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

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Abstract

The transcriptional repressor Bcl6 controls development of the follicular helper T cell (T_{FH}) lineage, however 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 alternative effector lineages, thus favoring T_{FH} differentiation. Analysis of T cell differentiation using Bcl6-deficient mice has been complicated by the strong pro-inflammatory phenotype of Bcl6-deficient myeloid cells. Here, we report data from a novel mouse model where Bcl6 is conditionally deleted in T cells ($Bcl6^{fl/fl}Cre^{CD4}$ mice). After immunization, PD-1^{high} T_{FH} cells in $Bcl6^{fl/fl}Cre^{CD4}$ mice are decreased over 90% compared to control mice, and antigen-specific IgG is sharply reduced. Residual PD-1^{high} CXCR5⁺ T_{FH} cells in $Bcl6^{fl/fl}Cre^{CD4}$ mice show a significantly higher rate of apoptosis than PD-1^{high} CXCR5⁺ T_{FH} cells in control mice. Immunization of $Bcl6^{fl/fl}Cre^{CD4}$ mice did not reveal enhanced differentiation into T_{H1} , T_{H2} or T_{H17} lineages, although IL-10 expression by CD4 T cells was markedly elevated. Thus, T cell extrinsic factors appear to promote the increased T_{H1} , T_{H2} and T_{H17} responses in germ-line 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.

Introduction

During an immune response, CD4 T helper cells can differentiate into several unique effector lineages that promote different immune responses via the secretion of distinct types of cytokines. Follicular T helper (T_{FH}) cells are a recently characterized CD4 lineage whose major function is to help B cells form germinal centers (GCs) and produce high-affinity antibodies (Abs) (reviewed in (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 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. While T_{FH} cells are critical for the proper production of high affinity Abs, the over-production 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 up-regulated in T_{FH} cells and is considered a master regulator for the T_{FH} 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, though three possible mechanisms have been proposed: a) Bcl6 inhibits the differentiation of CD4 T cells into other lineages (e.g. T_H1, T_H2, T_H17), thus indirectly favoring T_{FH} differentiation, b) Bcl6 inhibits terminal CD4 T cell differentiation by repressing Blimp1, again indirectly favoring the T_{FH} differentiation state, c) Bcl6 regulates a large number of microRNAs that directly control the T_{FH} fate (3). Bcl6 may promote T_{FH} 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 due 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, due to the difficulty of producing large numbers of consistently constituted chimeric mice for in-depth immunological studies. Further, these bone marrow chimeric mice cannot separate out the effects of hyper-inflammatory 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* reported a conditional KO model of Bcl6, and used it to analyze memory B cell development (16). Here, 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

Bcl6^{fl/fl} mice on a mixed C57BL/6-129Sv background were generated at the Indiana University School of Medicine (IUSM) 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. Cre^{EIIa} mice, obtained from Jackson Labs, 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 – TGAAGACGTGAAATCTAGATAGGC – 3)
- 5 loxP reverse (5 – ACCCATAGAAACACACTATACATC – 3)
- 3 loxP forward (5 –TCACCA ATCCCAGGTCTCAGTGTG–3)
- 3 loxP reverse (5 – CTTTGT CATATTTCTCTGGTTGCT–3).

Bcl6^{fl/fl} mice were mated to CD4-cre mice (17) to generate Bcl6^{fl/fl} Cre^{CD4} mice. Mice were bred under specific pathogen-free conditions at the laboratory animal facility at IUSM and were handled according to protocols approved by the IUSM Animal Use and Care Committee. Mice were immunized i.p. with 1×10^9 sheep red blood cells (SRBC; Rockland Immunochemicals Inc., 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 T cells and NKT cells were identified with CD1d tetramer obtained from the NIH tetramer core facility. Total spleen or thymus cells were incubated with anti-mouse CD16/CD32 (Fc

receptor) for 20 minutes, followed by surface staining for the indicated markers. For intracellular cytokine staining (ICS), total CD4⁺ T cells were isolated from spleen via bead separation (Miltenyi Biotec) and stimulated for 5 hours with PMA and ionomycin. After washing, cells were stained with a viability stain (Fixable Viability Dye eFluor[®] 780 from eBioscience), then stained for surface CD3 and CD4 and fixed in 2% formaldehyde for 10 minutes at RT in the dark. Cells were then washed twice in buffer containing 0.1% saponin to permeabilize. ICS was carried out in the saponin buffer. Annexin V staining and Caspase-3 staining were done on total splenocytes using the Annexin V Apoptosis detection Kit from eBioscience and the Caspase-3 Apoptosis kit from BD Biosciences, using manufacturers' instructions.

