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The Transcriptional Repressor Bcl-6 Directs T Follicular Helper Cell Lineage Commitment

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SUMMARY

Follicular helper T (Tfh) cells provide selection signals to germinal center B cells, which is essential for long-lived antibody responses. High CXCR5 and low CCR7 expression facilitates their homing to B cell follicles and distinguishes them from T helper 1 (Th1), Th2, and Th17 cells. Here, we showed that Bcl-6 directs Tfh cell differentiation: Bcl-6-deficient T cells failed to develop into Tfh cells and could not sustain germinal center responses, whereas forced expression of Bcl-6 in CD4$^+$ T cells promoted expression of the hallmark Tfh cell molecules CXCR5, CXCR4, and PD-1. Bcl-6 bound to the promoters of the Th1 and Th17 cell transcriptional regulators T-bet and RORγt and repressed IFN-γ and IL-17 production. Bcl-6 also repressed expression of many microRNAs (miRNAs) predicted to control the Tfh cell signature, including miR-17-92, which repressed CXCR5 expression. Thus, Bcl-6 positively directs Tfh cell differentiation, through combined repression of miRNAs and transcription factors.

INTRODUCTION

Naive T cells differentiate into distinct effector subsets, such as T helper 1 (Th1), Th2, and Th17 cells, producing specialized cytokines to provide protection from various types of pathogenic challenge (Ho and Glimcher, 2002; Murphy and Reiner, 2002; Reiner, 2007; Weaver et al., 2007) and expressing diverse chemokine receptors for homing to different tissue microenvironments (Bromley et al., 2008; Sallusto et al., 2000). T cells also provide help for B cell responses, and germinal centers (GCs) in lymphoid tissues serve as special sites where T cells select mutated high-affinity B cells and promote their differentiation to memory B cells and long-lived plasma cells during T cell-dependent antibody responses (Allen et al., 2007). Although the phenomenon of T cell help for B cells was described over 30 years ago, the nature of T helper cells for GC B cells has been controversial. The identification of the molecules responsible for follicular T cell-B cell interactions and colocalization, such as the costimulatory molecule CD40L (Casamayor-Palleja et al., 1995), ICOS (Hutloff et al., 1999), and the chemokine receptor CXCR5 (Ansel et al., 1999), has enabled the identification of a specialized CD4$^+$ helper cell subset that enters the follicles during T cell-dependent immune responses: follicular B helper T (Tfh) cells (Breitfeld et al., 2000; Kim et al., 2001; Schaerli et al., 2000). In both mice and humans, these cells are phenotypically distinct from other CD4$^+$ helper cell subsets (Chtanova et al., 2004; Kim et al., 2004; King et al., 2008; Vinuesa et al., 2005a; Vinuesa et al., 2005b). In particular, they express high amounts of CXCR5, which facilitates Tfh cell localization to follicles in which the ligand CXCL13 is expressed. The development of Tfh cells represents an important yet poorly understood step in T cell differentiation.

T cells differentiate to CD4$^+$ effector subsets through the actions of different transcription factors, such as T-bet for Th1 cell (Szabo et al., 2000), GATA-3 for Th2 cell (Zheng and Flavell, 1997), RORγt for Th17 cell (Ivanov et al., 2006), and FoxP3 for regulatory T (Treg) cell (Hori et al., 2003). The transcription factor responsible for Tfh differentiation, and the associated gene expression, remains undefined. Selective expression of the transcription factor Bcl-6 by human follicular T cells (Cattoretti et al., 1995; Chtanova et al., 2004) prompted us to hypothesize that Bcl-6 may regulate Tfh lineage commitment.

The Bcl-6 proto-oncogene encodes a POZ-zinc finger transcription factor that acts as a sequence-specific repressor of transcription through recruitment of a silencing mediator for retinoid and thyroid hormone receptors (SMRT) and histone deacetylase-containing complex (Dhordain et al., 1997). To date, Bcl-6 function has been mostly associated with B cell fate. Within the B lineage, Bcl-6 expression is largely confined to GC B cells (Allman et al., 1996; Cattoretti et al., 1995), and *Bcl6*$^{-/-}$ mice lack GCs and as a consequence display defective T cell-dependent antibody responses and demonstrate no antibody affinity maturation (Dent et al., 1997; Ye et al., 1997; Fukuda et al., 1997). Bcl-6 acts cell-autonomously in B cells to inhibit the terminal differentiation of GC B cells to plasma cells or memory cells, through the repression of *Prdm1*, the gene encoding Blimp-1 (Reljic et al., 2000; Shaffer et al., 2000). Bcl-6 also operates during T cell differentiation. For instance, Bcl-6 represses the Th2 cell transcription factor GATA-3 to inhibit Th2 cell differentiation (Kusam et al., 2003), controls long-term CD4$^+$ T cell memory (Ichii et al., 2007), and also regulates central memory CD8$^+$ T cell development (Ichii et al., 2002).

These findings suggest that the high expression of Bcl-6 found in Tfh cells might be important for Tfh cell lineage specification. However, one conundrum is the well-established repressor activity of Bcl-6. The transcriptional regulators of all the other T cell subsets positively promote transcription of their target genes, and in doing so specify cell fate. Here, we established the central role of Bcl-6 in Tfh cell lineage commitment and showed that Bcl-6 acted autonomously to promote Tfh cell differentiation and repress Th1 and Th17 cytokine production. Bcl-6 achieved this through direct repression of the transcription factors RORγt and T-bet together with repression of numerous microRNAs (miRNAs) including miR-17-92 that control the Tfh gene expression signature.

RESULTS

Bcl-6 Is Required for Tfh Cell Formation In Vivo

Bcl-6-deficient mice are unable to form GCs (Dent et al., 1997; Ye et al., 1997). In addition to its expression in GC B cells, Bcl-6 is also expressed in Tfh cells, which led us to ask whether Bcl-6 acts autonomously in T cells to induce Tfh cells. We generated mixed chimeras with fetal liver cells from *Bcl6*$^{+/+}$ CD45.1 and *Bcl6*$^{-/-}$ CD45.2 E16 embryos. A control group was generated with a mix of *Bcl6*$^{+/+}$ CD45.1 and *Bcl6*$^{+/+}$ CD45.2 fetal liver cells. Eight weeks after reconstitution, the recipient mice were immunized with sheep red blood cells (SRBCs). Control mice mounted robust GC responses after immunization (Figure 1A and Figure S1 available online) with comparable proportions of CD45.1- and CD45.2-derived GC B cells and Tfh cells. Analysis of GC formation in *Bcl6*$^{+/+}$ CD45.1 and *Bcl6*$^{-/-}$ CD45.2 mixed chimeras confirmed that *Bcl6*$^{-/-}$ B cells were unable to differentiate into GC cells (Figure 1A). Strikingly, *Bcl6*$^{-/-}$ T cells were also completely unable to differentiate into Tfh cells (Figure 1B), whereas *Bcl6*$^{+/+}$ T cells formed normal Tfh cell numbers in the same chimeric mice. To confirm the absence of *Bcl6*$^{-/-}$ T cells within GCs, we stained spleen sections from SRBC-immunized chimeric mice. *Bcl6*$^{+/+}$ CD45.2 CD4^{+} T cells could be identified within GCs (yellow cells in Figure 1C, right panel), but there was a complete absence of *Bcl6*$^{-/-}$ CD45.2 CD4^{+} T cells at these sites (Figure 1C, left panel). To assess a possible gene dose effect of Bcl-6 in Tfh cell differentiation, we performed a mixed bone marrow chimera experiment using a 1:1 mix of either *Bcl6*$^{+/+}$ CD45.1 and *Bcl6*$^{+/-}$ CD45.2 or control *Bcl6*$^{+/+}$ CD45.1 and *Bcl6*$^{+/+}$ CD45.2 bone marrow. Halving the gene dose of *Bcl6* resulted in 20%–30% reduction in the generation of Tfh cells ($p < 0.05$, Figure 1D). Together, these data indicate that Bcl-6 is essential for the generation of Tfh cells and that it functions in a gene dose-dependent manner.

