Lineage-specific functions of Bcl-6 in immunity and inflammation are mediated by distinct biochemical mechanisms

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The transcription factor Bcl-6 orchestrates germinal center (GC) reactions through its actions in B cells and T cells and regulates inflammatory signaling in macrophages. Here we found that genetic replacement with mutated Bcl6 encoding Bcl-6 that cannot bind corepressors to its BTB domain resulted in disruption of the formation of GCs and affinity maturation of immunoglobulins due to a defect in the proliferation and survival of B cells. In contrast, loss of function of the BTB domain had no effect on the differentiation and function of follicular helper T cells or that of other helper T cell subsets. Bcl6-null mice had a lethal inflammatory phenotype, whereas mice with a mutant BTB domain had normal healthy lives with no inflammation. The repression of inflammatory responses by Bcl-6 in macrophages was accordingly independent of the repressor function of the BTB domain. Bcl-6 thus mediates its actions through lineage-specific biochemical functions.

Bcl-6 is a transcriptional repressor originally identified as being encoded by a locus frequently translocated in diffuse large B cell lymphomas (DLBCLs). During normal development, Bcl-6 has critical functions in various cell types of the adaptive and innate compartments of the immune system. Bcl-6 expression undergoes substantial upregulation in B cells after challenge with T cell–dependent antigens, and Bcl-6 is required for the formation of germinal centers (GCs) in which B cells undergo affinity maturation of immunoglobulins. Bcl-6-deficient (Bcl6−/−) mice do not form GCs and thus are unable to generate high-affinity antibodies. The proposed biological function of Bcl-6 in B cells in the GC is to facilitate the simultaneous rapid proliferation and tolerance to the genomic damage that occurs during clonal expansion and somatic hypermutation by directly repressing genes encoding molecules involved in DNA-damage sensing and checkpoints, such as ATR, CHEK1 (ref. 7), EP300 (ref. 8), TP53 (ref. 9) and CDKN1A. Follicular helper T cells (T FH cells), specifically those in B cells, are specialized CD4+ helper T cells that provide help to B cells during the GC reaction. Bcl-6 expression is upregulated during the differentiation of T FH cells, and Bcl6−/− T cells do not differentiate into T FH cells in vivo. Constitutive expression of Bcl-6 enhances the differentiation of T FH cells. The requirement for Bcl-6 in both B cells and T FH cells in GCs is cell autonomous, and loss of Bcl-6 in either cell type leads to abrogation of the GC reaction. Bcl-6 also has an important role in macrophages, in which it mediates a dampening effect on inflammatory signaling through repression of chemokine expression and target genes of the transcription factor NF-κB. Bcl6−/− mice develop a lethal inflammatory disease caused by the interaction and crosstalk between macrophages and helper T cells.

Bcl-6 is a member of the BTF–zinc finger family of proteins. Its BTB domain forms an obligate homodimer and it contains C2H2 zinc fingers that bind to DNA. The interface between monomers of the Bcl-6 BTB domain creates two symmetrical extended lateral grooves that form docking sites for the corepressors SMRT, NCOR and BCOR. Those three corepressors bind to Bcl-6 via an unstructured 18–amino acid Bcl-6-binding domain. The Bcl-6-binding domains of NCOR and SMRT are identical, whereas that of BCOR is completely different, yet all three bind to the lateral groove of the Bcl-6 BTB domain in perfectly overlapping configurations. Substitution of lysine for asparagine at position 21 (N21K) and of alanine for histamine at position 116 (H116A) in the Bcl-6 BTB domain completely abrogates the binding of Bcl-6 to NCOR, SMRT and BCOR without impairing folding or dimerization. Mutant Bcl-6 with those N21K and H116A substitutions in its BTB domain is completely inactive, which indicates that the lateral groove–Bcl-6-binding domain interface is responsible for the repressor activity of the Bcl-6 BTB domain. However, Bcl-6 also has a middle autonomous repression region often called ‘repressor domain 2’ (RD2), which may recruit other corepressors, such as NuRD and CTBP. The sequence of the reported Bcl-6 consensus binding site (TTCC[T/A/C]GAA, where A/C indicates either adenosine or cytosine) overlaps that of STAT transcription factor–binding sites, and several lines of evidence indicate that Bcl-6 may antagonize STAT signaling, with potential relevance for inflammatory and innate immunological functions.
in which the endogenous Bcl6 locus encodes a mutant form of Bcl-6 protein with the N21K and H116A point substitutions in the BTB domain (Bcl6\textsuperscript{BTBMUT}). The facts that SMRT, NCOR, and BCOR are coexpressed with Bcl-6 in the relevant cell types and that the mechanism of the BTB domain is the only well-characterized biochemical function of Bcl-6 favor the proposal that the biological ‘readout’ of such a knock-in model would be most rigorously interpretable. Notably, our data suggest that the transcriptional mechanisms of action of Bcl-6 are specific to the lineage and biological function, with notable implications for the general understanding of how Bcl-6 and other transcription factors work, as well as for the ‘translation’ of such results into the clinical use of inhibitors of Bcl-6.

RESULTS

Viability of Bcl6\textsuperscript{BTBMUT} mice

To address the biological function of interactions between the Bcl-6 BTB domain and its corepressors in vivo, we introduced point mutations in exons 3 and 4 of Bcl6 that resulted in the desired N21K and H116A substitutions in the BTB domain (Bcl6\textsuperscript{BTBMUT}). The facts that SMRT, NCOR, and BCOR are coexpressed with Bcl-6 in the relevant cell types and that the mechanism of the BTB domain is the only well-characterized biochemical function of Bcl-6 favor the proposal that the biological ‘readout’ of such a knock-in model would be most rigorously interpretable. Notably, our data suggest that the transcriptional mechanisms of action of Bcl-6 are specific to the lineage and biological function, with notable implications for the general understanding of how Bcl-6 and other transcription factors work, as well as for the ‘translation’ of such results into the clinical use of inhibitors of Bcl-6.