In vitro stimulation

Total CD4⁺ T cells were isolated via magnetic bead separation (Miltenyi Biotec); naïve CD4⁺ T cells were isolated via FACS and gated as CD3⁺ CD4⁺ CD62L⁺ CD44^{low}. Cells were stimulated with plate-bound anti-CD3 (5 µg/ml) and anti-CD28 (10 µg/ml) Abs (BD Biosciences) for 24 hours at 1×10⁶ cells/ml. Th0 media conditions contain no cytokines or blocking antibodies, Th neutral (ThN) conditions contain anti-IFN γ and anti-IL-4 (10 µg/mL) (BD Biosciences) and T_{FH} conditions contain IL-6 and IL-21 (10 ng/ml each (R&D Systems), plus anti-IFN γ , -IL-4 and -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 antibodies were used (BD Biosciences). SRBC-specific IgG was measured as previously described (18). Briefly, wells were coated with SRBC membrane extract (prepared as described (18)) overnight at 4°C. Wells were blocked with 10% FCS and diluted serum was incubated in wells for 2 hours at RT. A peroxidase labeled Fc-specific anti-mouse IgG detection antibody was used (Sigma).

Gene expression analysis

Total cellular RNA was prepared using the Trizol method (Life Technologies), and cDNA 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 (ABI). QPCR assays were run with a Stratagene Mx3000P Real-Time QPCR machine. Levels of mRNA expression were normalized to beta-tubulin mRNA levels, and differences between samples analyzed using the ddCT method. Primers for SYBR Green assays were previously described (14, 19).

Statistical Analysis

Preliminary statistical analysis showed that the data was normally distributed and thus further statistical analysis was done using Student's *t* tests or ANOVA on SPSS Statistics 20 software.

Results

A conditional KO allele for Bcl6 (floxed Bcl6 or Bcl6^{fl}), where loxP sites flanked the zinc finger-encoding exons of Bcl6 (Figure 1A), was introduced into embryonic stem cells. After germline transmission of the floxed allele and deletion of the Neomycin gene, Bcl6^{fl/+} mice were mated to produce Bcl6^{fl/fl} offspring. Bcl6^{fl/fl} mice are born at an expected frequency, look normal and produce normal GC B cell and T_{FH} responses to immunization

(Supplemental Figure 1), thus indicating that the loxP targeting of the Bcl6 gene did not interfere with normal Bcl6 expression and function. CD4-Cre mice (17) were mated to Bcl6^{fl/fl} mice, to obtain Bcl6^{fl/+}Cre^{CD4} 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 (Figure 1B, Supplemental Figure 2). Bcl6^{fl/fl}Cre^{CD4} (conditional knockout or cKO) mice are born at an expected frequency, and in contrast to germline Bcl6 KO mice (13), 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 (Figure 2A, B). Consistent with our earlier findings (19), the number of FoxP3⁺ cells was unaffected by loss of Bcl6 (Figure 2C).

Next, we immunized Bcl6^{+/+}Cre^{CD4} (control) and Bcl6^{fl/fl}Cre^{CD4} (cKO) mice with sheep red blood cells (SRBCs) and analyzed GC B cell and T_{FH} responses after 10 days, at the peak of the response. Whereas control mice produced strong levels of Fas⁺GL7⁺PNA⁺ GC B cells and CXCR5⁺ICOS⁺PD-1^{high} T_{FH} cells, cKO mice had an almost complete loss of these cell populations (Figure 3A, B). These results confirm that Bcl6 controls T_{FH} cell development and/or survival, in a T cell intrinsic manner, and further, that T_{FH} cells are absolutely required to drive the GC reaction. To test whether loss of the T_{FH} cell population resulted in a functional defect in antibody production, we measured SRBC-specific IgG titers (Figure 3C). Antigen specific IgG was roughly 5-fold lower in the cKO mice, showing that loss of CXCR5⁺ICOS⁺PD-1^{high} T_{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 if PD-1^{high} T_{FH} cells were undergoing higher levels of apoptosis than T cells with lower levels of PD-1 expression, and if this apoptosis was regulated by Bcl6. Thus, we used two different markers of early apoptosis, active Caspase-3 and AnnexinV, to stain T cells from SRBC immunized control and cKO mice (Figure 4). We then analyzed apoptosis in non-T_{FH} cells and in CXCR5⁺ cells, focusing on the correlation between the level of PD-1 expression and degree of apoptosis. Non-T_{FH} cells had minimal apoptotic cells, whereas apoptosis increased in the CXCR5⁺ cells in parallel with PD-1 expression, with the highest levels of apoptotic cells in the CXCR5⁺PD-1^{high} T_{FH} fraction (Figure 4B, C). In cKO mice, non-T_{FH}, CXCR5⁺PD-1⁻, and CXCR5⁺PD-1^{low} populations exhibited similar or lower levels of apoptosis compared to control mice (Figure 4B, C). 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^{high} T_{FH} fraction (Figure 4B, C). These data indicate that Bcl6 regulates apoptosis in PD-1^{high} T_{FH} cells. Therefore, one novel mechanism for how Bcl6 controls T_{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_{FH} cell development by inhibiting the differentiation of CD4 T cells into other T_H lineages. We reasoned that if Bcl6 inhibited T helper cell differentiation, following a potent immune stimulus, CD4 T cells would differentiate more readily into T_H1, T_H2 or T_H17 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 Figure 5A–C, we observed no significant increase in the expression of the signature cytokines of T_H1, T_H2 and T_H17 cells in cKO T cells compared to control T cells, while 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_{FH} cells, without a compensatory increase in T cell differentiation into other helper lineages. We next wished to test whether Bcl6 directly regulates the expression of key transcription factors that regulate T cell differentiation (Tbet (*Tbx21*), Gata3, Ror- γ (*Rorc*) and Blimp1 (*Prdm1*)), as has been