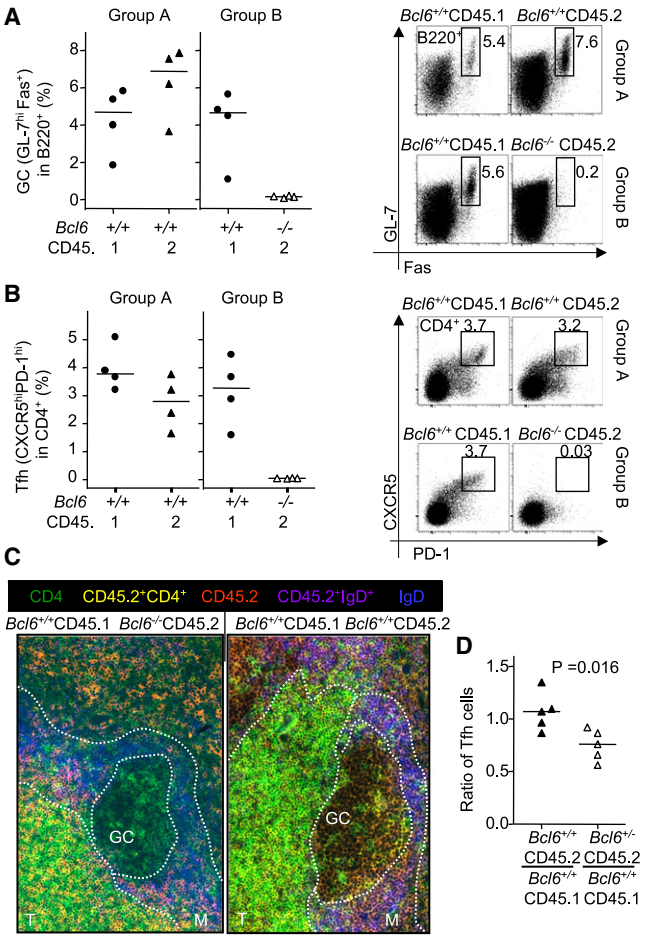

Figure 1. Bcl-6 Is Required for Tfh Cell Formation In Vivo

(A and B) Percentages of (A) GC cells and (B) Tfh cells 1 week after SRBC immunization of *Bcl6*$^{+/+}$ CD45.1: *Bcl6*$^{+/+}$ CD45.2 (group A) or *Bcl6*$^{+/+}$ CD45.1: *Bcl6*$^{-/-}$ CD45.2 fetal liver chimeric mice (group B).

(C) Photomicrographs of spleen sections from the same mice as in (A) and (B). The GC areas within IgD^{+} (blue) mantle zones have been delineated. *Bcl6*$^{+/+}$ CD45.2 T cells within GCs (right) appear yellow. *Bcl6*$^{-/-}$ Cd45.2 T cells (yellow) are absent from GCs (left), which only contain *Bcl6*$^{+/+}$ CD45.1 CD4^{+} T cells (green). T, T cell zone; M, mantle zone.

(D) Ratio of Tfh cells 1 week after SRBC immunization of *Bcl6*$^{+/+}$ CD45.1: *Bcl6*$^{+/+}$ CD45.2 (left) or *Bcl6*$^{+/+}$ CD45.1: *Bcl6*$^{+/-}$ CD45.*2* (right) mixed bone marrow chimera mice. The ratio was calculated by dividing the percentage of Tfh cells among CD45.2 T cells (*Bcl6*$^{+/+}$ or *Bcl6*$^{+/-}$) by the percentage of Tfh cells among CD45.1 T cells (*Bcl6*$^{+/+}$) in each individual mouse.

Each symbol represents one mouse. The lines are drawn through the median values in each group. p value was calculated by Student's t test.

Bcl-6-Deficient T Cells Fail to Support GC Reactions

We next examined whether deficiency of Bcl-6, specifically in T cells, results in a deficiency in GC formation. For these experiments, CD28-deficient fetal liver cells were chosen as a source for T cells unable to differentiate into Tfh cells (Linterman et al., 2009) and a source of B cells competent to differentiate into GC B cells. Sublethally irradiated *Rag1*$^{-/-}$ mice were reconstituted with a 1:1 mix of *Bcl6*$^{-/-}$ *Cd28*$^{+/+}$ CD45.2^{+} and *Bcl6*$^{+/+}$ *Cd28*$^{-/-}$ CD45.1 fetal liver cells (Figure 2, group A). In these chimeric mice, Tfh cells are not expected to form from *Cd28*$^{-/-}$ T cells or *Bcl6*$^{-/-}$ T cells; thus, if Bcl-6 in T cells is essential to sustain GC reactions, GCs derived from *Cd28*$^{-/-}$ B cells will not be supported. As a control, *Rag1*$^{-/-}$ mice were reconstituted with a 1:1 mix of *Bcl6*$^{+/+}$ *Cd28*$^{+/+}$ CD45.2 and *Bcl6*$^{+/+}$ *Cd28*$^{-/-}$ CD45.1 fetal liver cells (group B). In this group of mice, Tfh cells derived from the *Bcl6*$^{+/+}$ fetal liver cells are expected to support GCs derived from both *Cd28*$^{-/-}$ and *Cd28*$^{+/+}$ B cells. A parallel set of control chimeras consisted of a mix of *Bcl6*$^{-/-}$ *Cd28*$^{+/+}$ CD45.2 and *Bcl6*$^{+/+}$.*Cd28*$^{+/+}$ CD45.1 (group C) and a mix of *Bcl6*$^{+/+}$ *Cd28*$^{+/+}$ CD45.2 and *Bcl6*$^{+/+}$.*Cd28*$^{+/+}$ CD45.1