Impaired GC formation in Bcl6\textsuperscript{BTBMUT} mice

Phenotypic analysis showed normal early development of B cells in the bone marrow and spleens of Bcl6\textsuperscript{BTBMUT} mice, as well as normal distribution of peripheral T cells (Supplementary Fig. 3a–c). Bcl6\textsuperscript{BTBMUT} mice also formed normal splenic primary lymphoid follicles (data not shown). Analysis of the spontaneous formation of GCs in spleens of uninjected mice by staining with peanut agglutinin showed a few small GCs scattered in Bcl6\textsuperscript{+/-} mice, whereas none were present in Bcl6\textsuperscript{BTBMUT} mice (data not shown). After immunization with sheep red blood cells (SRBCs), a T-cell–dependent antigen, Bcl6\textsuperscript{BTBMUT} mice developed only a few, scattered and small peanut agglutinin–positive and Bcl6–6” cell clusters (Fig. 1a). Serial examination of GCs by immunohistochemistry at days 7, 10, and 14 after immunization with SRBCs showed that Bcl6\textsuperscript{BTBMUT} mice had considerably smaller and fewer GCs than Bcl6\textsuperscript{+/-} mice had at all three time points (Fig. 1b,c). Lymph nodes in Peyer’s patches of in Bcl6\textsuperscript{BTBMUT} mice showed a similar loss of GCs and the presence of residual cell clusters (Supplementary Fig. 4a), which emphasized the general requirement for Bcl-6 BTB domain–mediated repression in the formation of GCs.

To better understand the mechanism of the GC impairment in Bcl6\textsuperscript{BTBMUT} mice, we first compared the frequency of GC B cells (CD38\textsuperscript{lo–neg} Fas\textsuperscript{+}/B220\textsuperscript{+}) among splenic B cells from Bcl6\textsuperscript{+/-}, Bcl6–6” and Bcl6\textsuperscript{BTBMUT} mice before and at day 10 after immunization with SRBCs. The frequency of GC B cells in uninhibited Bcl6\textsuperscript{+/-} mice
Impaired immunoglobulin affinity maturation in Bcl6BTBMUT mice

Bcl-6 is not required for the response to T cell–dependent antigens, and accordingly, Bcl6BTBMUT mice had normal low-affinity antibody responses to the T cell–independent antigen of NP conjugated to the hydrophilic polysaccharide Ficoll (data not shown). The T cell–dependent B cell immune response triggers both an extrafollicular response, which generates short-lived plasma cells and an early wave of low-affinity antibody production, and a GC response, which gives rise to long-lived plasma cells or memory cells and a later wave of high-affinity antibodies. Immunization with NP-CGG induced a normal extrafollicular response in Bcl6BTBMUT mice, with the expected production of low-affinity immunoglobulin specific to NP26-BSA (NP conjugated to bovine serum albumin (BSA)) at a molecular ratio of 26:1 (Fig. 2a). In contrast, at day 21 after immunization with NP-CGG, the titers of NP-specific immunoglobulin G1 (IgG1) and IgG2a were significantly lower in Bcl6BTBMUT mice than in Bcl6+/+ mice, with a trend toward lower titers of other immunoglobulins (Fig. 2a). Early after immunization (at day 7 d), Bcl6BTBMUT and Bcl6+/+ mice formed a similar number of antigen-specific IgM- or IgG-secreting cells (Fig. 2b) and plasma cells (NP+CD138+CD11c–CD4–CD8– spleen cells (top) from mice left unimmunized (UI) or at 7 d after immunization with NP-CGG (NP-CGG (7 d)), with further gating of the NP+ compartment above on total plasma cells (middle) and of NP-specific GC B cells on live splenic B220+ cells (bottom). Numbers above outlined areas indicate percent CD138+B220lo–neg cells (total plasma cells; top row) or NP+GL-7+ cells (NP-specific GC B cells; bottom row); numbers above bracketed lines (middle) indicate percent NP+ plasma cells. P values (NS or *) Bcl6BTBMUT versus Bcl6+/+. (d) Titors of NP-specific IgG1 and IgG2a, measured with NP26-BSA in serum from mice 21 d after immunization with NP-CGG. (e) Ratio of the titer of IgG1 or IgG2a detected with NP26-BSA to that detected with NP26-BSA. Each symbol (a,b,d,e) represents an individual mouse; small horizontal lines indicate the mean. NS, not significant; *P < 0.05, **P < 0.01 and ***P < 0.001 (two-tailed t-test). Data are from three independent experiments with four to six mice (a,b,d,e) or are representative of two independent experiments (c, infected Bcl6+/+ or Bcl6BTBMUT mice; mean ± s.e.m. of three mice) or one experiment (c, uninfected Bcl6+/+ mice).

Figure 2 Intact extrafollicular responses but impaired GC responses in Bcl6BTBMUT mice. (a) Titors of NP-specific immunoglobulins, measured with NP26-BSA in serum from mice 8 d and 21 d (horizontal axes) after immunization with NP-CGG and presented in relative units (RU) as serial dilution of serum relative to antibody end-point titors. (b) Enzyme-linked immunosorbent assay of the frequency of NP-specific IgM- or IgG-secreting cells among splenocytes from mice 7 d after immunization with NP-CGG. (c) Flow cytometry analysis of CD138 and B220 on live CD11c–CD4–CD8– spleen cells (top) from mice left unimmunized (UI) or at 7 d after immunization with NP-CGG (NP-CGG (7 d)), with further gating of the NP+ compartment above on total plasma cells (middle) and of NP-specific GC B cells on live splenic B220+ cells (bottom). Numbers above outlined areas indicate percent CD138+B220lo–neg cells (total plasma cells; top row) or NP+GL-7+ cells (NP-specific GC B cells; bottom row); numbers above bracketed lines (middle) indicate percent NP+ plasma cells. P values (NS or *), Bcl6BTBMUT versus Bcl6+/+. (d) Titors of NP-specific IgG1 and IgG2a, measured with NP26-BSA in serum from mice 21 d after immunization with NP-CGG. (e) Ratio of the titer of IgG1 or IgG2a detected with NP26-BSA to that detected with NP26-BSA. Each symbol (a,b,d,e) represents an individual mouse; small horizontal lines indicate the mean. NS, not significant; *P < 0.05, **P < 0.01 and ***P < 0.001 (two-tailed t-test). Data are from three independent experiments with four to six mice (a,b,d,e) or are representative of two independent experiments (c, infected Bcl6+/+ or Bcl6BTBMUT mice; mean ± s.e.m. of three mice) or one experiment (c, uninfected Bcl6+/+ mice).
in the chimeras, GC B cells and T<sub>H</sub> cells derived from CD45.2<sup>+</sup> Bcl6<sup>–/–</sup> donor cells were effectively absent after immunization with SRBCs (Fig. 3b,c). In contrast, whereas CD45.2<sup>+</sup> donor Bcl6<sup>–/–</sup> cells showed profound deficiency in formation of GC B cells, they formed normal numbers of T<sub>H</sub> cells (Fig. 3b,c). The lower abundance of T<sub>H</sub> cells noted in the Bcl6<sup>–/–</sup> setting was thus secondary to the lack of GC B cells.