reported (9–11, 21, 22). Thus, we isolated naïve CD4⁺CD44^{low}CD62L⁺ T cells from control and cKO mice, activated them under T_{H0}, T_{H1}N and T_{FH} conditions for 24 hours, and analyzed gene expression (Figure 5D). Of the four factors, only *Gata3* was repressed by Bcl6 under all activation conditions, although the repression of *Gata3* by Bcl6 was less than two-fold. *Tbx21* was strongly increased in the cKO T cells under T_{H0} but not other conditions. *Rorc* trended towards an increase in the cKO T cells under T_{FH} conditions, but the increase was not statistically significant. Thus, the regulation of the T_{H1} and T_{H17} master factors by Bcl6 is dependent on specific stimulation conditions. *Prdm1* was increased about 2-fold under T_{FH} conditions, but not with other conditions. Thus, Bcl6 does not acutely repress the expression of *prdm1* following TCR- and CD28-mediated activation of naïve CD4 T cells. Repression of *prdm1* by Bcl6 occurs under T_{FH}-priming conditions, likely because IL-6 and IL-21 under these conditions strongly induce Stat3. However, the increase in *prdm1* in the cKO under T_{FH}-priming conditions does not correlate with enhanced differentiation into effector T_{H1}, T_{H2} and T_{H17} cells. Importantly, these data with conditional loss of Bcl6 in T cells indicate that much of the increased T_{H1}, T_{H2} and T_{H17} 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 (Figure 6A). IL-10 secretion was dramatically increased from cKO T cells, over 20-fold, compared to control T cells. We then tested *Il10* mRNA expression, and determined that it was significantly higher in the cKO T cells under T_{FH} conditions (Figure 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 (Figure 6C). As shown in Supplemental Figure 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 (Figure 6D). Taken together, these data show that Bcl6 critically regulates IL-10 expression in CD4 T cells by a T cell intrinsic manner, and moreover, that Bcl6 is required to repress IL-10 expression during T_{FH} differentiation.

Discussion

T_{FH} cells have emerged as the critical T cell subset that promotes the germinal center reaction and thus the high affinity B cell response to antigen. Bcl6 is a master regulator of the T_{FH} cell lineage, and there is great interest in understanding T_{FH} cells and the role of Bcl6 in T_{FH} cells. Here we have developed and characterized a novel mouse model for the study of T_{FH} cells: Bcl6^{fl/fl}Cre^{CD4} mice. In these mice, Bcl6 is deleted specifically in the T cell lineage. In contrast to germline Bcl6 knockout mice, Bcl6^{fl/fl}Cre^{CD4} mice do not develop inflammatory disease and do not die at an early age. Bcl6^{fl/fl}Cre^{CD4} mice thus have great advantage over germline Bcl6 knockout mice for the analysis of T_{FH} cells. Here we show that Bcl6^{fl/fl}Cre^{CD4} mice have normal T cell development in the thymus and can produce T_{H1}, T_{H2} and T_{H17} cells, but specifically lack T_{FH} cells. Thus, Bcl6^{fl/fl}Cre^{CD4} mice are a novel model of T_{FH} cell-deficiency, and may be a more specific system for studying immune responses in the absence of T_{FH} cells, compared to other available mice strains in which T_{FH} cells do not develop.

The lack of exaggerated differentiation of T_{H1}, T_{H2} and T_{H17} cells in Bcl6^{fl/fl}Cre^{CD4} (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 T_H1 , T_H2 and T_H17 differentiation observed in germline Bcl6-deficient mice is due to indirect or non-T cell intrinsic effects. The over-production of pro-inflammatory cytokines by Bcl6-deficient myeloid cells (14, 15, 23, 24) undoubtedly contributes to the increased T_H1 , T_H2 and T_H17 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 T helper cell differentiation towards the T_H1 , T_H2 and T_H17 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 helper T cell differentiation.

