Cutting Edge

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Cutting Edge: T Follicular Helper Cell Differentiation Is Defective in the Absence of Bcl6 BTB Repressor Domain Function

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T follicular helper (Tfh) cells are essential for germinal centers (GCs) and most long-term humoral immunity. Differentiation of Tfh cells depends on the transcriptional repressor B cell CLL/lymphoma 6 (Bcl6). Bcl6 mediates gene repression via the recruitment of corepressors. Currently, it is unknown how Bcl6 recruits corepressors to regulate gene expression of Tfh cells. In this article, we demonstrate, using a mutant form of Bcl6 with two BTB (bric-a-brac, tramtrack, broad-complex) mutations that abrogate corepressor binding, that the Bcl6 BTB domain is required for proper differentiation of Tfh and GC-Tfh cells in vivo. Importantly, we also observe a significant defect in GC B cell development. These results are consistent in multiple contexts, including a novel lymphocytic choriomeningitis virus nucleoprotein-specific TCR-transgenic mouse model. Taken together, these data suggest that the Bcl6 BTB domain is a key mediator of the differentiation of Tfh cells. ***The Journal of Immunology*****, 2015, 194: 5599–5603.**

The transcriptional repressor B cell CLL/lymphoma 6 (Bcl6) is essential for the differentiation of T follicular helper (Tfh) cells and germinal center (GC) B cells. Tfh cells are CD4 T cells specialized in providing help for B cells (1). The absence of Tfh cells results in the loss of GCs and, consequently, abrogated memory B cell, plasma cell, and neutralizing Ab responses. Thus, Tfh cells have critical roles in protective immune responses against pathogens, as well as deleterious roles in numerous autoimmune diseases (1, 2).

Bcl6 consists of a bric-a-brac, tramtrack, broad-complex (BTB/POZ) domain, a middle domain (also known as RDII), and a zinc finger domain consisting of six Kruppel-like zinc fingers (1). BTB domains are evolutionarily conserved protein-interaction domains that are widely present in transcription factors (3, 4). The BTB domain forms the interface of the obligate homodimer, and the corepressors BCOR, SMRT, and NCOR bind at the cleft formed by this interface (5–8). Although Bcl6 is required for Tfh cell differentiation (9–12), the contributions of its functional domains in $CD4^+$ T cells are not well understood. In this study, we sought to examine the role of the Bcl6 BTB domain in Tfh cell differentiation and function.

Materials and Methods

Mice and vectors

C57BL/6J (B6) and Cre^{CD4} mice were purchased from The Jackson Laboratory. $Bcl6^{fl/fl}$ (13), CD45.1-congenic, and Smarta TCR–transgenic (SM; specific for lymphocytic choriomeningitis virus [LCMV] gp66–77 on $I\text{-}A^b$) (14) mice were on a full B6 background and were bred at the La Jolla Institute for Allergy and Immunology. $Bcl6^{BTBMUT}$ mice, engineered to express the Bcl6 BTB domain mutant (BTBmut) from the endogenous *Bcl6* locus, were generously provided by Dr. Ari Melnick (15). They were crossed to homozygosity at the La Jolla Institute for Allergy and Immunology for use in all experiments. NIP TCR-transgenic mice were generated as described below and in Supplemental Fig. 1. TCR hybridomas were generated (J. White and P. Marrack, unpublished observations), and TCR sequences were cloned and sequenced using cDNA isolated from LCMV-reactive clones. TCR sequences were expressed in $58\alpha^-\beta^-$ T cell hybridomas and tested for reactivity against LCMV-infected dendritic cells. The TCRαβ pair showing the strongest reactivity (Vα1-Jα8 and Vβ6-Dβ1-Jβ2.3 rearrangements) was chosen and cloned into genomic TCR expression cassette vectors. Linearized DNA fragments were injected into fertilized C57BL/6 eggs at the University California, San Diego Transgenic Mouse Facility (La Jolla, CA). Pups were genotyped (Supplemental Fig. 1). A single α/β TCR-transgenic founder mouse (NIP) was selected and crossed to B6.SJL mice to generate $CD45.1^+$ NIP mice. All animal experiments were conducted in accordance with approved animal protocols. The GFP-expressing retroviral expression vector pMIG was used. BTBmut Bcl6 retrovirus (BTBmut-RV) was generated by inducing two point mutations in the protein interaction domain that do not affect dimerization (16). RV particles were produced as previously described (9). Cell transfers into host mice were performed as described (9) by i.v. injection via the retro-orbital sinus. Transferred cells were allowed to rest in host mice for 3–5 d before infection or immunization. 5×10^5 transduced Smarta cells were transferred into each mouse for day 3 analysis, and 25×10^3 transduced Smarta cells were transferred into each mouse for day 7 analysis. For protein immunization, 5×10^5 cells were transferred into each mouse. 5×10^3 naive CD4 T cells from NIP TCR-transgenic or retrogenic mice were transferred into each mouse.