To determine whether impaired affinity maturation of immunoglobulins in Bcl6<sup>BTKMUT</sup> mice was also intrinsic to B cells, we constructed chimeras by transferring bone marrow from CD45.1<sup>+</sup> mice (which lack B cells; 50%) along with bone marrow from Bcl6<sup>+/+</sup>, Bcl6<sup>BTKMUT</sup> or Bcl6<sup>–/–</sup> mice (50%) into sublethally irradiated Rag1<sup>–/–</sup> mice (Supplementary Fig. 5a). The μMT bone marrow provides a source of normal T cells but no B cells; thus, all B cells in these chimeras originate from Bcl6<sup>+/+</sup>, Bcl6<sup>BTKMUT</sup> or Bcl6<sup>–/–</sup> donor bone marrow cells. Chimeras given a mixture containing Bcl6<sup>BTKMUT</sup> bone marrow formed few GC B cells and GCs after SRBC immunization, similar to chimeras given a mixture containing Bcl6<sup>–/–</sup> bone marrow (Supplementary Fig. 5b-d). The titers of NP-specific IgG1 antibodies in chimeras given a mixture containing Bcl6<sup>BTKMUT</sup> bone marrow were 16% or 6% of those in chimeras given a mixture containing Bcl6<sup>+/+</sup> bone marrow after immunization with NP-CGG when NP<sub>26</sub>-BSA or NP<sub>4</sub>-BSA was used as capture antigen, respectively (Fig. 3d). The ratio of titers detected with NP<sub>4</sub>-BSA to those detected with NP<sub>26</sub>-BSA was significantly lower in chimeras given a mixture containing Bcl6<sup>BTKMUT</sup> bone marrow than in those given a mixture containing Bcl6<sup>+/+</sup> bone marrow (Fig. 3e). These results demonstrated a B cell–intrinsic requirement for BTB domain–mediated repression in the affinity maturation of immunoglobulins and the development of functional GC B cells.

**Lower proliferation and survival of Bcl6<sup>BTKMUT</sup> GC B cells**

Bcl-6 suppresses checkpoints that control cellular proliferation and survival, which may explain how GC B cells proliferate so rapidly and tolerate somatic hypermutation<sup>6,7,9,10</sup>. We reasoned that the inability to achieve that proliferative and prosurvival phenotype might explain the failure of Bcl6<sup>BTKMUT</sup> mice to form functional GCs. We determined the rate of cell proliferation in vivo by measuring incorporation of the thymidine analog BrdU. Less than 1% of non-GC B cells incorporated BrdU in either Bcl6<sup>+/+</sup> or Bcl6<sup>BTKMUT</sup> mice (Supplementary Fig. 6a). Notably, whereas 32% ± 4.3% of GC B cells in Bcl6<sup>+/+</sup> mice incorporated BrdU, only 16.8% ± 3.1% of GC B cells in Bcl6<sup>BTKMUT</sup> mice were BrdU<sup>+</sup> (Fig. 4a). Cell-cycle analysis showed a greater fraction of GC B cells arrested in the G1 phase with correspondingly fewer in S phase in Bcl6<sup>BTKMUT</sup> mice than in Bcl6<sup>+/+</sup> mice (Fig. 4a). We next assessed the abundance of apoptotic cells by evaluating active caspase and staining with the membrane-impermeable DNA-intercalating dye 7-AAD. Whereas 14.7% ± 1.1% of GC B cells in Bcl6<sup>+/+</sup> mice were positive for activation of total caspase, this fraction was higher (21% ± 1.75%) in Bcl6<sup>BTKMUT</sup> mice (Fig. 4b). Bcl6<sup>BTKMUT</sup> GC B cells also had more caspase-3 activity than did Bcl6<sup>+/+</sup> GC B cells (7-AAD<sup>–</sup> cells, 6% ± 1.1% (mutant) versus 3% ± 0.8% (wild-type); 7-AAD<sup>+</sup> cells, 10% ± 0.6% (mutant) versus 5% ± 1.2% (wild-type); Fig. 4b). Notably, after 3 h of incubation, Bcl6<sup>BTKMUT</sup> GC B cells still included a greater proportion of positive for active caspase-3 than did Bcl6<sup>+/+</sup> GC B cells (7-AAD<sup>–</sup> cells, 10% ± 1.5% (mutant) versus 6.7 ± 2.3%...
The lateral groove of the Bcl-6 BTB domain is required for the proliferation and survival of GC B cells. (a) BrdU incorporation (left) and DNA content (right) in GC B cells from Bcl6<sup>BTBMUT</sup> or Bcl6<sup>B6+/-</sup> mice immunized with SRBCs, assessed by flow cytometry. Numbers above bracketed lines (left) indicate percent BrdU+ (proliferating) cells; numbers at top (right) indicate percent cells in phases of the cell cycle. (b) Active total (pan-)- caspase (left) or caspase-3 (right) in fresh isolated splenocytes (0 h; left and middle) or splenocytes cultured for 3 h ex vivo (3 h; right), from mice as in a, assessed by incubation with fluorescein isothiocyanate–conjugated stain for caspase activity (VAD-fmk) or activated caspase-3 (DEVD-fmk), respectively, and flow cytometry with gating on splenic Fas<sup>+</sup>CD38<sup>neg</sup>B220<sup>+</sup> cells (GC B cells). Numbers above bracketed lines (left) indicate percent cells with active total caspase; numbers in outlined areas (right) indicate percent live cells (7-AAD<sup>-</sup>; bottom) or dead cells (7-AAD<sup>+</sup>; top) with active caspase-3. (c) ChIP-seq analysis of the presence of Bcl-6, SMRT or BCOR on CDKN1A, TP53 and ATR in human tonsil GC B cells. Below plots, coding sequences. Chr, chromosome. *P < 0.05 and **P < 0.01 (two-tailed t-test). Data are from two independent experiments with three to four mice per genotype (a, b; mean ± s.d.) or are representative of three independent experiments (c).

