memory B cell and plasma cell development. A key cytokine produced by T_{FH} cells is IL-21, which is a factor that potently promotes B cell activation and Ab secretion. Although T_{FH} cells are critical for the proper production of high-affinity Abs, the overproduction of T_{FH} cells can lead to autoimmunity; specifically, T_{FH} cells can help B cells produce self-reactive Abs (6–8). Thus, the proper regulation of T_{FH} cell differentiation is essential for normal immune function and preventing autoimmune disease.

The Bcl6 transcriptional repressor protein is upregulated in T_{FH} cells and is considered a master regulator for the T_{FH} cell lineage (9–11). Forced BCL6 expression promotes differentiation of CD4 T cells into T_{FH} cells, whereas Bcl6-deficient T cells cannot differentiate into T_{FH} cells. Relatively little is known about the mechanism by which Bcl6 promotes T_{FH} cell differentiation, although three possible mechanisms have been proposed: 1) Bcl6 inhibits the differentiation of CD4 T cells into other lineages (e.g., Th1, Th2, Th17), thus indirectly favoring T_{FH} cell differentiation; 2) Bcl6 inhibits terminal CD4 T cell differentiation by repressing Blimp1, again indirectly favoring the T_{FH} cell differentiation state; and 3) Bcl6 regulates a large number of microRNAs that directly control the T_{FH} cell fate (3). Bcl6 may promote T_{FH} cell differentiation and function by one or a combination of these mechanisms; alternatively, Bcl6 may act through an as yet unidentified mechanism. The evidence accumulated to date strongly supports an intrinsic role for Bcl6 in CD4 T cells in generating T_{FH} cells. However, experimental approaches using germline BCL6 knockout (KO) mice are problematic owing to the spontaneous inflammatory disease, early death, and non–T cell defects of the mice (12–15). Approaches using germline BCL6 KO mice for mixed bone marrow chimeras are limited owing to the difficulty of producing large numbers of consistently constituted chimeric mice for in-depth immunological studies. Furthermore, these bone marrow chimeric mice cannot separate out the effects of hyperinflammatory Bcl6-deficient myeloid cells. In contrast, a conditional KO mouse approach for BCL6 allows analysis of BCL6 function in specific cell lineages, in a consistent wild-type background. Recently, Kaji et al. (16) reported a conditional KO model of Bcl6 and used it to analyze memory B cell development. In this study, we report the generation of a second Bcl6 conditional KO mouse strain, and we have generated novel insights about the role of Bcl6 in CD4 T cell differentiation and in T_{FH} cells.
Materials and Methods

Mice and immunization

Bcl6loxP mice on a mixed C57BL/6-129Sv background were generated at the Indiana University School of Medicine Transgenic and Knockout Facility. LoxP sites were inserted into the Bcl6 gene locus, flanking exons 7–9 encoding the zinc finger domain of Bcl6, using standard molecular cloning and embryonic stem cell techniques. CreEIIa mice, obtained from The Jackson Laboratory, were used to remove the floxed Neomycin gene from the germline of knock-in mice. The floxed allele was genotyped by PCR using the following primers: 5’-loxP forward, 5’-TGAAGACGTGAAATCTATACTGATAGGC-3’; 5’-loxP reverse, 5’-ACCCATAGAAACACACTATAC-3’; 3’-loxP forward, 5’-TCACCAATCCCAGGTCTCAGTGTC-3’; and 3’-loxP reverse, 5’-TTTGTCAATTTTCTCTGTGGTGCCTGCT-3’.

Bcl6loxP mice were mated to CD4-cre mice (17) to generate Bcl6fl/fl CreCD4 mice. Mice were bred under specific pathogen-free conditions at the laboratory animal facility at Indiana University School of Medicine and were handled according to protocols approved by the Indiana University School of Medicine Animal Use and Care Committee. Mice were immunized i.p. with 1 × 10^6 sheep RBCs (SRBCs; Rockland Immunochemicals, Gilbertsville, PA) in PBS.