Infections and immunizations

LCMV Armstrong stocks were prepared and quantified as previously described (9). Infections were performed by i.p. injection of $0.5–2 \times 10^5$ PFU LCMV

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Abbreviations used in this article: B6, C57BL/6J; Bcl6, B cell CLL/lymphoma 6; BTBmut, BTB domain mutant; BTBmut-RV, BTBmut Bcl6 retrovirus; GC, germinal center; KLH, keyhole limpet hemocyanin; LCMV, lymphocytic choriomeningitis virus; NP, nucleoprotein; RV, retrovirus; SM, Smarta TCR transgenic; Tfh, T follicular helper; WT, wild-type.

Armstrong/mouse. gp61-keyhole limpet hemocyanin (KLH) was prepared in alum and injected as described previously (17). A total of 20 μg gp61-KLH was resuspended in alum for bilateral footpad injections.

Flow cytometry

Flow cytometry was done with mAbs against SLAM (CD150; BioLegend) and CD4, CD8, CD44, CD62L, CD25, B220, Fas, and GL7 (all from eBioscience). Stains were done for 30 min at 4°C in PBS supplemented with 0.5% BSA and 0.1% sodium azide, unless specified otherwise. CXCR5 staining was done as described (9, 18). Intracellular staining for Bcl6 was performed with an Alexa Fluor 647–conjugated mAb to Bcl6 (clone K112-91; BD Pharmingen) using the Foxp3 intracellular staining kit buffers and protocol (eBioscience).

Statistical analysis

Statistical tests were performed using Prism 5.0 (GraphPad). The p values were calculated by two-tailed unpaired Student t tests with a 95% confidence interval. Error bars depict the SEM.

Results and Discussion

The Bcl6 BTB domain regulates Tfh cell differentiation

Given that the Bcl6 BTB domain is known to interact with corepressors (such as BCOR) in B cells that are also expressed in CD4 T cells, we sought to determine the role of the Bcl6 BTB domain in Tfh cell differentiation. To this end, we generated N21K and H116A mutations in the Bcl6 BTB domain. These point mutations together were shown to prevent corepressor binding to the BTB domain without affecting the ability of Bcl6 to dimerize (16). Similar levels of Bcl6 protein expression were observed in CD4 T cells transduced with an RV expressing either wild-type (WT) Bcl6 or BTBmut, and the amount of Bcl6 expression was comparable to that of GC-Tfh cells (Fig. 1A). To examine the role of the Bcl6 BTB domain in vivo, we determined whether expression of the Bcl6 BTBmut protein in Bcl6-deficient cells could rescue Tfh cell development in response to acute LCMV infection. LCMV gp-specific *Bcl6*$^{fl/fl}$ Cre^{CD4} SM CD45.1 cells were transduced with Bcl6 WT, BTBmut, or an empty GFP vector (GFP) and transferred to *Bcl6*$^{fl/fl}$ Cre^{CD4} hosts. We first examined early Tfh cell development 3 d following an acute LCMV infection. Cells transduced with the empty vector (GFP$^+$) did not differentiate into Tfh cells, confirming the requirement of Bcl6 for Tfh cell differentiation. Ectopic expression of Bcl6 WT was sufficient to rescue Tfh cell development (Fig. 1B). We observed a defect in early Tfh cell differentiation in BTBmut$^+$ SM cells compared with Bcl6-WT$^+$ SM cells ($p < 0.0001$, Fig. 1B). We then examined Tfh and GC B cell development at 7 d following acute LCMV infection. Again, there was a significant impairment in Tfh cell differentiation in BTBmut$^+$ cells ($p < 0.0001$, Fig. 1C) compared with WT. This was coupled with an even larger reduction in GC-Tfh cells ($p = 0.006$, Fig. 1D). GC-Tfh cells are the more polarized Tfh cells that are present in the GCs and are identifiable as CXCR5^{hi}PD1^{hi}PSGL1^{lo}Bcl6^{hi} cells (1). In parallel, we observed a significant defect in GC B cell development ($p = 0.005$, Fig. 1E. Thus, Bcl6 BTB domain functions appeared to be necessary for the majority of Tfh differentiation and B cell help.