Given our previous studies showing a strong bias of Bcl6-deficient T cells to the T_H2 lineage (12–14, 19), a highly unexpected result in the cKO mice was significantly decreased T_H2 differentiation (as measured by IL-4 expression) compared to control mice. Thus, Bcl6-deficient T cells in the absence of Bcl6-deficient myeloid cells have a defect in IL-4 production and/or T_H2 differentiation. This result was further surprising given that Gata3 mRNA was increased in cKO T cells (Figure 5D). We previously showed that in mixed bone marrow chimeras with wild-type and germline Bcl6 knockout cells, that Bcl6-deficient T cells still have a significant intrinsic T_H2 bias compared to wild-type T cells within the same chimera (19). However, Bcl6-deficient myeloid cells are still present in these mixed bone marrow chimeras, meaning the T_H2 bias of Bcl6 knockout T cells may only manifest in the presence of the inflammatory cytokines. Thus, the ability of Bcl6 to regulate T_H2 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 T_H2 differentiation.

An essential question regarding T_{FH} cells is how Bcl6 controls the development of this lineage. Three basic mechanisms have been proposed: a) Bcl6 inhibits differentiation of CD4 T cells into T_H1 , T_H2 and T_H17 cells, thus indirectly favoring T_{FH} differentiation, b) Bcl6 inhibits terminal CD4 T cell differentiation by repressing Blimp1, thus favoring a relatively undifferentiated T_{FH} state, and c) Bcl6 represses a large number of microRNAs that directly promote the T_{FH} 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 T_{FH} cell differentiation is not well understood.

Our data in this study indicate that Bcl6-deficient T cells, in an otherwise wild-type immune environment, do not undergo enhanced differentiation into T_H1 , T_H2 and T_H17 cells. Thus, Bcl6 does not generally repress CD4 T cell differentiation into T_H1 , T_H2 and T_H17 cells, and the increased T_H1 , T_H2 and T_H17 responses in germ-line 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 T_{FH} lineage by repressing T_H1 , T_H2 and T_H17 differentiation, as proposed. However, we cannot rule out that a small number of T cells in the cKO mice that would normally become T_{FH} cells following antigen stimulation (if they could induce Bcl6 expression) actually undergo enhanced differentiation into T_H1 , T_H2 and/or T_H17 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 T_H1 , T_H2 and T_H17 responses in the cKO as in the control mice.

We do not detect repression of *Blimp1* by Bcl6 under T_H0 or T_HN conditions, indicating that Bcl6 does not generally repress *Blimp1* transcription. However, under specific T_{FH} 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 T_{FH} differentiation by Bcl6. However, the two-fold increase in Blimp1 in cKO T cells is

unlikely to fully account for the near complete loss of T_{FH} differentiation we observe in the cKO mice, and other Bcl6-regulated pathways are bound to be critical for normal T_{FH} differentiation.

Indeed, our study highlights two novel mechanisms for how Bcl6 controls the T_{FH} lineage: 1) by repressing IL-10 expression, and 2) by inhibiting apoptosis of T_{FH} cells. IL-10 has been shown recently to suppress T_{FH} 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 T_{FH} differentiation. IL-10 is a potent suppressor of T cell activation, by acting on APCs (28). Since T_{FH} differentiation requires high affinity interaction between the T cell and the APC (29), suppression of IL-10 may therefore be a critical mechanism for the control of T_{FH} differentiation by Bcl6. In cKO mice, T cells would receive the same signals to express Bcl6, but would not be able to up-regulate Bcl6 or suppress IL-10 gene transcription following 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, since 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 T_{FH} cells by Bcl6 is suppression of apoptosis. While there are extensive associations between PD-1 expression and T cell apoptosis (20), there has been little investigation into the apoptosis of PD-1^{high} T_{FH} cells. Here, we assessed whether PD-1^{high} T_{FH} cells were undergoing higher levels of apoptosis than PD-1^{low/neg} T cells, and observed that PD-1^{high} T_{FH} cells expressed both activated Caspase-3 and AnnexinV, markers of early apoptosis, at a very significant level. Thus, PD-1^{high} T_{FH} 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-1^{high} T_{FH} cells, which is a previously unappreciated mechanism for the function of Bcl6 in T_{FH} cells. By analogy, Bcl6 inhibits the apoptosis of B cells within the GC, in part by repressing the DNA damage sensor ATR and inhibiting apoptotic pathways activated by the extensive DNA alterations that occur in GC B cells (30). While T_{FH} cells are not known to undergo DNA rearrangements analogously to GC B cells, there may be pro-apoptotic stress signals for T_{FH} cells in the GC that are inhibited by Bcl6 expression. This pro-survival function of Bcl6 in T_{FH} cells is a new pathway that provides an important insight into T_{FH} cell biology, and clearly warrants further exploration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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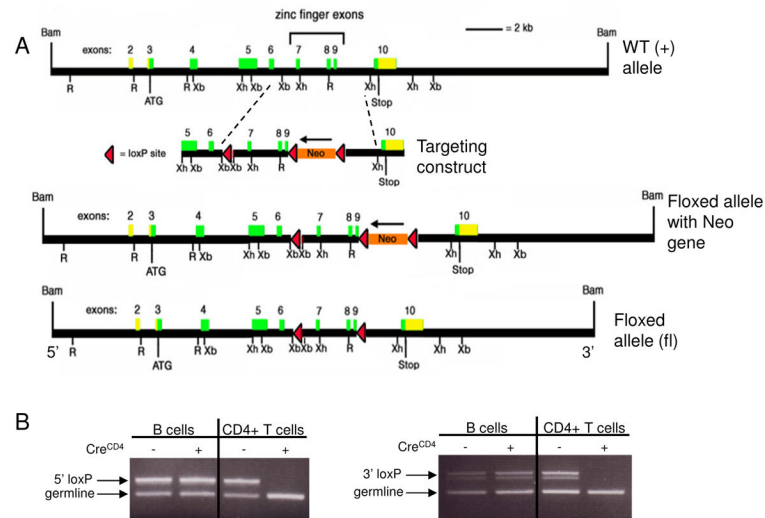