Flow cytometry

All Abs were purchased from eBioscience (San Diego, CA), BD Biosciences (San Jose, CA), or BioLegend (San Diego, CA). The GL3 Ab (BioLegend) was used to detect γδ T cells and NKT cells were identified with CD1d tetramer staining (eBioscience), total CD4+ T cells were isolated from total spleen or thymus cells were incubated with anti-mouse CD16/CD32 (FcγR) Ab, obtained from The Jackson Laboratory, were used to remove the floxed Neomycin gene from the germline of knock-in mice. The floxed allele was genotyped by PCR using the following primers: 5’-loxP forward, 5’-TGAAGACGTGAAATCTATACTGATAGGC-3’; 5’-loxP reverse, 5’-ACCCATAGAAACACACTATAC-3’; 3’-loxP forward, 5’-TCACCAATCCCAGGTCTCAGTGTC-3’; and 3’-loxP reverse, 5’-TTTGTCAATTTTCTCTGTGGTGCCTGCT-3’.

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Gene expression analysis

Total cellular RNA was prepared using the TRIzol method (Life Technologies), and cDNA was prepared with the Transcriptor First Strand cDNA synthesis kit (Roche). Quantitative PCR (QPCR) reactions were run by assaying each sample in triplicates using the Fast Start Universal SYBR Green mix (Roche Applied Science) with custom primers or specific TaqMan assays (Applied Biosystems). QPCR assays were run with a Stratagene Mx3000P real-time QPCR machine. Levels of mRNA expression were normalized to β-tubulin mRNA levels, and differences between samples were analyzed using the cycle threshold method. Primers for SYBR Green assays were previously described (14, 19).

Statistical analysis

Preliminary statistical analysis showed that the data were normally distributed, and thus further statistical analysis was done using Student t tests or ANOVA on SPSS Statistics 20 software.

In vitro stimulation

Total CD4+ T cells were isolated via magnetic bead separation (Miltenyi Biotec); naive CD4+ T cells were isolated via FACS and gated as CD3+ CD4+CD62L+CD44+. Cells were stimulated with plate-bound anti-CD3 (5 μg/ml) and anti-CD28 (10 μg/ml) Abs (BD Biosciences) for 24 h at 1 × 10^6 cells/ml. Th cell media alone (Th0) conditions contain no cytokines or blocking Abs. Th cell–neutral (ThN) conditions contain anti–IFN-γ and anti–IL-4 (10 μg/ml) (BD Biosciences). TFH cell conditions contain IL-6 and IL-21 (10 ng/ml each (R&D Systems), plus anti–IFN-γ, anti–IL-4, and anti–TGF-β Abs (10–20 μg/ml each).

ELISA

Cytokines and Ab titers were measured via ELISA. Kits from BD Biosciences were used to measure cytokines, except IL-17, in which purified and biotin-labeled Abs were used (BD Biosciences). SRBC-specific IgG was measured as previously described (18). Briefly, wells were coated with SRBC membrane extract (prepared as described in Ref. 18) overnight at 4˚C. Wells were blocked with 10% FCS and diluted serum was incubated in wells for 2 h at room temperature. A peroxidase-labeled Fc-specific anti-mouse IgG detection Ab was used (Sigma-Aldrich).

