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

J. Philip Nance,* Simon Bélanger,* Robert J. Johnston,† Toshitada Takemori,‡ and Shane Crotty*•

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

Mouse and vectors

C57BL/6J (B6) and CreCD4+ mice were purchased from The Jackson Laboratory. B6.Tg(Bcl6)13 (13), CD45.1-congenic, and Smarta TCR–transgenic (SM; specific for lymphocytic choriomeningitis virus [LCMV] gp66–77 on I-Ak) (14) mice were on a full B6 background and were bred at the La Jolla Institute for Allergy and Immunology. Bcl6BTBmut+ 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 586β 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 B6.3JL 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 injection or immunization. 5 × 103 transduced Smarta cells were transferred into each mouse for day 3 analysis, and 25 × 103 transduced Smarta cells were transferred into each mouse for day 7 analysis. For protein immunization, 5 × 106 cells were transferred into each mouse. 5 × 105 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 × 106 PFU LCMV

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.

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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, Fax, 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 Bc16 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 Bc16 BTB domain in Tfh cell differentiation. To this end, we generated N21K and H116A mutations in the Bc16 BTB domain. These point mutations together were shown to prevent corepressor binding to the BTB domain without affecting the ability of Bc16 to dimerize (16). Similar levels of Bc16 protein expression were observed in CD4 T cells transduced with an RV expressing either wild-type (WT) Bc16 or BTBmut, and the amount of Bc16 expression was comparable to that of GC-Tfh cells (Fig. 1A). To examine the role of the Bc16 BTB domain in vivo, we determined whether expression of the Bc16 BTBmut protein in Bc16-deficient cells could rescue Tfh cell development in response to acute LCMV infection. LCMV gp-specific Bc16+Cre+CD4+ SM cells transduced with Bc16 WT, BTBmut, or an empty GFP vector (GFP) and transferred to Bc16+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 Bc16 for Tfh cell differentiation. Ectopic expression of Bc16 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 Bc16-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 CXCR5hiBDCA2hiPSGL1hiBcl6hi cells (1). In parallel, we observed a significant defect in GC B cell development (p = 0.005, Fig. 1E). Thus, Bc16 BTB domain functions appeared to be necessary for the majority of Tfh differentiation and B cell help.

**Impaired Tfh differentiation by germline Bc16**

These results are different from a recent report using mixed bone marrow chimeric mice with Bcl6+Cre+CD4+ T cells infected with LCMV and transferred to Bcl6+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 Bc16 BTB domain in the generation of Tfh cells.

**FIGURE 1.** The Bc16 BTB domain is necessary for proper Tfh cell differentiation. (A) Similar levels of Bc16 expression in Bc16-RV, BTBmut-RV, and GC-Tfh cells. Bc16 mean fluorescence intensity of Bc16+Cre+CD4+ CD4 T cells transduced with either BTBmut-RV or Bc16-RV compared with ex vivo GC-Tfh cells isolated from mice at 7 d following LCMV infection. (B–E) Bc16+Cre+CD4+ SM cells were retrovirally transduced with empty GFP vector, Bc16 WT, or BTBmut; transferred to Bc16+Cre+CD4+ mice; and analyzed at day 3 or 7 following an acute LCMV infection. (B) Early Tfh cell differentiation (CXCR5hiSLAMlo) by transduced Bc16+Cre+CD4+ SM cells at day 3. (C) Tfh cell differentiation (CXCR5hiSLAMlo) by transduced Bc16+Cre+CD4+ SM cells at day 7. (D) GC-Tfh cell differentiation (CXCR5hiBDCA2hiPSGL1hiBcl6hi cells) (1). In parallel, we observed a significant defect in GC B cell development (p = 0.005, Fig. 1E). Thus, Bc16 BTB domain functions appeared to be necessary for the majority of Tfh differentiation and B cell help.

**FIGURE 2.** The Bc16 BTB domain regulates Tfh and GC B cell development at day 7 following an acute LCMV infection. (A) The Bc16 BTB domain regulates Tfh cell differentiation. (B) Similar levels of Bc16 expression in Bc16-RV, BTBmut-RV, and GC-Tfh cells. Bc16 mean fluorescence intensity of Bc16+Cre+CD4+ CD4 T cells transduced with either BTBmut-RV or Bc16-RV compared with ex vivo GC-Tfh cells isolated from mice at 7 d following LCMV infection. (B–E) Bc16+Cre+CD4+ SM cells were retrovirally transduced with empty GFP vector, Bc16 WT, or BTBmut; transferred to Bc16+Cre+CD4+ mice; and analyzed at day 3 or 7 following an acute LCMV infection. (B) Early Tfh cell differentiation (CXCR5hiSLAMlo) by transduced Bc16+Cre+CD4+ SM cells at day 3. (C) Tfh cell differentiation (CXCR5hiSLAMlo) by transduced Bc16+Cre+CD4+ SM cells at day 7. (D) GC-Tfh cell differentiation (CXCR5hiBDCA2hiPSGL1hiBcl6hi cells) (1). In parallel, we observed a significant defect in GC B cell development (p = 0.005, Fig. 1E). Thus, Bc16 BTB domain functions appeared to be necessary for the majority of Tfh differentiation and B cell help.

