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Bcl6 Mediates the Development of T Follicular Helper Cells

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Abstract

A fundamental function of CD4⁺ helper T (T_H) cells is the regulation of B cell-mediated humoral immunity. Development of T follicular helper (T_{FH}) cells that provide help to B cells is mediated by the cytokines interleukin-6 and interleukin-21 but is independent of T_H1, T_H2, and T_H17 effector cell lineages. Here, we characterize the function of Bcl6, a transcription factor selectively expressed in T_{FH} cells. Bcl6 expression is regulated by interleukin-6 and interleukin-21. Bcl6 overexpression induced T_{FH}-related gene expression and inhibited other T_H lineage cell differentiation in a DNA binding-dependent manner. Moreover, Bcl6 deficiency in T cells resulted in impaired T_{FH} cell development and germinal center reactions, and altered production of other effector T cell subsets. Our data thus illustrate that Bcl6 is required for programming of T_{FH} cell generation.

A critical function of CD4⁺ helper T (T_H) cells is to provide “help” to B cells, especially in the germinal center structures where activated B cells proliferate and undergo antibody affinity maturation. Recently, T follicular helper (T_{FH}) cells have been characterized by their expression of chemokine (C-X-C motif) receptor 5 (CXCR5) (1–3). We, as well as others, recently reported that T_{FH} cell development is mediated by interleukin (IL)–6 or IL-21 but is independent of T_H1, T_H2, and T_H17 cells (4,5).

The B cell lymphoma 6 (Bcl6) transcription factor is selectively expressed by T_{FH} cells (2,3). Bcl6 was previously shown to be inhibitory to T_H2 responses by blocking signal transducer and activator of transcription 6 (STAT6) binding to DNA (6,7), whereas Bcl6-deficient mice developed multiorgan inflammatory diseases, enhanced immunoglobulin E (IgE) production, and defective germinal center reaction (6,8). It is not clear whether the germinal center defect in these mice is caused by lack of proper T and/or B cell function because Bcl6 is also expressed by germinal center B cells (9). To analyze the function of Bcl6 in T_{FH} cells, we activated naïve CD4⁺ T cells (CD44^{low}CD62L^{high}CD25[–]) from C57BL/6 mice with antibodies to CD3 and CD28 in the presence or absence of various cytokines for 1 or 2 days, and Bcl6 mRNA expression was assessed by real-time reverse transcription polymerase chain reaction (RT-PCR) analysis (10). Treatment with IL-6 or IL-21 significantly up-regulated Bcl6 expression, which was strongly inhibited by the addition of exogenous transforming growth factor beta (TGFβ) (Fig. 1A). These results correlate with our previous observations that IL-6 or IL-21 alone induces T_{FH} cell development and Bcl6 expression, whereas treatment, together with TGFβ, promotes T_H17

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Supporting Online Material

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Materials and Methods

Figs. S1 to S11

References

differentiation instead (4). To determine whether IL-21 is necessary for IL-6–induced Bcl6 expression, we activated naïve wild-type and IL-21– or IL-21 receptor (IL-21R)–deficient CD4⁺ T cells in the presence of IL-6. IL-21– and IL-21R–deficient T cells showed significantly reduced expression of Bcl6 (fig. S1).

We next assessed whether overexpression of Bcl6 promoted T_{FH} cell development in the absence of exogenous cytokines. Bcl6 overexpression led to increased expression of endogenous Bcl6 mRNA as well as IL-21R, IL-6R, and CXCR5 mRNA, similar to cells treated with IL-6 or IL-21 (Fig. 1B and fig. S2A). Interestingly, IL-21 expression was not up-regulated by Bcl6 overexpression.

Bcl6 has multiple zinc finger (ZF) domains, and the mutation of two of these (ZF3 and ZF5) was previously shown to abolish DNA binding but not nuclear localization (11). We thus assessed the function of Bcl6 with a mutation in either domain in the induction of T_{FH}–specific genes. ZF3 and ZF5 mutations completely abrogated the ability of Bcl6 to up-regulate endogenous Bcl6, IL-21R, and CXCR5 expression, whereas the ZF3 exhibited less efficient inhibition of IL-6R expression than ZF5 (Fig. 1C). Thus, the regulation of T_{FH} gene expression by Bcl6 appears to depend largely on its ability to bind DNA.