FIGURE 1. Development of a conditional deletion system for BCL6. (A) A targeting construct containing BCL6 exons 7 through 9 and a Neo gene was inserted into the wild-type (+) allele. The Neo gene was removed by mating with a CreEIIa mouse, which resulted in a BCL6 floxed allele (fl). (B) B220+ CD19+ B cells and CD3+CD4+ T cells were sorted via FACS from mice heterozygous for the floxed allele. DNA from the cells was used to PCR amplify the loxP-containing sites. In B cells, both the 5’ and 3’ loxP sites were detected. However, in CD4+ T cells expressing Cre, only the germline allele of BCL6 was amplified, signaling deletion of the floxed region.
Results
A conditional KO allele for Bcl6 (Bcl6\textsuperscript{fl}), where loxP sites flanked the zinc finger–encoding exons of Bcl6 (Fig. 1A), was introduced into embryonic stem cells. After germline transmission of the floxed allele and deletion of the Neomycin gene, Bcl6\textsuperscript{fl/+} mice were mated to produce Bcl6\textsuperscript{fl/0} offspring. Bcl6\textsuperscript{fl/0} mice are born at an expected frequency, look normal, and produce normal GC B cell and T\textsubscript{FH} cell responses to immunization (Supplemental Fig. 1), thus indicating that the loxP targeting of the Bcl6 gene did not interfere with normal Bcl6 expression and function. CD4\textsuperscript{-Cre} mice (17) were mated to Bcl6\textsuperscript{fl/0} mice to obtain Bcl6\textsuperscript{fl/+CreCD4} offspring. We used these mice to test deletion of the floxed Bcl6 allele in response to CD4-Cre and saw efficient deletion of the floxed Bcl6 allele in CD4 T cells, but not in B cells (Fig. 1B, Supplemental Fig. 2). Bcl6\textsuperscript{fl/+CreCD4} (conditional KO [cKO]) mice are born at an expected frequency, and in contrast to germline Bcl6 KO mice (13), they look normal and have no apparent signs of disease (not shown). Thymuses and spleens of cKO mice contain normal T cell numbers and CD4 and CD8 populations (Fig. 2A,B). Consistent with our earlier findings (19), the number of Foxp3\textsuperscript{+} T cells was unaffected by loss of Bcl6 (Fig. 2C).

Next, we immunized Bcl6\textsuperscript{+/+CreCD4} (control) and Bcl6\textsuperscript{fl/+CreCD4} (cKO) mice with SRBCs and analyzed GC B cell and T\textsubscript{FH} cell responses after 10 d, at the peak of the response. Whereas control mice produced strong levels of Fas\textsuperscript{GL7} PNA\textsuperscript{+} GC B cells and CXCR5\textsuperscript{+}ICOS\textsuperscript{+}PD-1\textsuperscript{high} T\textsubscript{FH} cells, cKO mice had an almost complete loss of these cell populations (Fig. 3A, 3B). These results confirm that Bcl6 controls T\textsubscript{FH} cell development and/or survival in a T cell–intrinsic manner and, furthermore, that T\textsubscript{FH} cells are absolutely required to drive the GC reaction. To test whether loss of the T\textsubscript{FH} cell population resulted in a functional defect in Ab production, we measured SRBC-specific IgG titers (Fig. 3C). Ag-specific IgG was ∼5-fold lower in the cKO mice, showing that loss of CXCR5\textsuperscript{+}ICOS\textsuperscript{+}PD-1\textsuperscript{high} T\textsubscript{FH} cells and/or Bcl6 expression in T cells leads to a dramatic defect in help for B cells.