Figure 1. Development of a conditional deletion system for BCL6

(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 Cre^{EIIa} 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.

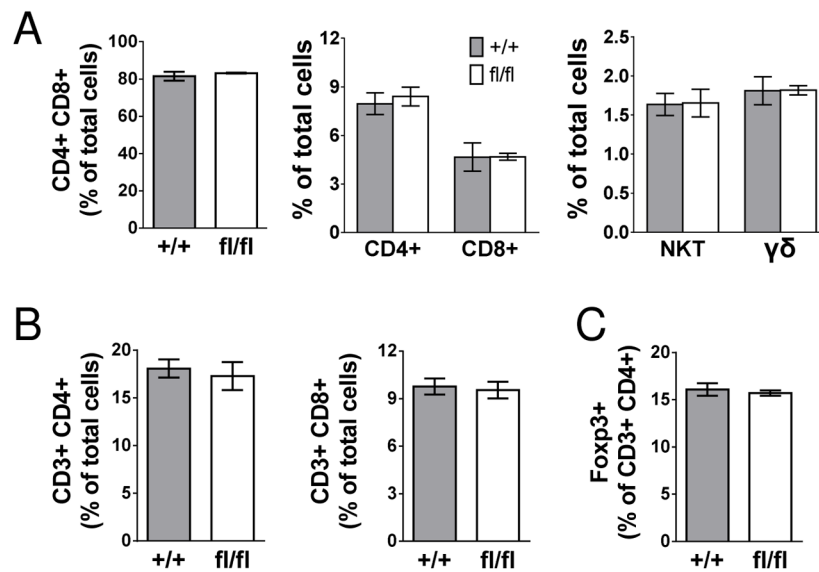


Figure 2. Normal T cell development in mice with conditional deletion of Bcl6 in T cells
 (A) Thymus cell percentages in 8 week old unimmunized $Bcl6^{+/+} Cre^{CD4}$ and $Bcl6^{fl/fl} Cre^{CD4}$ mice were enumerated via flow cytometry. From left, double positive CD4 CD8 cells, single positive CD4 and CD8 cells, natural killer T cells, and gamma delta T cells. $n = 4$, mean \pm SE. (B) T cell percentages in spleen of 7 – 9 week old mice immunized with SRBC and sacrificed on day 10. $n = 4$, mean \pm SE. (C) Percentage of T_{reg} cells in spleen of 7 – 8 week old unimmunized mice. Gated on $CD3^+ CD4^+$. $n = 4$, mean \pm SE.