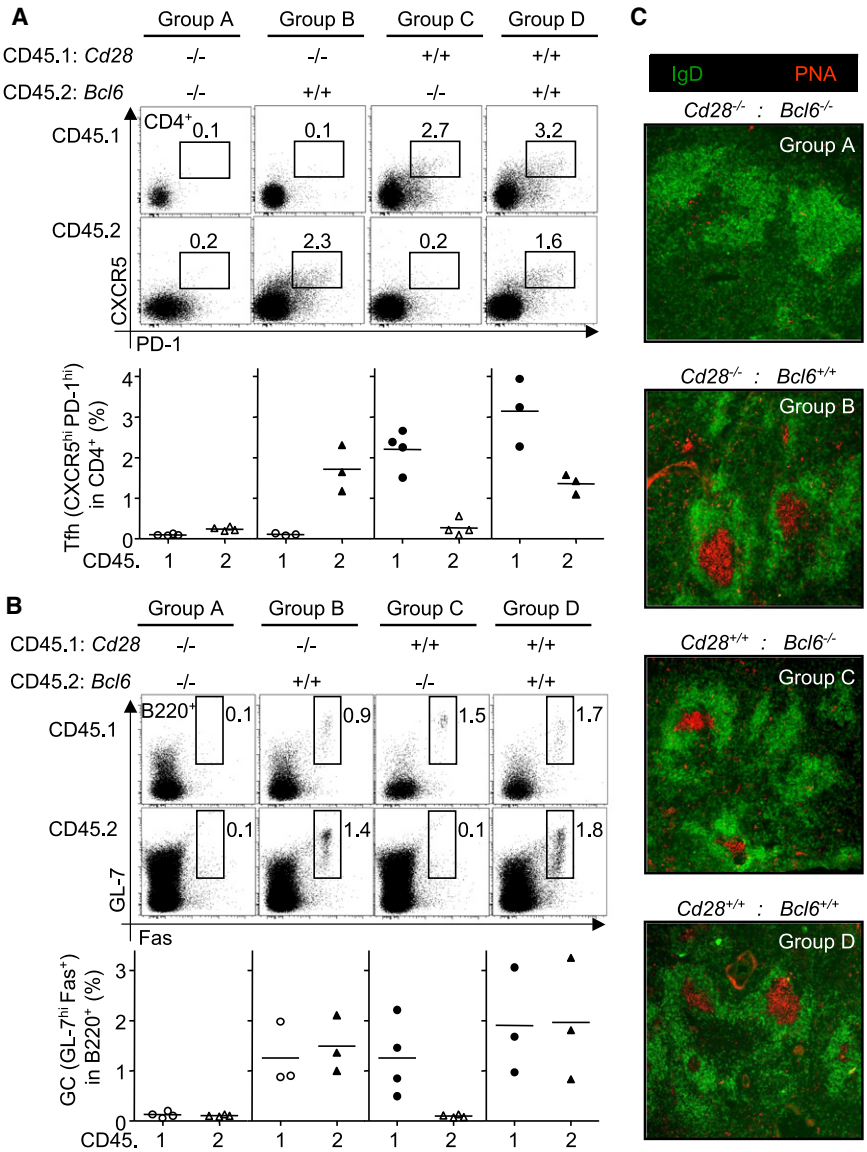

Figure 2. Bcl-6-Deficient T Cells Cannot Support GC Reaction

(A and B) Percentages of (A) Tfh cells and (B) GC cells among CD45.1 or (A) CD45.2 $CD4^+$ cells or (B) $B220^+$ cells 8 days after SRBC immunization of fetal liver chimeric mice reconstituted with the following 1:1 mixes: $Bcl6^{-/-}$ $Cd28^{+/+}$ CD45.2: $Bcl6^{+/+}Cd28^{-/-}$CD45.1 (group A), $Bcl6^{+/+}$ $Cd28^{+/+}$ CD45.2: $Bcl6^{+/+}$ $Cd28^{-/-}$ CD45.1 (group B), $Bcl6^{-/-}$ $Cd28^{+/+}$ CD45.2: $Bcl6^{+/+}$ $Cd28^{+/+}$ CD45.1 (group C), or $Bcl6^{+/+}$ $Cd28^{+/+}$ CD45.2: $Bcl6^{+/+}$ $Cd28^{+/+}$ CD45.1 (group D).

(C) Photomicrographs of spleen sections from the same mice as in (A) and (B). GC are PNA binding areas (red) within IgD^+ (green) mantle zones. Each symbol represents one mouse. The lines are drawn through the median values in each group. The p value was calculated by Student's t test.

(group D). The latter two groups of chimeric mice are constructed in the same way as those in Figure 1 and allowed us to rule out potential effects of halving the number of Tfh precursors, given that 50% of T cells in groups A and B were derived from $Cd28^{-/-}$ fetal liver. Eight weeks after reconstitution, mice were immunized with SRBCs and the percentage of Tfh cells and GC B cells derived from each type of donor fetal liver were determined by flow cytometry 8 days after immunization. In chimeras reconstituted with $Bcl6^{+/+}$ $Cd28^{-/-}$ CD45.1 and $Bcl6^{-/-}$ $Cd28^{+/+}$ CD45.2 cells (group A), no $CXCR5^{hi}$ $PD\text{-}1^{hi}$ Tfh cells could be identified in either the CD45.1 or CD45.2 compartment, consistent with the requirement for CD28 and Bcl-6 in T cells to induce Tfh cell formation. Strikingly, GC B cells derived from $Bcl6^{+/+}$ $Cd28^{-/-}$ CD45.1 fetal liver were virtually absent in these mice (Figures 2B and 2C, group A). By contrast, GC B cells derived from $Bcl6^{+/+}$ $Cd28^{-/-}$ CD45.1 fetal liver formed normally in mice reconstituted with a mix of $Bcl6^{+/+}$ $Cd28^{-/-}$ CD45.1 and $Bcl6^{+/+}$ $Cd28^{+/+}$ CD45.2 fetal liver cells (Figures 2B and 2C, group B), indicating that Bcl-6 expression in T cells is essential to support GCs. Immunofluorescent staining of frozen spleen sections from immunized chimeric mice confirmed abundant PNA^+ GCs in recipients of $Bcl6^{+/+}$ $Cd28^{-/-}$ CD45.1 and $Bcl6^{+/+}$ $Cd28^{+/+}$ CD45.2 fetal liver cells (group B), but a virtual absence of GC B cells in recipients of $Bcl6^{+/+}$ $Cd28^{-/-}$ CD45.1 and $Bcl6^{-/-}$ $Cd28^{+/+}$ CD45.2 fetal liver cells (group A, Figure 2C). Thus, Bcl-6-deficient T cells are functionally incapable of providing help to GC B cells.

Bcl-6 Is Not Required for Th1, Th17, and Treg Cell Differentiation and Cytokine Production

We next assessed whether the impaired $CD4^+$ T cell differentiation that occurred in the absence of Bcl-6 was specific for the Tfh cell subset or whether Bcl-6 deficiency affected other $CD4^+$ T cell effector subsets. Effector and memory ($CD25^-CD44^{hi}CD62L^{lo}$) $CD4^+$ T cells were quantified in the SRBC-immunized $Bcl6^{+/+}$.CD45.1 and $Bcl6^{-/-}$.CD45.2 fetal liver chimeric mice described in Figure 1, and $Bcl6^{-/-}$ $CD4^+$ T cells were found to differentiate normally into $CD44^{hi}$ effector populations (Figure S2A). The ratio of effector cells between CD45.2 and CD45.1 populations (within an individual mouse) was 30% lower in mice reconstituted with $Bcl6^{-/-}$.*Cd45.2* fetal liver (Figures S2B and S2C), partially because of a deficiency in Tfh cells and the reported deficiency in long-term memory cells (Ichii et al., 2007). Bcl-6 has been shown to repress rather than support Th2 cell differentiation (Dent et al., 1997; Ye et al., 1997). To investigate the effects of Bcl-6 deficiency on Th1 and Th17 cells, we measured their signature cytokines, IFN-γ and IL-17, in $CD4^+$ T cells from the chimeric mice 1 week after immunization with SRBCs. We normalized the percentages of IFN-γ- and IL-17-producing cells among $CD45.2^+$ cells to those of $CD45.1^+$ cells to investigate whether Bcl-6 acts