Because PD-1 is associated with T cell exhaustion and apoptosis (20), we wondered whether PD-1\textsuperscript{high} T\textsubscript{FH} cells were undergoing higher levels of apoptosis than T cells with lower levels of PD-1 expression, and whether this apoptosis was regulated by Bcl6. Thus, we used two different markers of early apoptosis, active caspase-3 and annexin V, to stain T cells from SRBC-immunized control and cKO mice (Fig. 4). We then analyzed apoptosis in non–TFH cells and in CXCR5\textsuperscript{+} cells, focusing on the correlation between the level of PD-1 expression and degree of apoptosis. Non–T\textsubscript{FH} cells had minimal apoptotic cells, whereas apoptosis increased in the CXCR5\textsuperscript{+} cells in parallel with PD-1 expression, with the highest levels of apoptotic cells in the CXCR5\textsuperscript{+}PD-1\textsuperscript{high} T\textsubscript{FH} cell fraction (Fig. 4B, 4C). In cKO mice, non–T\textsubscript{FH} cell, CXCR5\textsuperscript{+}PD-1\textsuperscript{low}, and CXCR5\textsuperscript{+}PD-1\textsuperscript{low} populations exhibited similar or lower levels of apoptosis compared with control mice (Fig. 4B, 4C). However, in the cell populations expressing higher PD-1, the cKO mice showed a marked increase in apoptotic markers, reaching significance in the PD-1\textsuperscript{high} T\textsubscript{FH} cell fraction (Fig. 4B, 4C). These data indicate that Bcl6 regulates apoptosis in PD-1\textsuperscript{high} T\textsubscript{FH} cells. Therefore, one novel mechanism for how Bcl6 controls T\textsubscript{FH} cell development is by stabilizing their survival and inhibiting them from excess apoptosis as a result of high PD-1 expression.

We then tested the idea that Bcl6 promotes T\textsubscript{FH} cell development by inhibiting the differentiation of CD4 T cells into other Th cell lineages. We reasoned that if Bcl6 inhibited Th cell differentiation, following a potent immune stimulus, CD4 T cells would differentiate more readily into Th1, Th2, or Th17 cells in cKO mice than in control mice. We therefore analyzed IFN-γ, IL-4, and IL-17 expression by both intracellular cytokine staining and ELISA from CD4 T cells isolated from SRBC-immunized control and cKO mice. As shown in Fig. 5A–C, we observed no significant increase in the expression of the signature cytokines of Th1, Th2, and Th17 cells in cKO T cells compared with control T cells, whereas, strikingly, IL-4 was significantly lower in the cKO T cells. These data suggest that loss of Bcl6 in CD4 T cells leads to a loss of T\textsubscript{FH} cells, without a compensatory increase in T cell differentiation into other helper lineages. We next wanted to test whether Bcl6 directly regulates the expression of key transcription factors that regulate T cell differentiation (Tbet [Tbx21], Gata3, retinoic acid–related orphan receptor -γ [Rorc], and Blimp1 [Prdm1]), as has been reported (9–11, 21, 22). Thus, we isolated naïve CD4\textsuperscript{+}CD44\textsuperscript{low}CD62L\textsuperscript{+} T cells from control and cKO mice, activated them under Th0, ThN, and T\textsubscript{FH} cell conditions for 24 h, and analyzed gene expression (Fig. 5D). Of the four factors, only Gata3 was repressed by Bcl6 under all activation conditions, although the repression of Gata3 by Bcl6 was <2-fold. Tbx21 was strongly increased in the cKO T cells under Th0 but not other conditions. Rorc trended toward an increase in the cKO T cells under T\textsubscript{FH} cell conditions, but the increase was not statistically significant. Thus, the regulation of the Th1 and Th17 master...
factors by Bcl6 is dependent on specific stimulation conditions. Prdm1 was increased ∼2-fold under TFH cell conditions, but not with other conditions. Thus, Bcl6 does not acutely repress the expression of Prdm1 following TCR- and CD28-mediated activation of naive CD4 T cells. Repression of Prdm1 by Bcl6 occurs under TFH cell–priming conditions, likely because IL-6 and IL-21 under these conditions strongly induce Stat3. However, the increase in Prdm1 in the cKO under TFH cell–priming conditions does not correlate with enhanced differentiation into effector Th1, Th2, and Th17 cells. Importantly, these data with conditional loss of Bcl6 in T cells indicate that much of the increased Th1, Th2, and Th17 differentiation observed in germline Bcl6-deficient mice can be attributed to T cell–extrinsic effects, possibly due to loss of Bcl6-mediated repression of inflammatory cytokines in myeloid cells (14, 15, 23, 24).