We then assessed whether Bcl6 overexpression antagonizes the differentiation of other T_H lineage cells. We first overexpressed Bcl6 in cells undergoing T_H17 differentiation in the presence of TGFβ and IL-6. We found that Bcl6 overexpression induced endogenous Bcl6 expression and also moderately increased the expression of CXCR5, IL-21R, and IL-6R (Fig. 2A); however, the expression of these genes was significantly lower than that induced by Bcl6 under neutral conditions or in T_{FH} cells (Fig. 1B). Bcl6 also inhibited IL-17 protein expression when measured by intracellular staining, as well as IL-17 and IL-17F mRNA expression, in a DNA binding–dependent manner (Fig. 2, A and B). In contrast, RORγt or IL-21 expression was not affected by Bcl6. We further analyzed whether Bcl6 might influence RORγt-dependent transcription using a luciferase reporter driven by the IL-17 gene promoter and the conserved noncoding sequence 2 (CNS2) element (12). Wild-type Bcl6, but not the DNA-binding mutants ZF3 and ZF5, strongly inhibited RORγt-induced luciferase activity (Fig. 2C). Thus, similar to Foxp3, Bcl6 inhibits RORγt function but not its expression. Unlike Foxp3 (13), however, Bcl6 function appears to be dependent on its binding to DNA.

In addition to T_H17 cells, we also analyzed the effect of Bcl6 in developing T_H1 and T_H2 cells and found that overexpression of Bcl6 increased endogenous Bcl6 expression and CXCR5 (Fig. 2D and fig. S2, B and C). Bcl6, but not ZF3 and ZF5 mutants, also significantly inhibited the expression of T_H1 (IFNγ and T-bet) and T_H2 (IL-4 and GATA3) genes (Fig. 2, D and E, and fig. S2, B and C). Together, these data suggest that Bcl6 autoregulates its own expression. Moreover, Bcl6 not only induces T_{FH} gene expression in neutral conditions, similar to IL-6 or IL-21, but also inhibits the differentiation of other T_H lineage cells.

To understand whether Bcl6 is necessary for T_{FH} cell development, we used a previously reported Bcl6 knockout mouse (*Bcl6*^{−/−}) (14) and crossed it with OT-II T cell receptor (TCR) transgenic mice. The OT-II TCR is specific for a peptide derived from chicken ovalbumin presented by major histocompatibility complex class II; thus, the majority of T cells in these mice are CD4⁺. Naïve CD4⁺ T cells were first differentiated to promote T_{FH} cell differentiation in vitro (4). Bcl6 deficiency resulted in substantial reduction of CXCR5 and IL-6R expression, although the expression of IL-21R and IL-21 was partially reduced (Fig. 3A).

We also subjected control and *Bcl6*^{-/-} naïve T cells to T_H1 and T_H17 differentiation. As expected, absence of Bcl6 resulted in enhanced T_H1 and T_H17 differentiation and increased expression of T-bet and RORγt, respectively (Fig. 3, B and C, and fig. S3). Interestingly, IL-21 mRNA expression in *Bcl6*^{-/-} cells differentiated under T_H17 condition was enhanced, correlating with increased expression of IL-17 and RORγt (Fig. 3C). When activated in the presence of TGFβ to induce Foxp3 expression, *Bcl6*^{-/-} T cells exhibited reduced levels of Foxp3 expression but enhanced IL-4 and IFNγ expression. Moreover, Foxp3 expression was partially restored by treatment with antibodies to IFNγ and IL-4 (Fig. 3D).

To analyze the function of Bcl6 in T_{FH} cell generation in vivo, we first analyzed germline wild-type and *Bcl6*^{-/-} mice. We found that memory T cells from aged *Bcl6*^{-/-} mice produced significantly elevated amounts of IL-17, but their production of IFNγ and IL-4 was comparable to that of wild-type cells (fig. S4, A and B). IL-21 expression in IL-17⁺ T cells was also increased in these mice (fig. S4C). We then immunized wild-type and *Bcl6*^{-/-} mice with keyhole limpet hemocyanin (KLH) protein (4), which induces an immune response where B cell activation requires T cell help. *Bcl6*^{-/-} mice exhibited severely reduced CXCR5 expression on T cells and numbers of germinal center B cells (fig. S5) 7 days after immunization. Immunohistochemistry analysis also revealed that the germinal center B cells were greatly reduced in the *Bcl6*^{-/-} mice when compared with immunized wild-type mice (fig. S6A). The production of KLH-specific IgG, IgM, and IgG2a was also reduced in *Bcl6*^{-/-} mice, whereas IgE was enhanced (fig. S7A). In contrast, we observed increased expression of T_H1, T_H2, and T_H17 cytokines in *Bcl6*^{-/-} mice, whereas IL-21 production was not affected (fig. S5). To substantiate the above results, we also purified CD4⁺CD44^{high} cells from immunized mice and measured their cytokine secretion after activation ex vivo with KLH and irradiated wild-type splenic antigen-presenting cells. We observed greatly increased expression of IL-4, IFNγ, and IL-17 in *Bcl6*^{-/-} T cells when compared with wild-type T cells (fig. S8A). Furthermore, *Bcl6*^{-/-} T cells exhibited defective expression of T_{FH}-related genes, but their expression of T_H1, T_H2, and T_H17 genes was significantly enhanced (fig. S8B). Interestingly, Bcl6 deficiency did not result in defective IL-21 production, and T_H17 cells are likely the source of IL-21 in knockout mice.