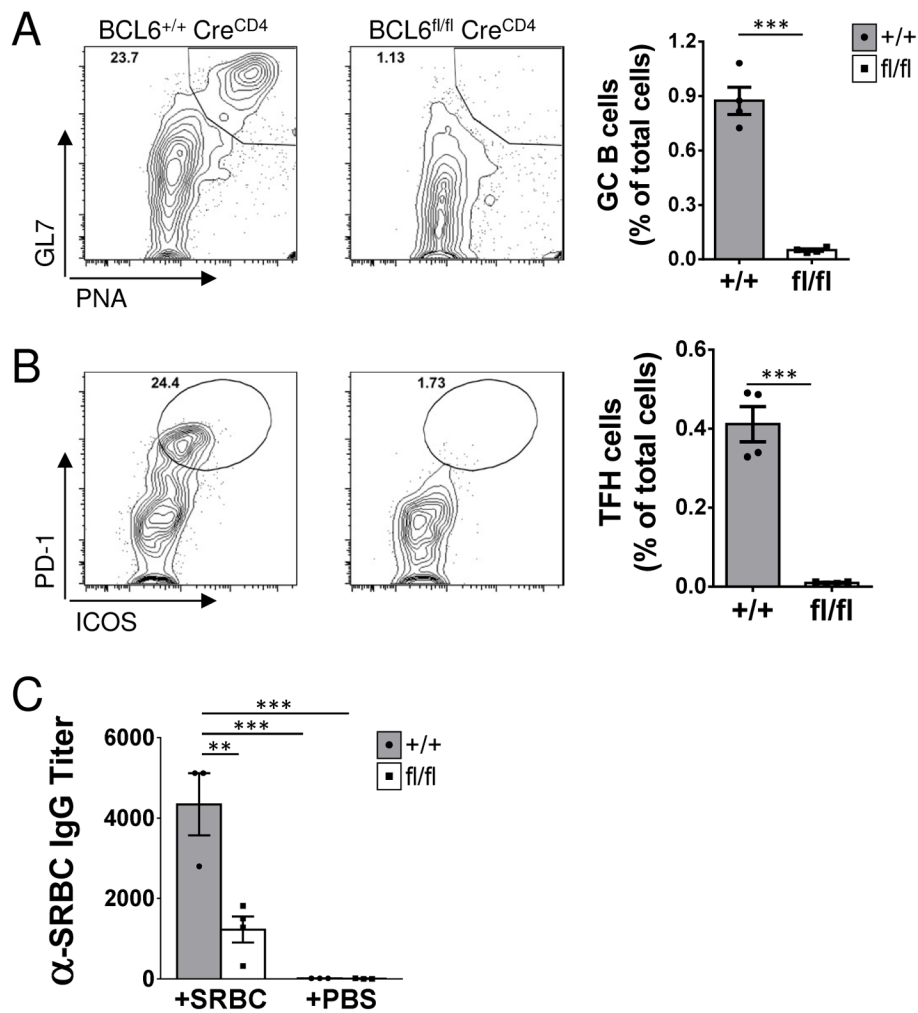


Figure 3. Conditional deletion of Bcl6 in T cells leads to loss of T_{FH} cells and loss of B cell helper activity

Representative flow plots of (A) GC B cell and (B) T_{FH} cell populations in spleen of 7 – 9 week old Bcl6^{+/+} Cre^{CD4} and Bcl6^{fl/fl} Cre^{CD4} mice immunized i.p. with SRBC and sacrificed on day 10. GC B cells gated on CD19⁺ B220⁺ Fas⁺. T_{FH} cells gated on CD3⁺ CD4⁺ CXCR5⁺. (C) Titers of IgG specific for SRBC, from serum of mice analyzed in (A) and (B) and unimmunized mice. n = 4 – 5, mean ± SE. (B and C) Symbols in bar graphs represent individual mice. (A-C) Data shown are representative of four to five separate experiments. *** p < 0.001