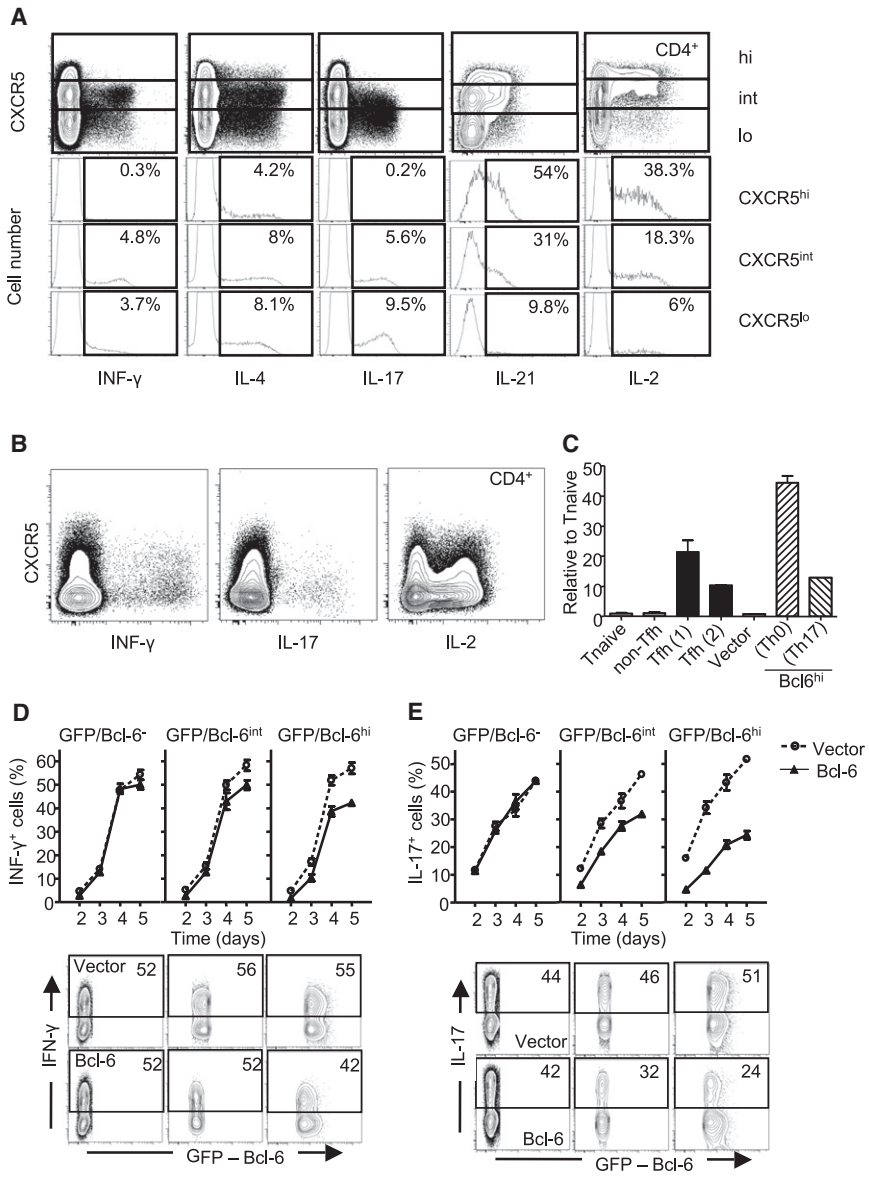

Figure 3. Bcl-6 Expression Suppresses Th1 and Th17 Cell Cytokine Production

(A) The upper panel shows cytokine production by tonsil human CD4$^+$ T cells (gates drawn around CXCR5^{hi}, CXCR5^{int} and CXCR5^{lo} populations). The lower panel presents histograms showing each cytokine expression in the three populations. Values are the percentages of cytokine-producing cells. Data are representative of more than three individual samples.

(B) Cytokine production by mouse splenic CD4$^+$ T cells 1 week after SRBC immunization. Data are representative of more than three experiments.

(C) *Bcl6* mRNA expression assessed by quantitative RT-PCR in mouse CD4$^+$ cell subsets (T naive, non-Tfh effectors, and Tfh cells), and sorted GFP^{hi} cells from CD4$^+$ T cells were transduced with either empty vector or Bcl-6 under Th0 or Th17 cell differentiation conditions. Data shown represent mean values ± SD, with n = 3.

(D and E) IFN-γ (D) and IL-17 (E) production by naive CD4$^+$ T cells transduced with either a Bcl-6-expressing retrovirus or a vector control and differentiated under Th1 (D) or Th17 (E) cell differentiation conditions for the times indicated. Transduced cells were divided into three groups—no (–), intermediate (int), and high (hi)—according to expression of GFP (Bcl-6). Day 5 is representative flow cytometry plots for each of the three groups are shown. Data shown represent mean values ± SD, with n = 3.

cell-autonomously to influence Th1 and Th17 cell differentiation. A near 3-fold increase in the ratio of IFN-γ-producing cells between CD45.2 and CD45.1 cells could be observed in mice reconstituted with *Bcl6*$^{-/-}$ CD45.2 fetal liver (Figure S3A, group B) compared with control chimeric mice (group A). A similar increase was observed in IL-17-producing cells (Figure S3B). IFN-γ and IL-17 production was also investigated among sorted effector CD25$^-$ CD44^{hi} CD62L^{lo} effector cells from the same chimeric mice restimulated ex vivo (sorting strategy shown in Figure S4A). A higher proportion of *Bcl6*$^{-/-}$ T cells produced IFN-γ compared with *Bcl6*$^{+/+}$ cells in the same mice (Figures S3C and S3D). The increase in the production of IL-17-producing cells was smaller (Figure S3D). As a complementary strategy, naive CD25$^-$CD44^{lo}CD62L^{hi} CD4$^+$ T cells were sorted and polarized ex vivo under Th1 or Th17 cell conditions for 3 days. Again, the proportion of *Bcl6*$^{-/-}$ CD4$^+$ T cells producing IFN-γ was higher than that of *Bcl6*$^{+/+}$ cells (Figure S3E) with a very modest increase in IL-17 (Figure S3F). In addition, there were no obvious differences between the percentages of Foxp3$^+$ Treg cells derived from *Bcl6*$^{-/-}$ or *Bcl6*$^{+/+}$ cells in chimeric mice, suggesting that Bcl-6 deficiency does not affect the generation of Treg cells (Figure S5). These results, together with work published by others on Bcl-6 in the context of Th2 cell responses, suggest that Bcl-6 specifically promotes Tfh cell differentiation and is not required for Th1, Th2, Th17, and Treg cell formation.

Forced Bcl-6 Expression Represses IFN-γ and IL-17 Production

In vivo and ex vivo data suggest that Bcl-6 expression induces a parallel specific suppression of Th1 and Th17 effectors. Because Tfh cells are the highest Bcl-6 expressers, we examined Th1, Th2, and Th17 cell cytokine production by Tfh cells. We chose to do this in human tonsils because of their enrichment in Th1, Th2, Th17, and Tfh cells. Tfh cells, expressing the highest amounts of CXCR5, produced minimal amounts of IFN-γ or IL-17 and low amounts of IL-4 compared to cells expressing intermediate or low amounts of CXCR5. By contrast, Tfh cells produced high amounts of IL-21 and IL-2 comparable to CXCR5^{int} cells (Figure 3A). These findings coincide with the cytokine production profiles in spleens from SRBCs immunized mice: compared to

CXCR5$^{lo\ or\ int}$ cells, CXCR5^{hi} Tfh cells produced the lowest amounts of IFN-γ and IL-17 but were still able to produce substantial amounts of IL-2 (Figure 3B).