Impaired Tfh differentiation by germline Bcl6^{BTBMUT} *CD4 T cells*

These results are different from a recent report using mixed bone marrow chimeric mice with *Bcl6*^{BTBMUT} and *Tcrb*$^{-/-}$ donor cells, in which no difference in total Tfh cell frequencies was observed upon immunization with SRBCs, although Ag-specific cells were not directly assessed in that study (19).

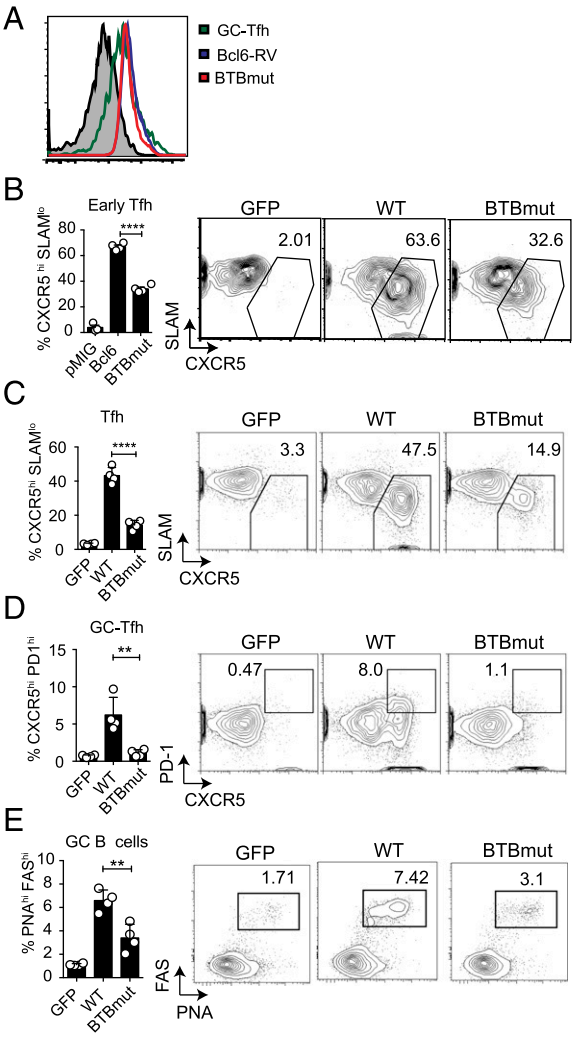

FIGURE 1. The Bcl6 BTB domain is necessary for proper Tfh cell differentiation. (**A**) Similar levels of Bcl6 expression in Bcl6-RV, BTBmut-RV, and GC-Tfh cells. Bcl6 mean fluorescence intensity of *Bcl6*$^{fl/fl}$ Cre^{CD4} CD4 T cells transduced with either BTBmut-RV or Bcl6-RV compared with ex vivo GC-Tfh cells isolated from mice at 7 d following LCMV infection. (**B**–**E**) *Bcl6*$^{fl/fl}$ Cre^{CD4} SM cells were retrovirally transduced with empty GFP vector, Bcl6 WT, or BTBmut; transferred to *Bcl6*$^{fl/fl}$ Cre^{CD4} mice; and analyzed at day 3 or 7 following an acute LCMV infection. (**B**) Early Tfh cell differentiation (CXCR5^{hi}SLAM^{lo}) by transduced *Bcl6*$^{fl/fl}$ Cre^{CD4} SM cells at day 3. (**C**) Tfh cell differentiation (CXCR5^{hi}SLAM^{lo}) by transduced *Bcl6*$^{fl/fl}$ Cre^{CD4} SM cells at day 7. (**D**) GC-Tfh cell differentiation (CXCR5^{hi}PD1^{hi}) by transduced *Bcl6*$^{fl/fl}$ Cre^{CD4} SM cells at day 7. (**E**) GC B cells (PNA^{hi}Fas^{hi}) at day 7. At least three mice were used for each condition. Experiments were repeated at least three times. $^{**}p < 0.01$, $^{****}p < 0.0001$.