Therefore, in light of those data, it was incumbent upon us to explore the role of the Bc16 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 Bc16+Cre+CD4+ mice for further studies. CD4 T cells were isolated from Bc16+Cre+CD4+ SM cells transduced with either BTBmut-RV or Bc16-RV compared with ex vivo GC-Tfh cells isolated from mice at 7 d following 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 Bc16 BTB domain in the generation of Tfh 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 TCRa and TCRb 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$^{fl/fl}$ CreCD4 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 2](http://www.jimmunol.org/)

**FIGURE 2.** Impaired Tfh cell differentiation by Bcl6$^{BTBMUT}$ CD4 T cells. Bcl6$^{BTBMUT}$ SM or WT SM cells were transferred to B6$^{CreCD4}$ mice and analyzed 7 d following acute LCMV infection. (A) Tfh (CXCR5hiSLAMlo) Bcl6$^{BTBMUT}$ SM cells. (B) GC-Tfh (CXCR5hiPSGL1lo) Bcl6$^{BTBMUT}$ SM cells. At least three mice were used for each condition. Experiments were repeated at least three times. *p < 0.05.

![Figure 3](http://www.jimmunol.org/)

**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$^{BTBMUT}$ CreCD4 mice and analyzed at day 7 following acute LCMV infection. (A) GC-Tfh cells (CXCR5hiPSGL1lo) among Bcl6$^{BTBMUT}$ NIP-TCR$^+$ cells. (B–E) Bcl6$^{BTBMUT}$ NIP or WT NIP cells were transferred to Bcl6$^{BTBMUT}$ CreCD4 mice and analyzed following acute LCMV infection. (B) Tfh (CXCR5hiSLAMlo) Bcl6$^{BTBMUT}$ NIP cells. (C) GC-Tfh (CXCR5hiPSGL1lo) Bcl6$^{BTBMUT}$ NIP cells. (D) GC B cells (PNAhiFashi). (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](http://www.jimmunol.org/)

**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 B6$^{CreCD4}$ mice and analyzed following gp61-KLH (alum) immunization on day 10. (A) Tfh (CXCR5hiSLAMlo) Bcl6$^{BTBMUT}$ SM cells. (B) GC-Tfh (CXCR5hiPSGL1lo) Bcl6$^{BTBMUT}$ SM cells. (C) GC B cells (PNAhiFashi). 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.
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 ∆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 CXC5 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 WT- ∆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 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 BCR), and, thus, repress target genes. Indeed, it was found that BCR-deficient CD4 T cells are defective for GC-Tfh cell differentiation and B cell help functions (26). Thus, BCR may be the main corepressor interacting with the Bcl6 BTB domain in Tfh cells. SMRT, NCoR, and BCR 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.

References


Supplementary Materials

T follicular helper cell (T\textsubscript{FH}) differentiation is defective in the absence of Bcl6 BTB repressor domain function

J. Philip Nance, Simon Bélanger, Robert J. Johnston, Toshitada Takemori and Shane Crotty†
Supplemental Fig. 1. Characterization of NP-specific NIP-transgenic CD4 T cells.

(A) 58αβ T cell hybridomas expressing the NP-specific TCRα and TCRβ chains were incubated with isolated dendritic cells loaded with the indicated peptides or infected with LCMV-Armstrong or pre-incubate with LCMV lysate. 4 hours after incubation, supernatants were harvested and subjected to IL-2 ELISA.

(B-H) NIP mice were genotyped for correct TCR expression (primers: Vα1 5’-GCACTGATGTCATCTTCTCT-3’ and Cα 5’-TGCGTGGTGTCTCTTTGAAG-3’ for the TCRα chain and Vβ6 5’-CTCTCAGTCATCTGCC-3’ plus Cβ 5’-)
CTTGGGTGGAGTCACATTTCTC-3′ for the TCRβ chain.) NIP TCRβ mice were then used to obtain cells for transfer experiments.

25,000 naïve CD45.1+ NIP-transgenic CD4 T cells were transferred into B6 mice. Mice were infected with 2x10⁵ PFU LCMV-Armstrong and analyzed 7 days later.

(B) NIP (CD45.1+) CD4 T cells were gated and quantified.

(C) Representative flow cytometry plot and quantification of Tfh (CXCR5+SLAMlo) in NIP CD4 T cells.

(D) Representative flow cytometry plot and quantification of GC Tfh (CXCR5*Bcl6+) in NIP CD4 T cells.

(E) Representative flow cytometry plot and quantification of Tfh (CXCR5*PSGL1+) and GC Tfh (CXCR5*PSGL1-) in NIP CD4 T cells.

(F) Representative flow cytometry plots and quantification of GC B cells (Fas+PNA+).

(G) Representative flow cytometry plots and quantification of plasma cells (CD138*IgD-).

(H) Quantification of LCMV-specific antibodies by ELISA in the serum of the recipient mice.
Supplemental Fig. 2. Characterization of CD4 T cells $Bcl6^{BTBmut}$ NIP-TCR$^+$ retrogenic mice after an acute LCMV infection. 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) Tfh (CXCR5$^{hi}$ SLAM$^{lo}$) among $Bcl6^{BTBmut}$ NIP-TCR$^+$ cells.

(B) GC Tfh cells (CXCR5$^{hi}$ PSGL1$^{lo}$) among $Bcl6^{BTBmut}$ NIP-TCR$^+$ cells. Quantitation is shown in Figure 3A.