We examined the effect of Bcl-6 overexpression on cells polarized to differentiate under Th1 or Th17 cell conditions. Sorted naive CD4$^+$ T cells were transduced by a Bcl-6-expressing retrovirus or a control vector. To assess Bcl-6 overexpression, we quantified Bcl-6 mRNA in transduced cells cultured in Th0 or Th17 cell conditions and found it to be 44.4 and 12.9-fold higher in cells overexpressing high levels of Bcl-6 compared to cells transduced with the empty vector, respectively (Figure 3C). This expression was comparable to that observed in Tfh cells (Figure 3C), which expressed 10- to 20-fold higher amounts of *Bcl6* mRNA than naive or non-Tfh effector (CD44^{hi} CXCR5$^{lo\ or\ int}$ PD-1$^{lo\ or\ int}$, Figure S4C) cells.

Transduced cells were cultured under either Th1 or Th17 cell-inducing conditions. IFN-γ- and IL-17-secreting cells were measured after 2, 3, 4, and 5 days. Transduced cells were divided into Bcl-6^{hi}, Bcl-6^{int}, and Bcl-6$^-$ groups according to their expression of GFP from the bicistronic vector. Overexpression of Bcl-6 suppressed Th1 and Th17 cell differentiation in a dose-dependent manner: on day 5, high amounts of Bcl-6 expression, compared to empty vector, led to a 25% decrease in IFN-γ-producing cells (Figure 3D) and a 50% decrease in IL-17-producing cells (Figure 3E). Similar suppression of Th1 and Th17 cytokines could be seen even when the cells had been polarized for 2 days toward those lineages ex vivo, before being transduced with Bcl-6 (Figure S6). We also observed that despite strong Bcl-6-mediated suppression of IL-17, IL-2, a cytokine produced by all helper T cells, was produced at much higher amounts by the same Bcl-6^{hi} cells (Figure S7), indicating that Bcl-6 specifically repress IFN-γ and IL-17 production rather than causing a general suppression of cytokine production. In summary, Bcl-6 expression can antagonize IFN-γ and IL-17 production even in committed Th1 and Th17 cells, respectively, and raises the possibility that in vivo, Bcl-6 may direct Tfh cell differentiation from other helper T cell populations.

Bcl-6 Targets the Transcription Factors for Th1 and Th17 Cell Differentiation

In B cells, Bcl-6 determines GC B cell lineage commitment by repressing its target genes, including the transcriptional regulator of plasma cells, Blimp-1 (Reljic et al., 2000; Shaffer et al., 2000). In CD4$^+$ cells, Bcl-6 has been shown to repress GATA-3 expression (Kusam et al., 2003). Given our findings that Bcl-6 overexpression represses Th1 and Th17 cytokine production, we hypothesized that Bcl-6 might also repress the transcription factors directing Th1 and Th17 cell cytokine production. Bioinformatic analysis revealed the presence of high-affinity Bcl-6 binding sites within both mouse and human promoter regions of critical transcription factors required for Th1 (T-bet) and Th17 (RORγt) cell differentiation (Figure 4A and Table S1). To validate the predicted binding of Bcl-6 to these promoter regions, we performed Bcl-6 chromatin immunoprecipitation (ChIP) assays on human Tfh cells and B cells sorted from tonsils (Figure S5B) by using a high-quality ChIP grade Bcl-6 antibody (Parekh et al., 2007). Importantly, no promoter enrichment by Bcl-6 binding was detected in the B cell population (Figure 4B). Marked Bcl-6 enrichment with ChIP ratios of 80 and 180 on the T-bet and RORγt promoters, respectively, was seen in Tfh cells, but no specific enrichment was visible in tonsillar B cells, which are enriched for Bcl-6$^+$ cells. ChIP enrichment ratios greater than 2 are considered to be substantial binding above background (Pokholok et al., 2006); hence, the enrichment values observed across the *Tbx21* (encoding T-bet) and *Rorc* (encoding RORγt) promoters are highly relevant.

The results above suggest that *Tbx21* and *Rorc* mRNA might be reduced in Tfh cells (CXCR5^{hi}), which express the highest amounts of Bcl-6, compared with non-Tfh effectors (CXCR5^{int}). This was confirmed by quantitative RT-PCR on human tonsillar T cell subsets (Figure 4C).

Next, we quantified the effects of Bcl-6 overexpression on T-bet and GATA-3 protein expression. Naive mouse CD4$^+$ T cells, differentiated ex vivo under Th1 cell polarizing conditions and transduced by a Bcl-6-expressing retrovirus, displayed ~50% reduction in T-bet expression and ~20% of the transduced cells became T-bet negative (Figure 4D). Similarly, with Th2 cell polarization, GATA-3 expression was reduced by ~60% in the Bcl-6 overexpressing cells and ~30% of the cells became GATA-3 negative (Figure S9).

Finally, we investigated T-bet and GATA-3 protein expression on naive, Tfh, and non-Tfh effector cells that were Bcl-6 sufficient or Bcl-6 deficient, by using CD45 congenic fetal liver chimeric mice as shown in Figure 1. T-bet and GATA-3 were upregulated in non-Tfh effector (CD44^{hi}CXCR5$^{int\ or\ lo}$PD-1$^{int\ or\ lo}$) CD4$^+$ cells in both Bcl-6-sufficient and Bcl-6-deficient mice, compared with T naive (CD44^{lo}) cells (Figure 4E and Figure S9B). T-bet expression in Tfh cells was nearly as low as that in naive T cells, whereas GATA-3 was still expressed in Tfh cells. Interestingly, in the absence of Bcl-6, T-bet expression was higher in non-Tfh effector cells (Figure 4E), consistent with the increased IFN-γ expression shown in Figure S3.

Increased Expression of Bcl-6 Promotes the Tfh Cell Gene Expression Signature

Having shown that Bcl-6 is essential for Tfh cell formation and turning down Th1, Th2, and Th17 cell cytokine profiles, we next examined whether Bcl-6 expression could also actively induce the characteristic gene expression signature of Tfh cells (Chtanova et al., 2004; Kim et al., 2004; Vinuesa et al., 2005a). Upregulation of CXCR5 and downregulation of CCR7 on Tfh cells determine their follicular positioning and thus are important for their function (Hardtke et al., 2005; Haynes et al., 2007). CXCR4, the receptor for follicular stromal cell-derived chemokine CXCL12, is also upregulated on Tfh cells and may assist in their localization, proximal to the follicular dendritic cell network (Estes et al., 2004). The T cell costimulatory receptors ICOS and PD-1 are expressed at the highest levels on Tfh cells (Haynes et al., 2007; Hutloff et al., 1999).