**Bcl6<sup>BTBMUT</sup> TFH cells support normal GC responses**

The results presented above indicated that the Bcl-6 BTB domain was dispensable for the formation of TFH cells (Fig. 3c). To determine whether Bcl6<sup>BTBMUT</sup> TFH cells were able to support GC functions, including the affinity maturation of immunoglobulins, we generated chimeras by transferring bone marrow from Tcr<sup-beta</sup>–Tcr<sup-delta</sup>– mice (which lack the genes encoding the T cell antigen receptor β- and δ-chains; 80%) along with bone marrow from Bcl6<sup>+/+</sup>, Bcl6<sup>BTBMUT</sup> or Bcl6<sup>B6+/-</sup> mice (20%) into sublethally irradiated Rag<sup>1/-/-</sup> mice (Supplementary Fig. 7a). Tcr<sup-beta</sup>–Tcr<sup-delta</sup>– bone marrow cells provide a source of normal B cells but no T cells; thus, the chimeras would be able to form functional GCs only if the other donor bone marrow provided a source of normal TFH cells. After immunization with SRBCs, chimeras that received a mixture containing Bcl6<sup>BTBMUT</sup> bone marrow formed an abundance of GC B cells and normal GCs in spleens equal to that formed by chimeras that received a mixture containing Bcl6<sup>B6+/-</sup> bone marrow, but those that received a mixture containing Bcl6<sup>+/+</sup> bone marrow did not (Fig. 5a,b and Supplementary Fig. 7b). Moreover, titers of NP-specific IgG1 antibodies in NP-CGG-immunized chimeras that received a mixture containing Bcl6<sup>BTBMUT</sup> bone marrow were indistinguishable from those in chimeras that received a mixture containing Bcl6<sup>B6+/-</sup> bone marrow when either NP<sub>26</sub>-BSA or NP<sub>26</sub>-BSA was used as the capture antigen (Fig. 5c). Their ratio of titers detected with NP<sub>26</sub>-BSA to those detected with NP<sub>26</sub>-BSA was also similar to that in chimeras that received a mixture containing Bcl6<sup>B6+/-</sup> bone marrow (Fig. 5d). Thus, Bcl6<sup>BTBMUT</sup> mice formed fully functionally competent TFH cells.

**Normal differentiation of helper T cell subsets in Bcl6<sup>BTBMUT</sup> mice**

Bcl6<sup>-/-</sup> mice show universal skewing of T cells toward the T helper type 2 (Th2) and interleukin 17 (IL-17)-producing helper T cell (Th17) lineages, which contributes to aberrant inflammatory signaling and the eventual death of these mice<sup>3,33</sup>. That defect can be triggered and aggravated by immunization<sup>34</sup>. To better define the contribution of the lateral groove of the BTB domain to the differentiation of those effector helper T cell subtypes, we isolated splenocytes from age-matched Bcl6<sup>BTBMUT</sup> and Bcl6<sup>B6+/-</sup> mice, then measured the abundance of Th1, Th2 and Th17 cells. At baseline, Bcl6<sup>BTBMUT</sup> mice had an abundance of Th1 cells, Th2 and Th17 cells similar to that of their Bcl6<sup>B6+/-</sup> littermates (data not shown). We next analyzed helper T cell subtypes in mice immunized with SRBCs. As reported before<sup>3,33</sup>, Bcl6<sup>-/-</sup> mice had significantly more Th2 cells and Th17 cells than did Bcl6<sup>B6+/-</sup> mice (Th2 cells, 11.5% ± 0.4% (Bcl6<sup>+/+</sup>) versus 1% ± 0.2% (Bcl6<sup>-/-</sup>); Th17 cells, 4.8% ± 0.4% (Bcl6<sup>+/+</sup>) versus 0.6% ± 0.2% (Bcl6<sup>-/-</sup>); Fig. 6a,b). In contrast, Bcl6<sup>BTBMUT</sup> mice had an abundance of Th12 and Th17 cells similar to that of Bcl6<sup>B6+/-</sup> mice (0.98 ± 0.3% (Bcl6<sup>BTBMUT</sup>) versus 1 ± 0.2% (Bcl6<sup>+/+</sup>); 0.62 ± 0.3% (Bcl6<sup>BTBMUT</sup>) versus 0.6 ± 0.2% (Bcl6<sup>B6+/-</sup>); Fig. 6a,b). Thus, the lateral groove of the BTB domain was not involved in Bcl-6-mediated suppression of Th12 or Th17 differentiation in vivo.

**No Bcl6<sup>-/-</sup> inflammatory phenotype in Bcl6<sup>BTBMUT</sup> mice**

Bcl6<sup>-/-</sup> mice are born at lower than expected frequencies, are runted<sup>3,33</sup> and develop a severe Th2-type inflammatory syndrome that involves many organs and frequently includes the lungs and spleen<sup>3,34</sup>. However, we observed that Bcl6<sup>BTBMUT</sup> mice were born at the expected frequency, demonstrated normal growth rates, and were indistinguishable from littermate controls when housed under standard conditions.