To further understand the role of Bcl6 in the regulation of gene expression in T cells, we analyzed IL-10, a previously identified target of Bcl6 in T cells (25), in the cKO mice. We initially analyzed IL-10 secretion by activated CD4 T cells from SRBC-immunized control and cKO mice (Fig. 6A). IL-10 secretion was dramatically increased from cKO T cells (20-fold) compared with control T cells. We then tested Il10 mRNA expression and determined that it was significantly higher in the cKO T cells under TFH cell conditions (Fig. 6B). As assessed by ICS, the total percentage of IL-10–expressing T cells was slightly higher in T cells from cKO mice, although the difference was not statistically significant (Fig. 6C). As shown in Supplemental Fig. 3, exclusion of dead cells and staining of unstimulated T cells verified the specificity of the IL-10 ICS. Using ICS, we then measured the level of IL-10 expression per individual T cell and found it was significantly higher in the cKO T cells (Fig. 6D). Taken together, these data show that Bcl6 critically regulates IL-10 expression in CD4 T cells in a T cell–intrinsic manner and, moreover, that Bcl6 is required to repress IL-10 expression during TFH cell differentiation.

**Discussion**

TFH cells have emerged as the critical T cell subset that promotes the GC reaction and thus the high-affinity B cell response to Ag. Bcl6 is a master regulator of the TFH cell lineage, and there is great interest in understanding TFH cells and the role of Bcl6 in TFH cells. In this study, we have developed and characterized a novel mouse model for the study of TFH cells: Bcl6fl/flCreCD4 mice. In these mice, Bcl6 is deleted specifically in the T cell lineage. In contrast to germline Bcl6 KO mice, Bcl6fl/flCreCD4 mice do not develop inflammatory disease and do not die at an early age. Bcl6fl/flCreCD4 mice thus have great advantage over...
germline Bcl6 KO mice for the analysis of T<sub>FH</sub> cells. In this study, we show that Bcl6<sup>fl/fl</sup>Cre<sup>CD4</sup> mice have normal T cell development in the thymus and can produce Th1, Th2, and Th17 cells, but they specifically lack T<sub>FH</sub> cells. Thus, Bcl6<sup>fl/fl</sup>Cre<sup>CD4</sup> mice are a novel model of T<sub>FH</sub> cell deficiency, and they may be a more specific system for studying immune responses in the absence of T<sub>FH</sub> cells, compared with other available mice strains in which T<sub>FH</sub> cells do not develop.

The lack of exaggerated differentiation of Th1, Th2, and Th17 cells in Bcl6<sup>fl/fl</sup>Cre<sup>CD4</sup> (cKO) mice was unexpected in light of previous work indicating that Bcl6 negatively regulates the differentiation of these lineages (9–11, 21, 22). Our results with the cKO mice imply that much, if not all, of the increased Th1, Th2, and Th17 differentiation observed in germline Bcl6-deficient mice is due to indirect or non-T cell-intrinsic effects. The overproduction of proinflammatory cytokines by Bcl6-deficient myeloid cells (14, 15, 23, 24) undoubtedly contributes to the increased Th1, Th2, and Th17 differentiation in germline Bcl6-deficient mice. In the cKO mice, where loss of Bcl6 is specifically restricted to T cells, we observe no bias in Th cell differentiation toward the Th1, Th2, and Th17 lineages. Thus, much of the enhanced effector T cell phenotype previously seen with germline Bcl6-deficient mice was due to indirect effects masking the true phenotype of loss of Bcl6 in T cells on Th cell differentiation.