We first confirmed selective high CXCR4, CCR7, PD-1, and ICOS expression in human and mouse Tfh cells compared with other effector CD4$^+$ T populations. In resected human tonsils, most CD4$^+$ T cells are antigen experienced and therefore do not express CD45RA (Figure 5A). Among these activated cells, Tfh cells identified by the highest expression of CXCR5 also displayed the highest expression of PD-1, ICOS, and CXCR4 and the lowest expression of CCR7 (Figure 5A). In mice, robust Tfh cell responses were generated upon transfer of OVA-specific

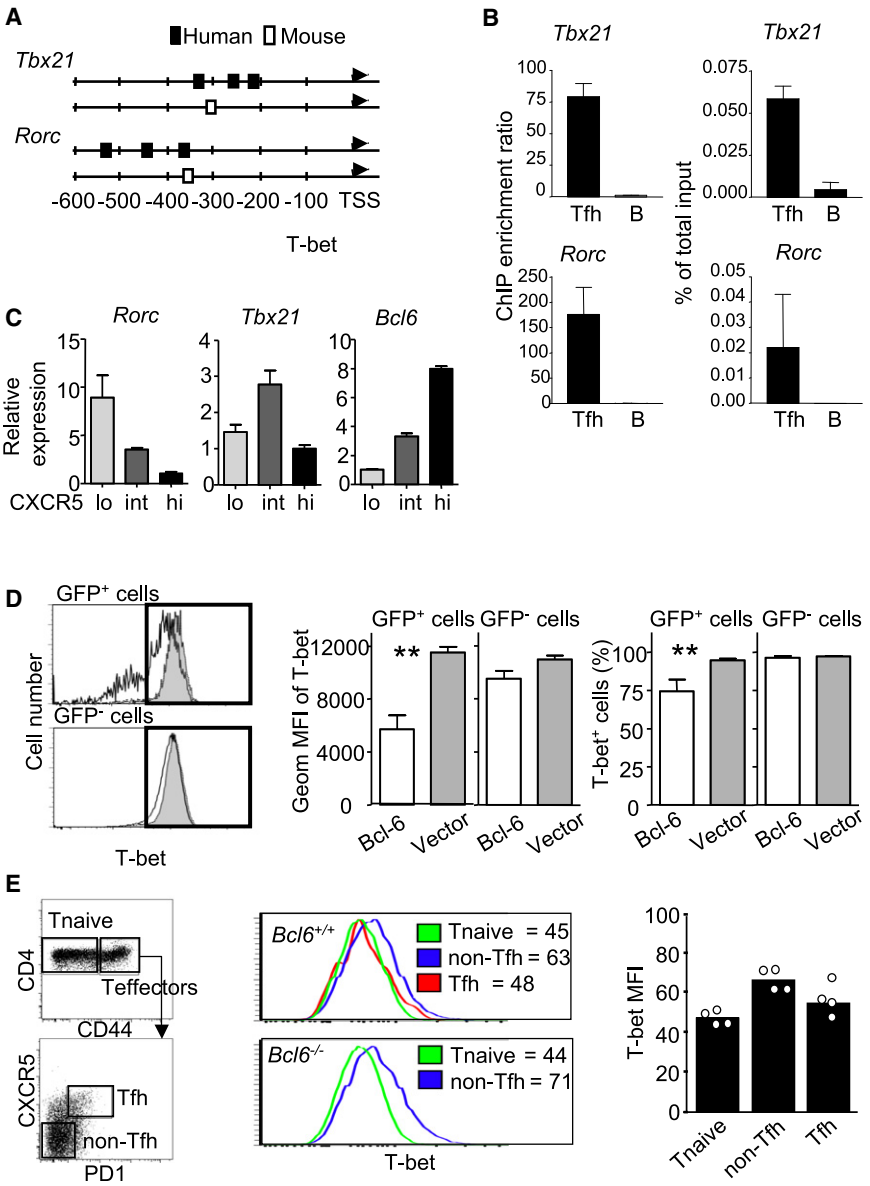

Figure 4. Bcl-6 Suppresses Transcription Factors for Th1, Th2, and Th17 Cell Differentiation

(A) Bcl-6 binding sites as predicted by MatInspector (Genomatix Software) within −600 bp to −100 bp, relative to the transcription start sites (TSS), of promoter regions in humans and mice. Detailed information listed in Table S1.

(B) Binding of Bcl-6 to putative high-affinity binding sites in the promoters regions. Bcl-6 ChIP assays were performed on human tonsil Tfh cells and B cells. The results are presented as either "ChIP enrichment ratio" or "percentage of total (genomic) input" and represent the mean of duplicate assays from two independent experiments, with error bars being SD.

(C) The mRNA expression of indicated genes assessed by quantitative real-time RT-PCR in sorted human tonsil $CD4^+$ T cell subsets as shown in Figure 3A ($CXCR5^{lo}$, $CXCR5^{int}$, and $CXCR5^{hi}$). Data shown represent mean values ± SD, with n = 3.

(D) Intracellular T-bet expression in sorted mouse naive $CD4^+$ T cells transduced with either a Bcl-6-expressing retrovirus or a vector control and cultured in Th1 cell differentiation conditions for 2 days (left panels). Bar graphs show geometric mean fluorescent intensity (Geom MFI) and positive percentages in transduced (GFP^+) and non-transduced (GFP^-) cells (right panels). Data shown represent mean values ± SD, with n = 3. p values were calculated by Student's t test.

(E) Representative flow cytometric profiles showing CD4 versus CD44 expression and CXCR5 versus PD-1 from $Bcl6^{+/+}$ CD45.1: $Bcl6^{-/-}$ CD45.2 mixed chimeric mice (described in Figure 1), used to gate T naive (Tn), Tfh, and non-Tfh effectors (left panel). The histograms (middle panel) show T-bet expression in $Bcl6^{+/+}$ (top panel) and $Bcl6^{-/-}$ (bottom panel) $CD4^+$ T cells from the chimeric mice; numbers indicate mean fluorescence intensity in each population. The graph (right panel) shows T-bet MFI in $Bcl6^{+/+}$ T_N, Tfh, and non-Tfh effectors from four individual mice.

Thy1.1 OT-II cells into Thy1.2 recipient mice and immunization with OVA in alum (Figure 5B). One week after immunization, more than 75% of the transferred $Thy1.1^+$ cells had become effectors, expressing high CD44 (Figure 5B). As observed in human Tfh cells, mouse Tfh cells—expressing the highest amounts of CXCR5—also expressed the highest amounts of PD-1 and CXCR4 and the lowest CCR7 (Figure 5B). ICOS expression was high in all transferred OT-II cells, regardless of CXCR5 expression (Figure 5B); this concurs with the report that ICOS can be expressed at high amounts by other effector T cell subsets (Lohning et al., 2003).