To specifically analyze the function of Bcl6 in T cells, we mixed wild-type or *Bcl6*^{-/-} naïve CD4⁺ T cells with wild-type B cells and transferred them into *Rag1*^{-/-} mice, which lack T and B cells, and immunized the mice with KLH. Absence of Bcl6 in T cells again resulted in greatly reduced numbers of CXCR5⁺ T cells (Fig. 4A). Moreover, germinal center B cells (GL7⁺ FAS⁺) were greatly reduced in these animals (Fig. 4A), which was consistent with our immunohistochemistry analysis (fig. S6B). KLH-specific IgG, IgM, and IgG2a production was also reduced (fig. S7B). We also observed that Bcl6 deficiency resulted in enhanced production of IL-4 by T cells and KLH-specific IgE by B cells, but only moderately decreased IL-17 and IL-21 expression by knockout T cells (Fig. 4A and fig. S7B). In another experiment, we transferred wild-type and *Bcl6*^{-/-} total CD4⁺ T cells with wild-type B cells into *Rag1*^{-/-} mice and immunized them with KLH. The analysis of sorted effector-phenotype CD4⁺CD44^{high} cells from the immunized mice revealed that Bcl6 deficiency in T cells resulted in the reduction of mRNA expression of most of the T_{FH}-related genes except for IL-21 (fig. S9), which was increased together with IL-17 and RORγt, suggesting that the source of IL-21 may be T_H17 cells.

Because Bcl6 deficiency also caused hyper-IL-4 production in the above experiment, we examined the effect of IL-4 neutralization. Treatment with antibody to IL-4 almost completely blocked IL-4 production by T cells, whereas defective CXCR5 expression was still observed in *Bcl6*^{-/-} T cells (Fig. 4B). We also observed reduced generation of germinal center B cells and KLH-specific IgG, IgM, and IgG2a production (Fig. 4B, fig. S6C, and fig. S7C). In addition, *Bcl6*^{-/-} T cells also exhibited increased IL-22, IL-17, and IFNγ

production (Fig. 4B). Despite the absence of T_{FH} cells in *Bcl6*^{-/-} mice, we detected IL-21 production that was comparable to wild-type mice, likely derived from T_H17 cells.

To further demonstrate the role of Bcl6 in T and B cells, we generated mixed bone marrow chimeras by transferring a mixture of congenic CD45.1⁺ wild-type and CD45.2⁺ Bcl6-deficient bone marrow cells into sublethally irradiated *Rag1*^{-/-} mice. Eight weeks after reconstitution, we immunized the mice with KLH. *Bcl6*^{-/-} CD4⁺ T cells did not mature into CXCR5⁺ T_{FH} cells that also express B and T lymphocyte attenuator (BTLA) (Fig. 4C), and did not exhibit germinal center B cells. CD4⁺CD44^{high} *Bcl6*^{-/-} cells exhibited greatly reduced T_{FH} gene expression; however, the expression of T_H1, T_H2, and T_H17 genes were all increased when compared with wild-type cells (fig. S10). Moreover, Bcl6-deficient B cells did not produce germinal center B cells. On the basis of the above data, we conclude that Bcl6 expression in both T and B cells is required for the germinal center reactions.

In summary, we have demonstrated that Bcl6 is regulated by IL-6 and IL-21 but that, similar to ROR γ t in T_H17 cells, it does not regulate the expression of IL-21. Bcl6 deficiency in vivo did not inhibit IL-21 expression, likely by T_H17 cells. Thus, IL-21 expression by non-T_{FH} cells is not sufficient to induce germinal center reactions. Our data overall indicate that Bcl6, selectively induced by IL-21 or IL-6 in the absence of TGF β signaling, serves as a master transcriptional factor in T_{FH} cell differentiation, analogous to ROR γ t and ROR α , both of which are induced by IL-6 or IL-21 in the presence of TGF β and function to promote T_H17 differentiation (fig. S11). IL-6 alone induces the expression of ROR γ t (15), whose function is inhibited by Bcl6, similar to regulatory T cells (T_{reg}), where the function of TGF β -induced ROR γ t is suppressed by Foxp3 (13,16). The combination of TGF β and IL-6 not only induces ROR γ t expression in T cells, but in the meantime, Foxp3 and Bcl6 expression is suppressed by IL-6 and TGF β signaling, respectively, allowing ROR γ t to function properly in promoting T_H17 cell differentiation.