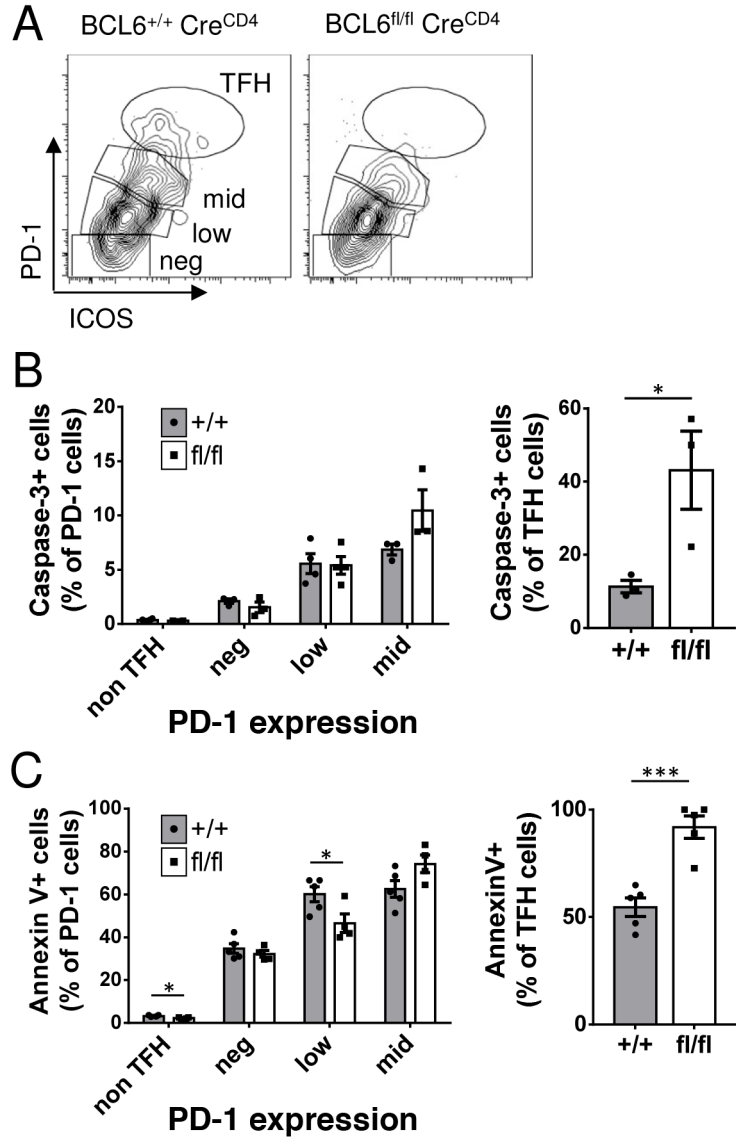


Figure 4. Bcl6 maintains the survival of PD-1^{high} T_{FH} cells
Mice were immunized with SRBC and sacrificed on day 10. Spleen cells were analyzed. (A) Representative flow plots of T_{FH} cells. Gated on CD3⁺ CD4⁺ CXCR5⁺. Gates for different levels of PD-1 expression are shown. (B) Percentage of Caspase-3⁺ cells in different populations of PD-1 subsets are shown. “Non T_{FH}” cells are gated on CD3⁺ CD4⁺ CXCR5^{neg} ICOS^{neg} PD-1^{neg}. n = 3 – 4, mean ± SE. (C) Percentage of AnnexinV⁺ cells in different populations of PD-1 subsets. Same gating as in (B). n = 4 – 5, mean ± SE. (B and C) Symbols in bar graphs represent individual mice. (A–C) Data shown are representative of three separate experiments. * p < 0.05, *** p < 0.001.