To test whether high Bcl-6 expression could induce the $PD\text{-}1^{hi}$ $CXCR5^{hi}$ $CXCR4^{hi}$ and $CCR7^{lo}$ Tfh cell signature, we transduced sorted mouse naive $CD4^+$ T cells with a retrovirus expressing Bcl-6 or empty vector and cultured them with anti-CD3 and anti-CD28 under Th0 (no cytokines or antibodies), Thn (anti-IFN-γ and anti-IL-4), Th1, Th2, and Th17 cell conditions for 2 days. Given our previous observation that Bcl-6 functions in a gene dose-dependent manner for both Tfh cell development (Figure 1D) and suppression of other helper T cell lineages (Figures 3D and 3E; Figures S6 and S7), transduced cells were again divided according to GFP-Bcl-6 expression into high and intermediate expressers. Even though PD-1 is normally upregulated after T cell stimulation, Bcl-6 overexpression further increased the expression of PD-1 in all culture conditions (Figure 5C and Figure S10). Similarly, Bcl-6 enhanced the expression of CXCR5 and CXCR4 by 2- to 3-fold across all conditions. Downregulation of CCR7 by Bcl-6 was also seen under Th17 cell differentiation conditions (Figure 5C and Figure S10). Thus, Bcl-6 can induce upregulation of PD-1 and the chemokine receptors required for follicular homing, in both unpolarized T cells, and in T cells differentiating under Th1, Th2, and Th17 cell conditions. These findings, together with the repression of Th1 and Th17 cell cytokines in polarized Th1 and Th17 cells

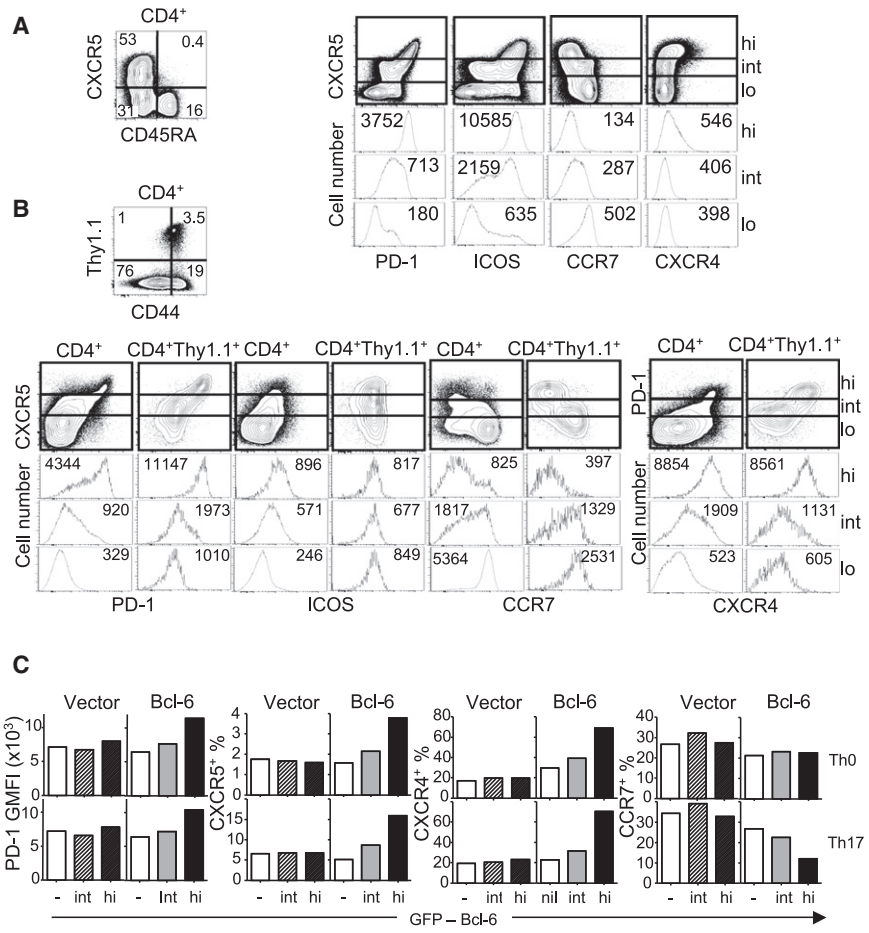

Figure 5. Bcl-6 Promotes the Tfh Cell Gene Expression Signature

(A) CXCR5 versus CD45RA expression by purified human tonsillar CD4$^+$ T cells (left). Expression (Geom MFI) of PD-1, ICOS, CCR7, and CXCR4 analyzed in CXCR5^{hi}, CXCR5^{int}, and CXCR5^{lo} populations (right). Data are representative from more than three individual samples.

(B) Thy1.1 versus CD44 expression on splenic CD4$^+$ T cells from C57BL/6 mouse transferred with Thy1.1$^+$ OT-II cells and immunized with OVA-alum (Hutloff et al., 1999). Expression (Geom MFI) of PD-1, ICOS, CCR7, and CXCR4 analyzed in CXCR5^{hi}, CXCR5^{int}, and CXCR5^{lo} populations is shown. Data are representative of more than three experiments.

(C) PD-1, CXCR5, CXCR4, and CCR7 expression on sorted mouse naive CD4$^+$ T cells transduced by either a Bcl-6-expressing retrovirus or a vector control and cultured under Th0 or Th17 cell differentiation conditions for 2 days.

Numbers beside the histograms in (A) and (B) represent Geom MFI values of PD-1 or percentages of chemokine receptors (CXCR5, CXCR4, and CCR7) on cells expressing no, intermediate, or high GFP (Bcl-6), respectively. Data are representative of two experiments.

shown above, suggest Bcl-6 expression may enable all types of T helper cells—including Th1 and Th17 effectors—to enter the follicles, change their cytokine production pattern, and become bona fide Tfh cells.

Bcl-6 Widely Represses miRNA Expression

To understand the mechanism by which Bcl-6, a transcription repressor, determines the Tfh cell signature, we performed microarray analyses to compare the gene expression profiles between naive CD4$^+$ T cells retrovirally transduced with Bcl-6 or an empty vector. In addition to increased CXCR5, CXCR4, and PD-1 mRNA and decreased CCR7 mRNA, intriguingly, a large number of primary miRNA transcripts were downregulated in cells overexpressing Bcl-6 (data not shown). miRNAs are ~23 nucleotide endogenous single-stranded noncoding RNAs that pair to mRNA mainly through the 3′ untranslated region (3′ UTR) to direct the posttranscriptional repression of these mRNAs by translational inhibition and/or mRNA decay (Baek et al., 2008). These results suggested that Bcl-6 might promote the Tfh gene expression signature by repressing the miRNAs that normally suppress these genes.

We used microarrays to profile mature miRNA expression in mouse CD4$^+$ T cells transduced with retroviruses expressing Bcl-6 or an empty vector. Bcl-6 overexpression caused more than 50% downregulation of 34 miRNAs out of the 65 that were abundantly expressed (i.e. with raw values >100, Table S2). There was not a single miRNA upregulated by more than 2-fold (Table S3). Of the 34, 31 were transcribed from clusters encoding other miRNA(s) that were also downregulated (Figure 6 and Table S2). In control, nontransduced cells (sorted GFP$^-$ cells from the same cultures), there was only one miRNA with raw values > 100 regulated by more than 2-fold (Figure S11). This global repression of miRNAs by Bcl-6 provides a putative mechanism by which Bcl-6 may induce the Tfh gene expression signature. This idea was further supported by a bioinformatic prediction of the multiple target sites within the 3′ UTRs of CXCR5, CXCR4, and PD-1 for those miRNAs markedly downregulated by Bcl-6 (Figure S12). Although 25% of these miRNAs are encoded from transcripts contained within protein-encoding transcripts, the majority are located in intergenic regions or within primary transcripts that are nonprotein coding (Table S4), arguing against the possibility that the observed downregulation is not specific for miRNAs but a consequence of Bcl-6 repressing other target genes. We have previously reported that two miRNAs, miR-101 and miR-103, repressed the Tfh cell feature molecule ICOS (Yu et al., 2007). Both miR-101 and miR-103 were downregulated in cells overexpressing Bcl-6 (Table S3, miR-101 was expressed at low levels and therefore not included).