Given our previous studies showing a strong bias of Bcl6-deficient T cells to the Th2 lineage (12–14, 19), a highly unexpected result in the cKO mice was significantly decreased Th2 differentiation (as measured by IL-4 expression) compared with control mice. Thus, Bcl6-deficient T cells in the absence of Bcl6-deficient myeloid cells have a defect in IL-4 production and/or

**FIGURE 5.** Loss of Bcl6 in T cells does not lead to increased Th1, Th2, or Th17 differentiation. (A) Mice were immunized with SRBCs and sacrificed on day 10. Total CD4<sup>+</sup> T cells were isolated via magnetic bead separation and stimulated with PMA and ionomycin for 5 h before being fixed and stained for flow cytometry. Representative flow plots for ICS of IFN-γ, IL-17A, and IL-4 are shown. Gated on CD3<sup>+</sup> CD4<sup>+</sup>. (B) Graphs of ICS (n = 3–4, mean ± SE). Data shown are representative of four separate experiments. (C) Total CD4<sup>+</sup> T cells were isolated as in (A) and stimulated with anti-CD3 and anti-CD28 Abs for 24 h in Th0 culture conditions. Cytokine levels in supernatants were measured via ELISA (n = 3–4, mean ± SE). Data shown are representative of four separate experiments. (D) Naïve CD4<sup>+</sup> T cells were sorted via FACS and stimulated with anti-CD3 and anti-CD28 Abs for 24 h in either Th0, ThN, or T<sub>FH</sub> cell culture conditions. Gene expression was measured by QPCR (n = 3–4, mean ± SE). This experiment was repeated once with similar results. Symbols in bar graphs represent individual mice. *p < 0.05, **p < 0.01.
Our data in this study indicate that Bcl6-deficient T cells, in an otherwise wild-type immune environment, do not undergo enhanced differentiation into Th1, Th2, and Th17 cells. Thus, Bcl6 does not generally repress CD4 T cell differentiation into Th1, Th2, and Th17 cells, and the increased Th1, Th2, and Th17 responses in germine Bcl6-deficient mice are due to T cell-extrinsic factors, as discussed above. A further possible interpretation for this result is that Bcl6 does not control differentiation into the Tfh cell lineage by repressing Th1, Th2, and Th17 differentiation, as proposed. However, we cannot rule out that a small number of T cells in the cKO mice that would normally become Tfh cells following Ag stimulation (if they could induce Bcl6 expression) actually undergo enhanced differentiation into Th1, Th2, and/or Th17 cells. We need to assume that this population is too small to markedly affect the total cytokine profile of effector cells in the cKO mice, since overall, we observe similar Th1, Th2, and Th17 responses in the cKO mice as in the control mice.

We do not detect repression of Blimp1 by Bcl6 under Th0 or ThN conditions, indicating that Bcl6 does not generally repress Blimp1 transcription. However, under specific Tfh cell activation conditions, we observe significant repression of Blimp1 by Bcl6. This specific regulation of Blimp1 by Bcl6 thus fits with one of the three proposed models for the control of Tfh cell differentiation by Bcl6. However, the 2-fold increase in Blimp1 in cKO T cells is unlikely to fully account for the near complete loss of Tfh cell differentiation we observe in the cKO mice, and other Bcl6-regulated pathways are bound to be critical for normal Tfh cell differentiation.