Therefore, in light of those data, it was incumbent upon us to explore the role of the Bcl6 BTB domain in Tfh cell differentiation in multiple contexts, directly examining Ag-specific Tfh cells in each case. Thus, we obtained the germline knock-in *Bcl6*^{BTBMUT} mice for further studies. CD4 T cells were isolated from *Bcl6*^{BTBMUT} SM CD45.1$^+$ or WT SM CD45.1$^+$ mice and transferred to *Bcl6*$^{fl/fl}$ Cre^{CD4} hosts. Ag-specific Tfh and GC-Tfh cell differentiation was examined at 7 d following acute LCMV infection. We observed a significant defect in both Tfh cell ($p = 0.017$, Fig. 2A) and GC-Tfh cell ($p = 0.014$, Fig. 2B) differentiation, confirming the requirement for the Bcl6 BTB domain in the generation of Tfh cells.

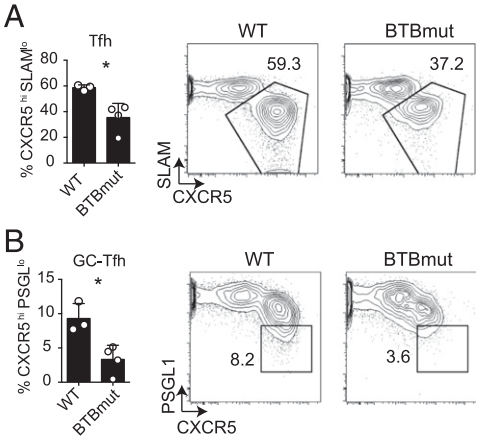

FIGURE 2. Impaired Tfh cell differentiation by *Bcl6*^{BTBMUT} CD4 T cells. *Bcl6*^{BTBMUT} SM or WT SM cells were transferred to *Bcl6*$^{fl/fl}$ Cre^{CD4} mice and analyzed 7 d following acute LCMV infection. (**A**) Tfh (CXCR5^{hi}SLAM^{lo}) *Bcl6*^{BTBMUT} SM cells. (**B**) GC-Tfh (CXCR5^{hi}PSGL1^{lo}) *Bcl6*^{BTBMUT} SM cells. At least three mice were used for each condition. Experiments were repeated at least three times. $^{*}p < 0.05$.

BTB-dependent Tfh cell differentiation in NIP-transgenic Bcl6^{BTBMUT} *CD4 T cells*

One possibility is that this observed phenotype may be selective to the SM CD4 T cell response, although SM CD4 T cells have been very informative in numerous contexts (9, 14, 17, 20). To this end, we developed a new LCMV-specific TCR-transgenic mouse. The LCMV nucleoprotein (NP) is the major target of the Ab response during LCMV infection, and the currently used gp-specific SM CD4 T cells do not allow for the study of the immunodominant Ab response, because gp-specific CD4 T cells do not provide help to NP-specific B cells (14). Epitope mapping defined a specific peptide (NP 311–325) of the NP that is efficiently recognized by CD4 T cells during LCMV infection (21, 22). For this reason, NP 311–325 was chosen as a target to generate a novel TCR-transgenic mouse model (NIP, Supplemental Fig. 1). NIP-transgenic CD4 T cells transferred into B6 mice exhibited robust expansion and differentiated into Tfh and GC-Tfh cells following LCMV infection (Supplemental Fig. 1B–E). NIP CD4 T cells transferred into B6 hosts were able to induce robust B cell responses, including the generation of GC B cells (Supplemental Fig. 1F), plasma cells (Supplemental Fig. 1G), and LCMV-specific Abs (Supplemental Fig. 1H).

We examined the role of the Bcl6 BTB domain in the context of a retrogenic mouse model in which bone marrow from *Bcl6*^{BTBMUT} or B6 (WT) mice was transduced with an NP-specific TCRα and TCRβ RV construct (NIP-RV), and the transduced bone marrow was transferred into irradiated WT recipients (23). WT or Bcl6^{BTBMUT} NP-specific naive CD4 T cells were isolated from these mice after reconstitution and transferred to *Bcl6*$^{fl/fl}$ Cre^{CD4} hosts. The NIP^{+} WT and NIP^{+} Bcl6^{BTBMUT} CD4 T cells expanded robustly in response to an acute LCMV infection. At 7 d following LCMV infection, we observed a significant defect in Tfh cell differentiation