**Supplementary information**

Figure 4. The lateral groove of the Bcl-6 BTB domain is required for the proliferation and survival of GC B cells. (a) BrdU incorporation (left) and DNA content (right) in GC B cells from Bcl6<sup>BTBMUT</sup> or Bcl6<sup>B6+/-</sup> mice immunized with SRBCs, assessed by flow cytometry. Numbers above bracketed lines (left) indicate percent BrdU<sup>+</sup> (proliferating) cells; numbers at top (right) indicate percent cells in phases of the cell cycle. (b) Active total (pan-)- caspase (left) or caspase-3 (right) in fresh isolated splenocytes (0 h; left and middle) or splenocytes cultured for 3 h ex vivo (3 h; right), from mice as in a, assessed by incubation with fluorescein isothiocyanate–conjugated stain for caspase activity (VAD-fmk) or activated caspase-3 (DEVD-fmk), respectively, and flow cytometry with gating on splenic Fas<sup>+</sup>CD38<sup>neg</sup>B220<sup>+</sup> cells (GC B cells). Numbers above bracketed lines (left) indicate percent cells with active total caspase; numbers in outlined areas (right) indicate percent live cells (7-AAD<sup>-</sup>; bottom) or dead cells (7-AAD<sup>+</sup>; top) with active caspase-3. (c) ChIP-seq analysis of the presence of Bcl-6, SMRT or BCOR on CDKN1A, TP53 and ATR in human tonsil GC B cells. Below plots, coding sequences. Chr, chromosome. *P < 0.05 and **P < 0.01 (two-tailed t-test). Data are from two independent experiments with three to four mice per genotype (a, b; mean ± s.d.) or are representative of three independent experiments (c).
Mendelian frequencies, were developmentally indistinguishable from their Bcl6+/+ littermates and had normal body weights (Fig. 7a). By 6 weeks of age, more than half of the Bcl6−/− mice had died, whereas all Bcl6^BTRMUT mice remained healthy even after 1 year of monitoring. Bcl6−/− mice had the expected infiltrates of inflammatory cells in their lungs, as well as multinodular lesions characterized by the infiltration of eosinophils into the spleen, whereas histological studies of Bcl6^BTRMUT organs (including lungs, spleen, heart, liver, thymus, kidney and intestine) showed no such infiltrates (Fig. 7b and Supplementary Fig. 8a). We confirmed that the mice had that phenotype in two independent founder lines with different genetic backgrounds (data not shown).

**Minimal effect on chemokines in Bcl6^BTRMUT macrophages**

Bcl6−/− macrophages have substantial upregulation of the expression of inflammatory chemokines such as CCL2, CCL3, CCL6, CCL7 and IL-1α, which are believed to be critical mediators of the lethal inflammatory response in Bcl6−/− mice.18,19 Bcl-6 also suppresses macrophage proliferation through repression of CCND2, a critical driver of the cell cycle.35 The unexpected lack of an inflammatory phenotype in Bcl6^BTRMUT mice prompted us to measure chemokine expression and the proliferation of macrophages isolated from these mice. We first measured the abundance of mRNA from genes encoding molecules involved in the inflammatory response (Ccl2, Ccl3, Ccl6, Il1a, Ccl7 and Ccnd2) in resting and lipopolysaccharide (LPS)-stimulated macrophages from Bcl6+/+^, Bcl6^BTRMUT and Bcl6−/− mice, by quantitative PCR. Most of these genes had 20-fold higher expression in resting Bcl6−/− macrophages than in resting Bcl6+/+ cells and were further induced by treatment with LPS (Fig. 7c). In contrast, Bcl6^BTRMUT macrophages showed no more than 1.4- to 4-fold higher basal expression of these genes relative to their expression in Bcl6+/+ cells, and that limited higher expression was not further enhanced by LPS treatment (Fig. 7c). As expected, Bcl6−/− macrophages divided faster than Bcl6+/+ macrophages did, whereas Bcl6^BTRMUT macrophages had a proliferation rate very similar to that of Bcl6+/+ macrophages (Fig. 7d).

To determine whether other Bcl-6 domains contribute to the repression of genes encoding inflammatory molecules to a greater extent than the BTB domain does, we transduced Bcl6−/− macrophages with retrovirus expressing wild-type Bcl6 or Bcl6 with various mutations. Bcl6−/− macrophages transduced to express wild-type Bcl6, Bcl6 with point mutations that result in the substitutions BN21K and H116A (as in Bcl6^BTRMUT mice), Bcl6 with mutations that result in inactivation of the RD2 repression domain36 or Bcl6 with both sets of mutations retained the ability to suppress the expression

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**Figure 5** Normal GC responses in chimeras given Tcrb−/−Tcrd−/− and Bcl6^BTRMUT bone marrow. (a) Flow cytometry of GC B cells from chimeras given Tcrb−/−Tcrd−/− bone marrow plus Bcl6+/+, Bcl6^BTRMUT or Bcl6−/− bone marrow (Supplementary Fig. 6a), assessed 10 d after immunization of chimeras with SRBCs, gated on live splenic B220^hi lymphocytes. Numbers in outlined areas indicate percent Fas^−/−CD8−/− B cells (GC B cells). (b) Staining of spleen sections from mice in a with peanut agglutinin. Scale bars, 200 μm. (c) IgG1 titers in serum from chimeras as in a, 21 d after immunization with NP-CGG, measured with NP4-BSA and NP2-BSA. (d) Ratio of the titer of IgG1 or IgG2a detected with NP2-BSA to that detected with NP4-BSA. Each symbol represents an individual mouse; small horizontal lines indicate the mean. *P < 0.05 (one-tailed t-test). Data are representative of three independent experiments (a, b) or two independent experiments with four mice (c, d).

**Figure 6** Normal T\(\text{H}2\) and T\(\text{H}17\) differentiation in Bcl6^BTRMUT mice. (a) Flow cytometry of splenic CD4+ T cells stimulated for 5 h with the phorbol ester PMA (20 ng/ml) and ionomycin (1 μg/ml) in the presence of the protein-transport inhibitor GolgiPlug (1 μg/ml), followed by staining for CD4 and IFN-γ, IL-4 or IL-17. Numbers adjacent to outlined areas indicate percent cells in each. (b) Frequency of T\(\text{H}2\) (IFN-γ+) cells, T\(\text{H}17\) (IL-4+) cells and T\(\text{H}17\) (IL-17+) cells among CD4+ T cells, based on flow cytometry as in a. Each symbol represents an individual mouse; small horizontal lines indicate the mean (± s.d. of two to three mice). *P < 0.001 (two-tailed t test). Data are representative of two independent experiments.
of genes encoding chemokines and inflammatory molecules (Fig. 7c).