Indeed, our study highlights two novel mechanisms for how Bcl6 controls the Tfh cell lineage: 1) by repressing IL-10 expression, and 2) by inhibiting apoptosis of Tfh cells. IL-10 has been shown recently to suppress Tfh cell differentiation and function (26, 27). Therefore, a key function of Bcl6 may be to suppress expression of IL-10 by activated T cells, thus aiding Tfh cell differentiation. IL-10 is a potent suppressor of T cell activation by acting on APCs (28). Because Tfh cell differentiation requires high-affinity interaction between the T cell and the APC (29), suppression of IL-10 activation, leading to suppression of APC activity and weaker T cell activation. In mice, Th2 cells produce high levels of IL-10, and one earlier model of repression of IL-10 by Bcl6 was that this was part of the repression of Th2 differentiation by Bcl6 (19, 25). However, our data show that the regulation of IL-10 in T cells by Bcl6 is a separate pathway from the regulation of Th2 differentiation by Bcl6, because we observe greatly enhanced IL-10 expression at the same time as significantly decreased Th2 differentiation in the cKO mice. Another novel mechanism we have described for the control of Tfh cells by Bcl6 is suppression of apoptosis. Although there are extensive associations between PD-1 expression and T cell apoptosis (20), there has been little investigation into the apoptosis of PD-L1high Tfh cells. In this study, we assessed whether PD-L1high Tfh cells were undergoing higher levels of apoptosis than PD-L1low/neg T cells, and we observed that PD-L1high Tfh cells expressed both activated caspase-3 and annexin V, markers of early apoptosis, at a very significant level. Thus, PD-L1high Tfh cells are more unstable and prone to apoptosis, and this apoptosis is accelerated in the absence of Bcl6. Thus, Bcl6 appears to stabilize the survival of PD-L1high Tfh cells, which is a previously unappreciated mechanism for the function of Bcl6 in Tfh cells. By analogy, Bcl6 inhibits the apoptosis of B cells within the GC, in part by repressing the DNA damage sensor ATR and

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**FIGURE 6.** Bcl6 is a critical repressor of IL-10 expression in Th cells. (A) Total CD4+ T cells from immunized mice were isolated and stimulated as in Fig. 5C. Levels of IL-10 secretion measured by ELISA (n = 3–4, mean ± SE). (B) IL-10 gene expression was measured by QPCR under Th0, ThN, and Tfh cell conditions, as in Fig. 5D (n = 3–4, mean ± SE). (C) IL-10–expressing cells as a percentage of total CD4+ T cells from immunized mice, isolated, and stimulated as described in Fig. 5A; dead cells were excluded by use of a viable cell staining gate (n = 3–5, mean ± SE). (D) IL-10 expression levels in IL-10+ cells measured by mean fluorescence intensity (MFI). (A–D) Symbols in bar graphs represent individual mice. Data shown are representative of three to four separate experiments. *p < 0.05, **p < 0.01.

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Th2 differentiation. This result was further surprising given that Gata3 mRNA was increased in cKO T cells (Fig. 5D). We previously showed that in mixed bone marrow chimeras with wild-type and germine Bcl6 KO cells that Bcl6-deficient T cells still have a significant intrinsic Th2 bias compared with wild-type T cells within the same chimera (19). However, Bcl6-deficient myeloid cells are still present in these mixed bone marrow chimeras, meaning that the Th2 bias of Bcl6 KO T cells may only manifest in the presence of the inflammatory cytokines. Thus, the ability of Bcl6 to regulate Th2 differentiation is clearly complex and is affected by inflammatory cytokine signals secreted by myeloid cells. Further work is required to completely understand how Bcl6 regulates Th2 differentiation.

An essential question regarding Tfh cells is how Bcl6 controls the development of this lineage. Three basic mechanisms have been proposed: 1) Bcl6 inhibits differentiation of CD4 T cells into Th1, Th2, and Th17 cells, thus indirectly favoring Tfh cell differentiation; 2) Bcl6 inhibits terminal CD4 T cell differentiation by repressing Blimp1, thus favoring a relatively undifferentiated Tfh cell state; and 3) Bcl6 represses a large number of microRNAs that directly promote the Tfh cell phenotype (3, 9–11). These three mechanisms are not mutually exclusive, and Bcl6 may use all of these mechanisms, as well as other mechanisms not yet understood. Importantly, the extent to which each of these three known pathways control Thfh cell differentiation is not well understood.
inhibiting apoptotic pathways activated by the extensive DNA alterations that occur in GC B cells (30). Although TFH cells do not undergo DNA rearrangements analogously to GC B cells, there may be proapoptotic stress signals for Tfh cells in the GC that are inhibited by Bcl6 expression. This prosurvival function of Bcl6 in Tfh cells is a new pathway that provides an important insight into Tfh cell biology, and clearly warrants further exploration.

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Disclosures

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