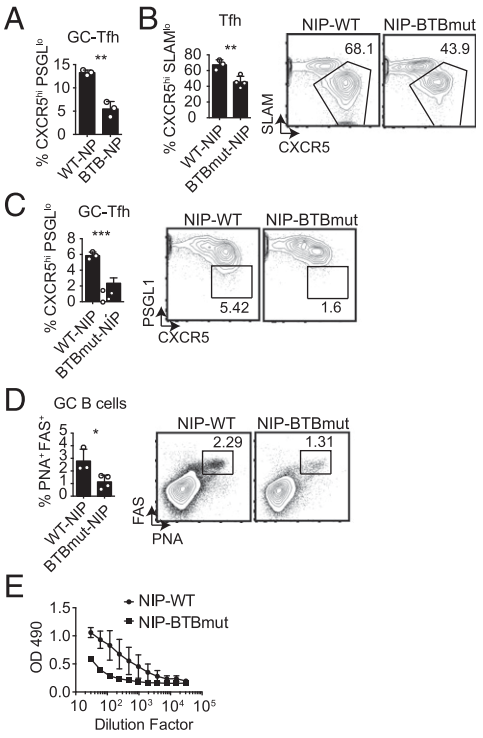

FIGURE 3. *Bcl6*^{BTBMUT} LCMV-specific NIP-transgenic CD4 T cells are defective in Tfh cell differentiation and function. CD4 T cells from *Bcl6*^{BTBMUT} NIP-TCR^{+} retrogenic mice were transferred to *Bcl6*$^{fl/fl}$Cre^{CD4} mice and analyzed at day 7 following acute LCMV infection. (**A**) GC-Tfh cells (CXCR5^{hi}PSGL1^{lo}) among *Bcl6*^{BTBMUT} NIP-TCR^{+} cells. (**B–E**) *Bcl6*^{BTBMUT} NIP or WT NIP cells were transferred to *Bcl6*$^{fl/fl}$ Cre^{CD4} mice and analyzed following acute LCMV infection. (B) Tfh (CXCR5^{hi}SLAM^{lo}) *Bcl6*^{BTBMUT} NIP cells. (C) GC-Tfh (CXCR5^{hi}PSGL1^{lo}) *Bcl6*^{BTBMUT} NIP cells. (D) GC B cells (PNA^{hi}Fas^{hi}). (E) LCMV-specific IgG. At least three mice were used for each condition. Experiments in (B)–(D) were repeated at least three times. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

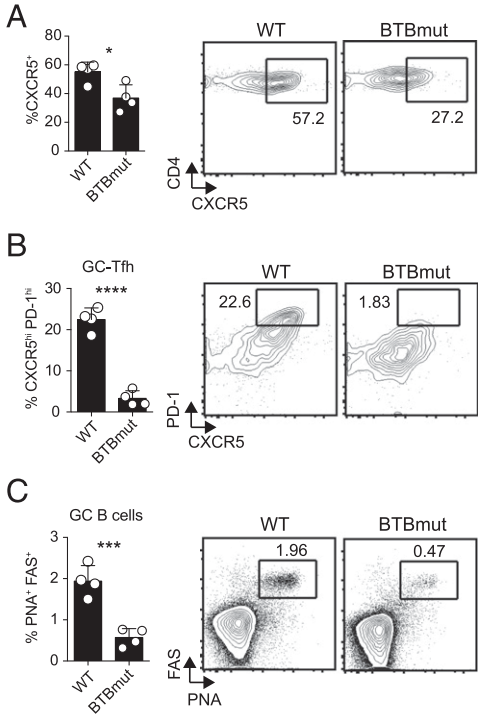

FIGURE 4. Defective Tfh cell differentiation and function in the absence of a functional Bcl6 BTB domain following protein immunization. *Bcl6*^{BTBMUT} SM or WT SM cells were transferred to *Bcl6*$^{fl/fl}$ Cre^{CD4} mice and analyzed following gp61-KLH (alum) immunization on day 10. (**A**) Tfh (CXCR5^{hi}SLAM^{lo}) *Bcl6*^{BTBMUT} SM cells. (**B**) GC-Tfh (CXCR5^{hi}PD1^{hi}) *Bcl6*^{BTBMUT} SM cells. (**C**) GC B cells (PNA^{hi}Fas^{hi}). At least three mice were used for each condition. Experiments were repeated at least three times. $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