In contrast, those transduced to express Bcl6 with a mutation that affects the third C2H2 zinc finger of Bcl-6 that abolishes DNA binding without affecting localization to the nucleus37 were completely unable to repress those target genes (Fig. 7c). Hence, whereas DNA binding was required for the repression of chemokine expression, the lateral groove of the BTB domain was dispensable for that function, consistent with the lack of inflammatory phenotype of Bcl6^{BTTMUT} mice.

ChIP-seq analysis of Bcl6 in macrophages has shown that Bcl-6-binding sites show considerable enrichment for STAT-binding motifs, and reciprocal changes in the binding of STAT5 and Bcl-6 at the promoters and enhancers of the Ccl2, Ccl3, Ccl6, Ccl7, and Ccnd2 genes (Fig. 7d).

**DISCUSSION**

Given that Bcl-6 and coexpressors of its BTB domain are expressed together in most Bcl-6-expressing tissues, the biochemical function of Bcl-6 would be expected to be similar among cell types, and its tissue-specific function would be expected to be perhaps mediated by binding to distinct sets of target genes. Indeed, genome-localization studies of Bcl-6 in various cell types have suggested that its transcriptional targets are partially cell type specific19,28,29. However, evidence of a more profound, biochemical level of functional diversification has been hinted at by studies of cell-penetrating peptides designed to dissociate the BTB domain of Bcl-6 from its coexpressors30,31. The administration of such peptides to mice bearing human lymphoma xenografts induces growth arrest and apoptosis in lymphoma cells but does not induce the Bcl6^{−/−} inflammatory phenotype32,33. Unfortunately, the interpretation of such peptide studies has been limited by the unknown kinetics and degree of inhibition of Bcl-6 in various cell types, as well as a relatively time-limited exposure. Our introduction of point mutations in the native Bcl6 locus in mice afforded constitutive loss of the repressor function of the BTB domain in all tissues while preserving proper timing and amount of expression, which allowed us to gain critical insights into the function of this unique biochemical mechanism of Bcl-6.

Bcl6^{BTTMUT} mice had a normal early extrafollicular response but an impaired late GC response after immunization with a T cell–dependent antigen. Notably, loss of the repression function of the BTB domain manifested as failure of GC B cells to proliferate and survive, even in the presence of wild-type T \(_{H12}\) cells and a wild-type microenvironment. Hence, the function of the BTB domain of Bcl-6 was specifically...
to enable the proliferative and DNA damage–tolerant phenotype of GC B cells, possibly by repressing ATR, TP53 and CDKN1A. Blockade of the BTB domain with peptide inhibitors in primary GC B cells and DLBCL cells results in derepression of those genes6,32. One implication of these results is that the same biochemical function through which Bcl-6 specifically mediates the GC B cell phenotype also drives the survival and proliferation of DLBCL cells. Targeting the BTB domain of Bcl-6 with peptides or small-molecule inhibitors kills DLBCL cell lines in vitro and in vivo, as well as primary human DLBCL cells ex vivo39–41. Thus, DLBCLs are essentially driven by a normal transcriptional mechanism derived from their cell of origin.

Similar to Bcl6−/− mice, Bcl6<sup>BTBMUT</sup> mice had fewer CXCR5<sup>PD-1</sup> T<sub>FH</sub> cells and GC T<sub>FH</sub> cells. However, in contrast to the T cell development in Bcl6−/− mice, Bcl6<sup>BTBMUT</sup> mice generated perfectly functional T<sub>FH</sub> cells after immunization in the presence of normal cognate B cells. As cognate B cells are absolutely required for the differentiation and maintenance of GC T<sub>FH</sub> cells42–44, the defect in GC T<sub>FH</sub> cells in Bcl6<sup>BTBMUT</sup> mice was probably secondary to the defect in the B cell compartment. The finding that the defect in CXCR5<sup>PD-1</sup> T<sub>FH</sub> cells was less pronounced was probably related to the preservation of the early, extrafollicular phase of the immune response that we observed in Bcl6<sup>BTBMUT</sup> mice. The mechanisms underlying the deficiencies in the T<sub>FH</sub> cell phenotype in Bcl6<sup>BTBMUT</sup> and Bcl6−/− mice are completely different. Further research is thus needed to explain the biochemical mechanism through which Bcl-6 mediates the T<sub>FH</sub> cell phenotype. Perhaps different sets of corepressors that bind to different sites on Bcl-6 are involved.

Another salient finding of our phenotypic analysis of Bcl6<sup>BTBMUT</sup> mice was the absence of the lethal inflammatory disease of Bcl6−/− mice, which indicated that the lateral groove of the BTB domain was dispensable for the functions of Bcl-6 in the innate immune system. In macrophages, Bcl-6 attenuates inflammatory responses through the repression of chemokines and NF-κB repressors and activators can compete for promoter occupancy by Bcl-6 and STAT proteins, rather than the interaction of Bcl-6 with its corepressors, may be the dominant biochemical function of Bcl-6 in innate immunity.

Finally, our study has implications for the clinical use of inhibitors of Bcl-6 designed to disrupt the binding of corepressors to its BTB domain39,40, which are now being ‘translated’ for use in the treatment of patients with Bcl-6-dependent tumors. The potential for such drugs to cause systemic inflammation and atherosclerosis is a potential concern for humans treated with such compounds.