Although we proposed in the past year that T_{FH} cell differentiation mediated by IL-6 and IL-21 is a novel lineage of T_H cell differentiation, subsequently several groups reported that CXCR5⁺ T cells also express T_H2 or T_H17 cytokines in vivo (17,18). Our current study indicates that Bcl6 promotes the expression of T_{FH}-related genes but inhibits the differentiation of T_H1, T_H2, and T_H17 cells. Our data thus support Bcl6-dependent T_{FH} cell generation as a pathway that is independent of other T_H cell lineages.

Supplementary Material

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Reference and Notes

1. Vinuesa CG, Tangye SG, Moser B, Mackay CR. Nat. Rev. Immunol 2005;5:853. [PubMed: 16261173]
2. Fazilleau N, Mark L, McHeyzer-Williams LJ, McHeyzer-Williams MG. Immunity 2009;30:324. [PubMed: 19303387]
3. King C, Tangye SG, Mackay CR. Annu. Rev. Immunol 2008;26:741. [PubMed: 18173374]
4. Nurieva RI, et al. Immunity 2008;29:138. [PubMed: 18599325]
5. Vogelzang A, et al. Immunity 2008;29:127. [PubMed: 18602282]
6. Dent AL, Shaffer AL, Yu X, Allman D, Staudt LM. Science 1997;276:589. [PubMed: 9110977]
7. Harris MB, et al. Mol. Cell. Biol 1999;19:7264. [PubMed: 10490661]
8. Ye BH, et al. Nat. Genet 1997;16:161. [PubMed: 9171827]
9. Klein U, Dalla-Favera R. Nat. Rev. Immunol 2008;8:22. [PubMed: 18097447]
10. Materials and methods are available as supporting material on *Science Online*

11. Mascle X, Albagli O, Lemerrier C. *Biochem. Biophys. Res. Commun* 2003;300:391. [PubMed: 12504096]
12. Yang XO, et al. *Immunity* 2008;28:29. [PubMed: 18164222]
13. Yang XO, et al. *Immunity* 2008;29:44. [PubMed: 18585065]
14. Ye BH, et al. *Nat. Genet* 1997;16:161. [PubMed: 9171827]
15. Ivanov II, et al. *Cell* 2006;126:1121. [PubMed: 16990136]
16. Zhou L, et al. *Nature* 2008;453:236. [PubMed: 18368049]
17. Bauquet AT, et al. *Nat. Immunol* 2009;10:167. [PubMed: 19098919]
18. Reinhardt RL, Liang H-E, Locksley RM. *Nat. Immunol* 2009;10:385. [PubMed: 19252490]
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Fig. 1.

Bcl6 regulates T_{FH} -specific gene expression. (A) Naïve T cells were activated with antibodies to CD3 and CD28, and with or without indicated cytokines for 1 or 2 days. Bcl6 mRNA expression was analyzed by real-time RT-PCR. The graph shows means $\pm$ SD. * $P < 0.005$; ** $P < 0.001$ when comparing IL-6 and IL-6 plus TGF β to nontreated sample, analysis of variance (ANOVA) test. ++ $P < 0.001$ when comparing IL-21 and IL-21 plus TGF β to nontreated sample, ANOVA test. (B) Naïve OT-II T cells were activated with antibodies to CD3 and CD28 alone or together with IL-6 or IL-21 and with antibodies to IL-4, IFN γ , and TGF β , and infected with a bicistronic retrovirus containing internal ribosomal entry site green fluorescent protein (GFP) expressing Bcl6 or a vector control

virus. mRNA expression of indicated genes was assessed by real-time RT-PCR. (C) Naïve OT-II T cells were activated with antibodies to CD3 and CD28 alone and infected with retroviruses expressing Bcl6, Bcl6 mutants (ZF3 and ZF5), or vector alone. GFP⁺ cells were sorted from (B) and (C) and restimulated for 4 hours with antibody to CD3. mRNA expression of indicated genes was analyzed by real-time RT-PCR. The data shown were normalized to the expression of a reference gene, *Actb*. The graph shows means $\pm$ SD. * $P < 0.005$; ** $P < 0.001$, t test. The data represent at least three independent experiments with consistent results.

Fig. 2.