(Supplemental Fig. 2) and GC-Tfh cell differentiation (Fig. 3A, Supplemental Fig. 2) for $Bcl6^{BTBMUT}$ NIP-RV$^+$ mice compared with WT NIP-RV$^+$ mice ($p = 0.01$ and $p = 0.0014$). We then made use of germline NIP TCR-transgenic mice crossed with $Bcl6^{BTBMUT}$ mice. $Bcl6^{BTBMUT}$ NIP or WT NIP CD4 T cells were transferred into $Bcl6^{fl/fl}$ Cre^{CD4} recipients followed by LCMV infection. We observed severe defects in Tfh cell ($p = 0.0088$, Fig. 3B) and GC-Tfh cell ($p = 0.0006$, Fig. 3C) differentiation in $Bcl6^{BTBMUT}$ NIP CD4 T cells. The consequence of the substantial loss of GC Tfh cells was a significant decrease in GC B cell development (Fig. 3D) and a reduced anti-LCMV IgG response (Fig. 3E). Taken together, these data demonstrate that the Bcl6 BTB domain is important for Tfh cell differentiation and function in response to at least two Ags.

The Bcl6 BTB domain regulates Tfh cell differentiation and GC B cell development following protein immunization

To examine the role of the Bcl6 BTB domain in another experimental context, we used a protein immunization model: KLH conjugated with gp61–80 peptide in alum (17). We transferred $Bcl6^{BTBMUT}$ SM or WT SM CD4 T cells into $Bcl6^{fl/fl}$ Cre^{CD4} hosts, followed by immunization with gp61-KLH in alum. At 10 d following immunization, lymph nodes were harvested and examined for Ag-specific Tfh cell differentiation and function. We observed a significant reduction in CXCR5 expression by $Bcl6^{BTBMUT}$ CD4 T cells ($p = 0.0195$, Fig. 4A) and a severe defect in GC-Tfh cell differentiation ($p < 0.0001$, Fig. 4B). $Bcl6^{BTBMUT}$ CD4 T cells were functionally deficient because they were unable to promote GC B cell responses ($p = 0.0008$, Fig. 4C). We also observed defects in Tfh and GC-Tfh cell differentiation in $Bcl6^{fl/fl}$ Cre^{CD4} mice receiving BTBmut-RV$^+$ SM CD4 T cells compared with Bcl6-WT-RV$^+$ SM CD4 T cells at 10 d following immunization (data not shown). Thus, the Bcl6 BTB domain is needed for optimal Tfh cell differentiation and function in both the context of protein immunizations and an acute viral infection.

Our results demonstrate the importance of the Bcl6 BTB repressor domain in CD4 T cells in the differentiation of Tfh cells and in the ability of Tfh cells to provide help to B cells. A previous study did not observe a Tfh cell defect in $Bcl6^{BTBMUT}$ CD4 T cells (19), possibly because Ag-specific Tfh cells were not analyzed directly or possibly as the result of a confounding negative-feedback Bcl6 autoregulatory mechanism involving cullin3, whereby cullin3 is most likely recruited via the Bcl6 BTB domain (24). Alternatively, the BTB domain of Bcl6 has more prominent roles for Bcl6 activity in some in vivo contexts than others. Bcl6 functions in Tfh cells via binding to thousands of genes, demonstrating that Bcl6 is deeply integrated into Tfh cell biology (25). In this study, Tfh cell defects in the absence of BTB domain function were consistent when assessing Ag-specific Tfh cells using two Ag specificities, as well as in the context of both acute viral infection and protein immunization. The defect in Tfh cell differentiation and function that was observed is likely due to the inability of Bcl6 to recruit corepressors (e.g., SMRT, NCOR, and BCOR), and, thus, repress target genes. Indeed, it was found that BCOR-deficient CD4 T cells are defective for GC-Tfh cell differentiation and B cell help functions (26). Thus, BCOR may be the main corepressor interacting with the Bcl6 BTB domain in Tfh cells. SMRT, NCOR, and BCOR are all expressed in CD4 T cells, and further investigation of the comparative roles of each of these Bcl6 BTB domain–interacting transcription factors is needed. This report highlights the importance of the repressive ability of the Bcl6 BTB domain in Tfh cells for successful GC reactions and robust Ab responses.

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Disclosures

The authors have no financial conflicts of interest.

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