Our data have shown that such side effects would be unlikely to occur, consistent with reported toxicity studies of such inhibitors in mice39. In summary, by constructing a knock-in mutation that results in constitutive disruption of the binding of corepressors to the BTB domain, we were able to demonstrate that Bcl-6 mediated immunological lineage-specific effects through various protein interactions and thus provide a new paradigm for the functional diversification of transcription factors.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. GEO: microarray data, ChIP-seq data, GSE43350.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

C.H. designed and did most of the experiments; K.H. did and analyzed ChIP-seq experiments; and A.M. conceived of the project and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Generation of Bcl6<sup>BTBMUT</sup> mice. The mutations encoding N21K and H116A were introduced into exon 3 and exon 4 of a Bcl6 bacterial artificial chromosome (RP24-371N16; Children’s Hospital Oakland Research Center) by a Galkk-positive-counterselection strategy. A neomycin-resistance (neo<sup>+</sup>) cassette flanked by two loxp sites was inserted into the Bcl6 intron 3.800 base pairs upstream to the residue encoding His116. Finally, 2.0-kb diphtheria toxin α-chain cassette replaced the 1.0-kilobase genomic fragment 2.0 kilobases downstream of the 3’ loxP site (Supplementary Fig. 1a). The targeting vectors were made linear and were transfected by electroporation into 129 × C57Bl/6 embryonic stem cells. Embryonic stem cell clones were screened for integration by PCR (primers F1 and R1, Supplementary Table 1). Two clones confirmed to contain the homologous-targeted mutation were injected into C57Bl/6 blastocysts, and those blastocytes were implanted into pseudopregnant female mice. Germline transmission resulted in the generation of Bcl6<sup>BTBMUT</sup> mice with the neo<sup>+</sup> cassette in the knocked-in allele. Those mice were mated with C57Bl/6 EIIa-Cre mice (with transgenic expression of Cre recombinase controlled by the EIIa promoter, which targets Cre expression to the early mouse embryo, to remove the loxp-flanked neo<sup>+</sup> cassette); this generated Bcl6<sup>BMTMUT</sup> mice. Those mice were further bred to C57Bl/6 mice for at least five generations and were intercrossed to obtain homozygous mice for most experiments. The genotyping primers are shown in Supplementary Table 1 for study of the development of inflammatory diseases in Bcl6<sup>BMTMUT</sup> mice, Bcl6<sup>BMTMUT</sup> mice were bred with Sv129 mice (Jackson Laboratory) for three generations and were intercrossed to obtain homozygous mice.

Mice and mixed–bone marrow chimera studies. Bcl6<sup>BMTMUT</sup> mice were provided by H. Ye. For the generation of mixed bone marrow chimera, 4 × 10<sup>6</sup> cells from a mixture of B6.129S2-P<sup>Bcl6</sup> +/+; C57Bl/6J bone marrow (CD45.1<sup>+</sup>; Jackson Laboratory) plus Bcl6<sup>BMTMUT</sup> bone marrow, at a ratio of 1:1, or a mixture of μMT bone marrow (Jackson laboratory) plus Bcl6<sup>BMTMUT</sup> bone marrow, at a ratio of 1:1, or a mixture of C57Bl/6bone marrow (Jackson Laboratory) plus Bcl6<sup>BMTMUT</sup> bone marrow, a ratio of 4:1, were transferred intravenously into sublethally irradiated Rag1<sup>-/-</sup> mice (Jackson Laboratory). Then, 8 weeks later, the recipient mice were immunized and killed for further analysis of GC formation and antibody production. Mice were housed in the specific pathogen–free animal facility at Weill Cornell Medical College and animal experiments were done with protocols approved by the Institutional Animal Care and Use Committee.

Antibodies and flow cytometry. Phycoerythrin–fluorescein isothiocyanate–or allophycocyanin-conjugated antibody to mouse B220 (RA3-6B2), allophycocyanin–conjugated antibody to mouse IgM (II/41), phycoerythrin–conjugated antibody to mouse IgG (11-26c.2a), phycoerythrin–indotricarbocyanine–conjugated antibody to Fas (anti-Fas; jo2), phycoerythrin–indotricarbocyanine–conjugated antibody to mouse CXCR5 (2G8), phycoerythrin–conjugated anti-IL-4 (11b11), fluorescein isothiocyanate–conjugated anti–GL-7 (GL-7), Pacific blue–conjugated anti–CD138– (181-2), allophycocyanin–indotricarbocyanine–conjugated anti–CD11c (N418) and fluorescein isothiocyanate–conjugated anti–IFN–γ (XM1G.1.2) were from BD Bioscience. Fluorescein isothiocyanate–conjugated anti–CD45.2 FITC (104), Phycoerythrin– or fluorescein isothiocyanate–conjugated antibody to mouse PD-1 (J43), Alexa Fluor 488–conjugated antibody to mouse CD1d (1B1), fluorescein isothiocyanate–conjugated antibody to mouse CD4 (GK1.5), phycoerythrin–conjugated antibody to mouse CD23 (B23B4), fluorescein isothiocyanate– or allophycocyanin–indotricarbocyanine–conjugated anti–mouse CD43 (EbioR2/60) or to mouse CD80 (55.67), allophycocyanin–conjugated antibody to mouse CD38 (90), and Alexa Fluor 488–conjugated antibody to mouse IL-7A (ebioCT11-18H10.1) were from ebiosciences. NP-phycoerythrin was from Biosearch Technologies. All flow cytometry data were acquired on a BD LSR II and were analyzed with FlowJo software package (Tri-Star).

Intracellular cytokine staining. For analysis of cytokine secretion by flow cytometry, spleen cells were stimulated for 5 h with PMA (phorbol 12-myristate 13-acetate; 20 ng/ml; Invitrogen) and ionomycin (1 μg/ml; Invitrogen) in the presence of GolgiPlug (1 μg/ml, Invitrogen) and were stained with allophycocyanin–conjugated antibody to mouse CD4, followed by permeabilization in Fix/Perm buffer and intracellular staining with fluorescein isothiocyanate–conjugated anti–IFN–γ, phycoerythrin–conjugated anti–IL-4 or Alexa Fluor 488–conjugated antibody to mouse IL-17A in Perm/Wash buffer (BD Pharmingen).