Bcl6 suppresses TH17 and TH1 differentiation. (A and B) Naïve OT-II T cells were activated under TH17 conditions and infected with retroviruses expressing Bcl6, Bcl6 mutants, or vector alone. GFP⁺ cells sorted by fluorescence-activated cell sorting (FACS) were restimulated with antibody to CD3, and mRNA expression of indicated genes was analyzed by real-time RT-PCR (A). The data shown were normalized to the expression of reference gene *Actb*. The graph shows means $\pm$ SD. * P < 0.005; ** P < 0.001 when comparing Bcl6 and ZF3 to vector alone, ANOVA test. *** P < 0.001 when comparing Bcl6 and ZF5 to vector alone, ANOVA test. IL-17-expressing cells were measured by intracellular staining of the sorted GFP⁺ population (B). Numbers in dot-plot quadrants represent the percentages. (C)

EL-4 cells were transfected with a vector containing the firefly luciferase gene under the control of the IL-17 promoter and the CNS2 region, a vector expressing Renilla luciferase, and vectors expressing ROR γ t, Bcl6 wild-type, various Bcl6 mutants, or vector alone. The firefly luciferase activity was determined and normalized to Renilla luciferase. Values were also normalized to vector alone. The graph shows means $\pm$ SD. (**D** and **E**) Naïve OT-II T cells were activated under T_H1 conditions and infected with the indicated viruses. GFP⁺ cells were restimulated with antibody to CD3, and mRNA expression of the indicated genes was analyzed by real-time RT-PCR (D). The data shown were normalized to the expression of reference gene *Actb*. The graph shows means $\pm$ SD. * $P < 0.005$; ** $P < 0.001$, t test. IFN γ expression was analyzed by intracellular staining on FACS-sorted GFP⁺ cells (E). Numbers in dot-plot quadrants represent the percentages. The data represent at least three independent experiments with consistent results.

Fig. 3. Bcl6 deficiency causes defective T cell differentiation in vitro. Naïve CD4⁺ T cells from *Bcl6*^{-/-} mice and their littermate control mice carrying the OT-II TcR transgene were activated under T_{FH} (A), T_{H0} and T_{H1} (B), T_{H17} (C), and T_{reg} (TGF β or TGF β with antibodies to IL-4 and IFN γ) (D) conditions. Five days later, cells were assessed for IFN γ and IL-17 production [(A) to (C)] or Foxp3 expression (D) using intracellular staining. Numbers in FACS plot quadrants represent the percentages. mRNA expression of various genes was analyzed by real-time RT-PCR, and the data shown were normalized to the expression of reference gene β -actin. The graph shows means $\pm$ SD. *P* values were calculated using a *t* test comparing wild-type and *Bcl6*^{-/-} CD4⁺ T cells and are indicated as

follows: $*P < 0.005$; $**P < 0.001$. The experiments were repeated three times with consistent results.

Fig. 4.

Bcl6 is necessary for the generation of T_{FH} cells in vivo. (**A** and **B**) Naïve $CD4^{+}$ cells from wild-type (WT) or *Bcl6*^{-/-} mice were mixed with WT B cells and transferred into *Rag1*^{-/-} mice (3 mice per group). The recipient mice were immunized subcutaneously with KLH emulsified in Freund's complete adjuvant (FCA). In (**B**), recipient mice were also treated with antibody to IL-4. Seven days after the immunization, germinal center B cells were determined by staining with GL-7, FAS and B220 antibodies, and T_{FH} cells by CD4 and CXCR5 antibodies. Numbers in dot-plot quadrants represent the percentages in gated T and B cells, respectively. Spleen cells from immunized mice were stimulated with the indicated concentration of KLH. Effector cytokines were measured after 4 days of treatment. The

graph shows means $\pm$ SD. *P* values were calculated using a *t* test comparing CXCR5 expression on T cells or GL7/Fas expression on B cells between mice transferred with WT or Bcl6-deficient T and B cells, respectively, and are indicated as follows: **P* < 0.005; ***P* < 0.001. The results are representative of multiple mice (*n* = 6 per group) from two independent experiments with similar results. (C) Mixed-bone marrow chimeric mice (*n* = 15) were generated and immunized with KLH in FCA. Spleen cells were stained with antibody to CD45.2 to distinguish WT and Bcl6 knockout compartments and then analyzed for the germinal center B cells and T_{FH} cells. *P* values were calculated using a *t* test comparing CXCR5 and GL7/Fas expression between WT and Bcl6-deficient T and B cells, respectively, and are indicated as follows: **P* < 0.005; ***P* < 0.001. The results are a representative of multiple mice (*n* = 15) from three independent experiments with similar results.