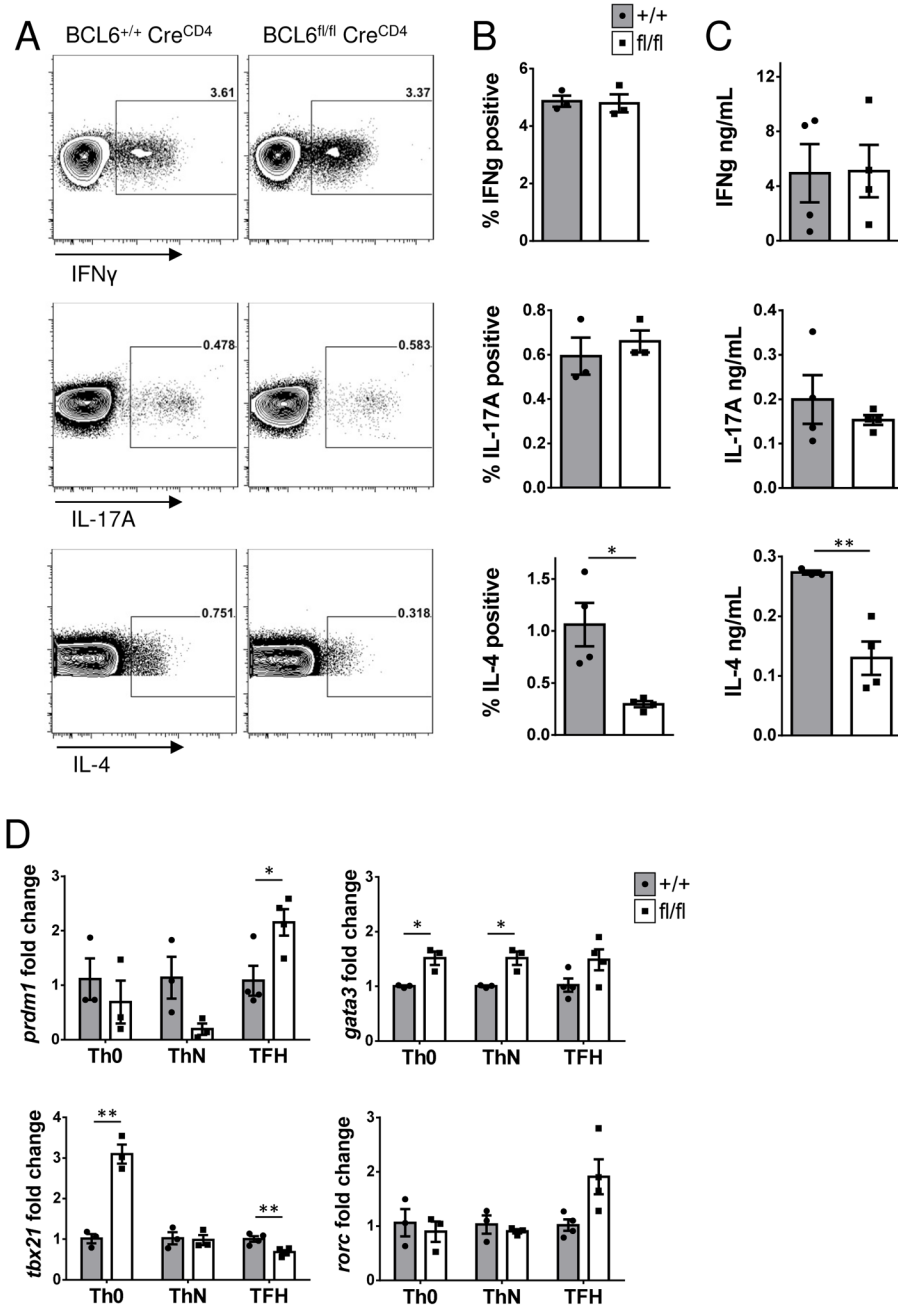


Figure 5. Loss of Bcl6 in T cells does not lead to increased Th1, Th2 or Th17 differentiation
 (A) Mice were immunized with SRBC and sacrificed on day 10. Total CD4⁺ T cells were isolated via magnetic bead separation and stimulated with PMA and ionomycin for 5 hours before being fixed and stained for flow cytometry. Representative flow plots for ICS of IFN γ , IL-17A, and IL-4 are shown. Gated on CD3⁺ CD4⁺. (B) Graphs of ICS. n = 3 – 4, mean \pm SE. Data shown is representative of four separate experiments. (C) Total CD4⁺ T cells were isolated as in (A) and stimulated with anti-CD3 and anti-CD28 antibodies for 24 hours in Th0 culture conditions. Cytokine levels in supernatants were measured via ELISA. n = 3 – 4, mean \pm SE. Data shown is representative of four separate experiments. (D) Naïve CD4⁺ T cells were sorted via FACS and stimulated with anti-CD3 and anti-CD28 Abs for 24 hours

in either T_{H0} , T_{HN} or T_{FH} culture conditions. Gene expression was measured by QPCR. $n = 3 - 4$, mean \pm SE. This experiment was repeated once with similar results. Symbols in bar graphs represent individual mice. (B–D) * $p < 0.05$, ** $p < 0.01$

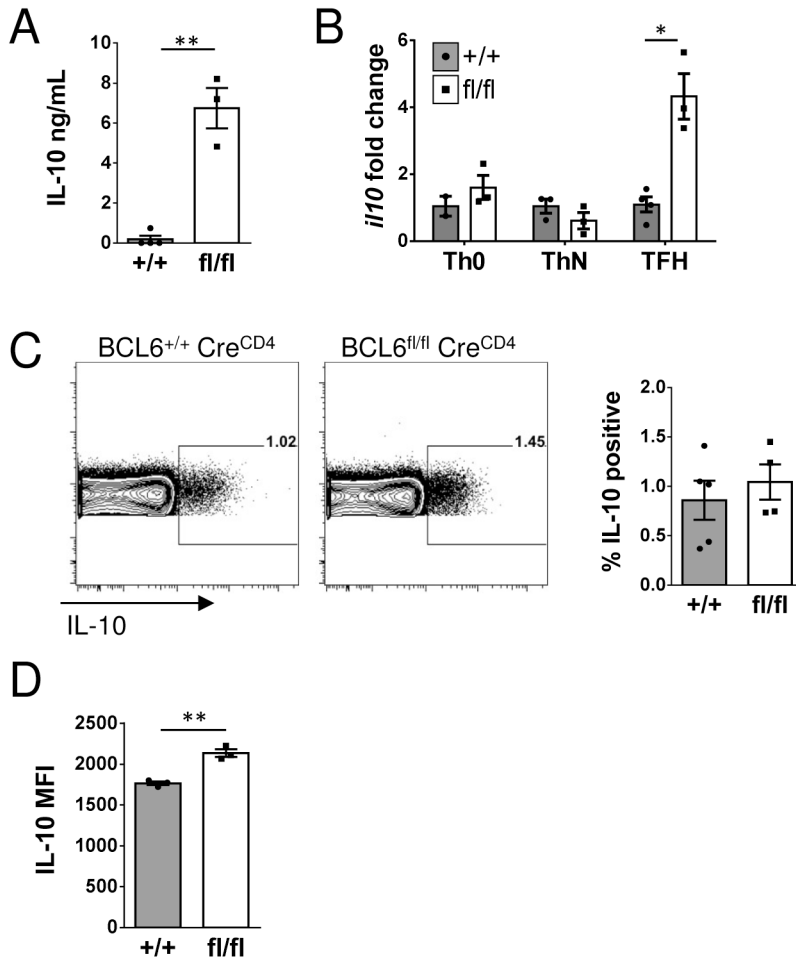


Figure 6. *Bcl6* is a critical repressor of IL-10 expression in T helper cells
 Total CD4⁺ T cells from immunized mice were isolated and stimulated as in Figure 5C. (A) Levels of IL-10 secretion measured by ELISA. n = 3 – 4, mean ± SE. (B) IL-10 gene expression was measured by QPCR, under T_H0, T_HN and T_{FH} conditions, as in Figure 5D. n = 3 – 4, mean ± SE. (C and D) ICS for IL-10 was performed on CD4⁺ T cells from immunized mice, isolated and stimulated as described in Figure 5A; dead cells were excluded by use of a viable cell staining gate. n = 3 – 5, mean ± SE (C) IL-10-expressing cells as a percent of total CD4 T cells. (D) IL-10 expression levels in IL-10⁺ cells measured by mean fluorescent intensity (MFI). (A–D) Symbols in bar graphs represent individual mice. Data shown are representative of 3 to 4 separate experiments. * p < 0.05, ** p < 0.01