BrdU detection, and cell-cycle and apoptosis assays. For BrdU labeling, mice were given intravenously injection of 2 mg BrdU (Sigma-Aldrich) 2 h before being killed. Splenocytes were prepared and stained with phycoerythrin–conjugated antibody to mouse B220, phycoerythrin–indotricarbocyanine–conjugated anti–Fas and allophycocyanin–conjugated antibody to mouse CD38. Then, BrdU<sup>+</sup> cells were detected with a BrdU Flow kit according to the manufacturer’s protocol (BD Pharmingen). For cell-cycle analysis, splenocytes were stained with phycoerythrin–conjugated antibody to mouse B220, phycoerythrin–indotricarbocyanine–conjugated anti–Fas and allophycocyanin–conjugated antibody to mouse CD38 and were fixed for 45 min in fixation/permeable buffer (FoxP3 staining set; eBioscience), followed by staining with DAPI (4,6-diamidino-2-phenylindole). Phase distribution was analyzed automatically with the Dean-Jett-Fox model (Flowjo). For detection of apoptosis in situ, fresh isolated splenocytes or splenocytes incubated for 3 h were maintained for 1 h at 37 °C in FITC-VAD-fmk (fluorescein isothiocyanate–Val-Ala–Asp–fluoromethylketone) or FITC-DEVD-fmk (fluorescein isothiocyanate–Asp-Glu-Val-Asp–fluoromethylketone; BioVision) in RPMI medium. Cells were washed according to the manufacturer’s protocol and then were labeled with phycoerythrin–conjugated antibody to mouse B220, phycoerythrin–indotricarbocyanine–conjugated anti–Fas and allophycocyanin–conjugated antibody to mouse CD38 for surface phenotyping.

Immunization, enzyme-linked immunosorbent assay and enzyme-linked immunospot assay. For analysis of the formation of GCs, mice were immunized intraperitonally for the appropriate number of days with SRBCs (1 × 10<sup>6</sup> cells per mouse) or NP21–GGG (Biosearch Technologies) in Injject alun (Pierce). For analysis of T cell–dependent antibody production, mice were immunized intraperitonally with 100 μg NP<sub>26</sub>Ficoll (Biosearch Technologies) and analyzed for 8 d. For analysis of T cell–dependent antibody production, mice were immunized intraperitonally with 100 μg NP21–GGG. On days 8 and 21 after immunization, serum was collected and titers of isotype-specific antibodies to NP were measured in plates coated with NP<sub>26</sub>BSA or NP<sub>26</sub>BSA with the SBA Clonotyping System, according to the manufacturer’s protocol (Southern Biotech). Titters are presented as the greatest serum dilution that provided an average absorbance exceeding 1.5-fold above the average background absorbance at 405 nm. For enzyme-linked immunospot assay, spleen cells were incubated for 20 h at 37 °C on NP<sub>26</sub>BSA–coated 96-well MultiScreen–HA filter plates (Millipore). Spots were visualized with goat antibody to mouse IgG (1034-05) or IgM (102105) conjugated to horseradish peroxidase (Southern Biotechnology), and color was visualized by the addition of 3,3’,5,5’-tetramethylbenzidine (Southern Biotechnology).

Immunoblot analysis. B220<sup>+</sup> cells were isolated from spleens with Mouse B220 MicroBeads (Miltenyi Biotec) and were analyzed by immunoblot with anti–Bcl6 (D8) and anti–actin (C-11; both from Santa Cruz). Quantitative RT-PCR. Total RNA was prepared with TriZol regent (Invitrogen) or an RNasasy Mini kit (Qiagen), then cDNA were synthesized with Superscript reverse transcriptase and random primers (Invitrogen). Quantitative PCR was done with Power SYBR Green PCR master mix (Applied Biosystems) and the appropriate primers (sequences, Supplementary Table 3).

BMDM culture, retrovirus production and transduction. BMDMs were cultured as described<sup>18</sup>. After 8 d of culture, mature macrophages were scraped...
off the dishes and washed with PBS, then were replated in complete DMEM. Cells were allowed to adhere overnight, and then fresh medium, with or without 5 µg/ml LPS (from Escherichia coli strain 055:B5; Sigma-Aldrich), was added for 6 h before collection of cells. Expression constructs for Bcl6+/+ and Bcl6 with point mutations that result in the substitutions BN21K and H116A have been described24. Expression constructs for the latter and for Bcl6 with mutations that result in inactivation of RD2 or Bcl6 with mutation affecting its third C2H2 zinc finger were generated with QuikChange II Site-Directed Mutagenesis kits (Agilent Technologies). The cDNA fragments for Bcl6+/+ and those mutated forms of Bcl6 were subcloned into MIGR1-GFP or MIGR1-puromycin retroviral expression vector. Viral supernatants were prepared with Plat-E cells according to a standard protocol. For retroviral infection, bone marrow cells were maintained in complete DMEM for 4 d and infected with viral supernatants in the presence of 8 µg/ml polybrene (Sigma). For MIGR1-GFP-infected cells, GFP+ cells were sorted 7 d after infection for analysis of gene expression by quantitative RT-PCR. For MIGR1-puromycin–infected cells, puromycin-resistant cells were selected by the addition of 2 µg/ml puromycin (Invitrogen) and used for quantitative ChIP assays.

**Immunohistology.** Spleens and Peyer’s patches of the terminal ileum were fixed in 4% paraformaldehyde and embedded in paraffin. Sections of each sample 6 µm in thickness were prepared, cleared in xylene and hydrated through a descending alcohol series to distilled water. Slides were boiled for 20 min in citrate antigen retrieval buffer, followed by washes under running water. Endogenous peroxidase activity was blocked by treatment of the sections for 20 min with 3% hydrogen peroxide in methanol. Tissue sections were then incubated overnight at 4 °C with biotin-conjugated peanut agglutinin (Vector Laboratories). After a further wash in TBS, streptavidin–horseradish peroxidase was added, followed by incubation for 30 min. Horseradish peroxidase activity was detected with a DAB kit (Vector Laboratories). Finally, sections were counterstained with hematoxylin if necessary. For double staining, sections were incubated overnight at 4 °C with anti-Bcl-6 (N3; Santa Cruz), followed incubation for 1 h with biotin-conjugated secondary antibody (sc-2030; Santa Cruz). Streptavidin–alkaline phosphatase was added after a further wash in TBS followed by incubation for 30 min. Alkaline phosphatase activity was detected with a Alkaline Phosphatase Substrate Kit III (Vector Laboratories). Sections were boiled for 10 min followed by incubation with biotin-conjugated anti-B220 (RA3-6B2; Caltag Metystems), then incubation with streptavidin–horseradish peroxidase. Horseradish peroxidase activity was detected with a DAB kit (Vector Laboratories).

**Statistical analysis.** Student’s t-test was used for statistical analysis with the software GraphPad Prism 5. P values above 0.05 were considered not significant.