The expression of miR-101 and miR-103 was confirmed to be downregulated by more than 50% in cells transduced with Bcl-6-expressing retroviral vector and also downregulated in Tfh cells by quantitative RT-PCR (Figure S13). Together, these data suggest Bcl-6 may promote Tfh cell differentiation through the repression of multiple miRNAs that normally prevent effector T cells from expressing maximal amounts of CXCR5, CXCR4, PD-1, and other Tfh cell signature molecules.

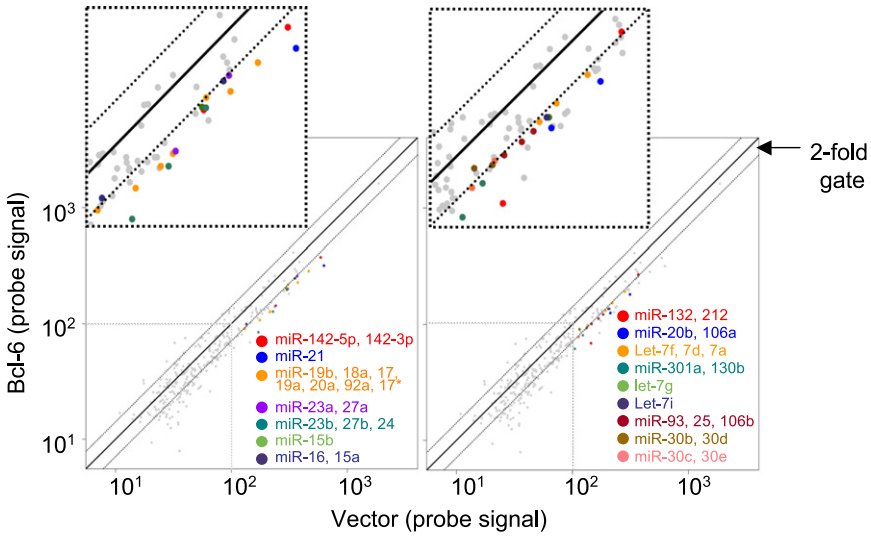

Figure 6. Bcl-6 Suppresses Expression of Multiple miRNAs

Comparison of miRNA probe-expression values in Bcl-6 transduced cells versus vector-transduced cells analyzed with Agilent miRNA microarrays. The probe values are the average of duplicate assays.

The miRNA Cluster 17-92 Represses CXCR5 Expression

In addition to the upregulation of CXCR5 by Bcl-6 overexpression (Figure 5C and Figure S10), a fundamental role for Bcl-6 in inducing upregulation of CXCR5 was seen in vivo. Indeed, $CXCR5^{int}$ and $CXCR5^{hi}$ $CD4^+$ T cells were virtually absent in the *Bcl6*$^{-/-}$ $CD4^+$ T cell population from mixed *Bcl6*$^{-/-}$ *Bcl6*$^{+/+}$ fetal liver chimeras (Figures 7A and 7B). Bcl-6 deficiency also reduced the levels of CXCR5 on B cells (Figures 7A and 7B, bottom panels), suggesting an important role for Bcl-6 in regulating CXCR5 on all lymphocytes.

Because of the important role of CXCR5 in the differentiation and function of Tfh cells, we examined the role of miRNAs in the regulation of CXCR5 expression. Many Bcl-6-downregulated miRNAs are predicted to target CXCR5 (Figure 7C). To confirm these findings, we performed quantitative real-time RT-PCR for several miRNAs in mouse $CD4^+$ cells overexpressing Bcl-6 or empty vector. A 71%, 70%, and 78% downregulation for miR-17, miR-18, and miR-20a, respectively, was observed in cells overexpressing Bcl-6 compared with cells expressing empty vector (Figure 7D). miR-17 and miR-20a were also downregulated in Tfh cells compared to naive T cells (Figure 7E).

Given the 3-fold increase in $CXCR5^+$ cells observed in Bcl-6-overexpressing $CD4^+$ cells (Figure 5C), we tested whether the downregulation of miRNAs, regulated by Bcl-6 and predicted to target CXCR5, could contribute to this change in CXCR5 expression. Three miRNAs in the miR17-92 cluster are predicted to target CXCR5: miR-17, miR-18a, and miR-20a (Figure 7C). All of these individual miRNAs were found to be downregulated upon Bcl-6 overexpression (Figure 6). We used a retroviral vector expressing the primary miRNA encoding the miR17-92 cluster to transduce mouse B cells. B cells were chosen because (1) they have high basal CXCR5 expression, which allows detection of downregulation (the low and transient levels of detectable CXCR5 expression on mouse T cells in culture and the proapoptotic nature of Tfh cells make these cells unsuitable for assays to asses downregulation and for retroviral transduction) and (2) we have shown that Bcl-6 also regulates CXCR5 expression in B cells (Figures 7A and 7B). Overexpression of miR-17-92 caused more than a 50% decrease in endogenous CXCR5 protein expression compared with cells transduced with empty vector or untransduced (GFP^- cells) in the same cultures (Figure 7F). As a control, no change in CXCR4 expression was observed in cells transduced with this miRNA cluster (Figure S14), consistent with the predicted absence of target sites for these miRNAs within the *Cxr4* 3′ UTR mRNA. Thus, the miR-17-92 cluster, which is repressed by Bcl-6, inhibits CXCR5 expression, suggesting that "repression of the repressors" may be a mechanism by which Bcl-6 positively induces Tfh cell differentiation.

DISCUSSION

The identification and characterization of Tfh cells as a T effector subset distinct from Th1, Th2, and Th17 cells represents an important new advance for understanding T cell-dependent antibody responses (King et al., 2008; Vinuesa et al., 2005b). Previous studies by us and others showed that Bcl-6 was selectively expressed by Tfh cells. Here, we established that Bcl-6 is essential for Tfh cell formation. Indeed, Bcl-6 specifies Tfh cell lineage commitment and achieves this by turning on a widespread gene repressor program by (1) directly binding to key transcriptional regulators of other T helper cell lineages, namely T-bet (Th1) and RORγt (Th17), and (2) repressing transcription of a large number of miRNAs, an effect that probably contributes to the upregulation of proteins associated with the Tfh cell phenotype. This combined strategy represents a powerful mechanism by which a single transcriptional repressor can "positively" specify a cellular phenotype and serve as a regulator for the development of an important lineage of effector T cells.

It is unclear whether Bcl-6 determines $CD4^+$ Tfh cell lineage commitment at the time of T cell priming, or reprograms pre-existing Th cell subsets at later stages during an immune response to become Tfh cells. Our finding that Bcl-6 overexpression can induce a Tfh cell phenotype not only under nonpolarizing conditions but also under Th1, Th2, and Th17 cell polarizing conditions suggests that Tfh cell lineage commitment may occur from developing T helper $CD4^+$ cells. We also showed that Bcl-6 suppressed IFN-γ and IL-17 secretion in cells prepolarized under Th1 and Th17 cell conditions. Moreover, Bcl-6 binds to the promoter regions of the *Tbx21* and *Rorc* genes in Tfh cells, suggesting that Bcl-6 is able to reprogram Th1 and Th17 cells. Indeed, few IFN-γ-producing cells (compared with abundant $IL\text{-}4^+$ cells) were identified inside GCs in a recent study (Reinhardt et al., 2009); most were found outside peanut agglutinin-binding areas. Also, there is evidence that accumulation of

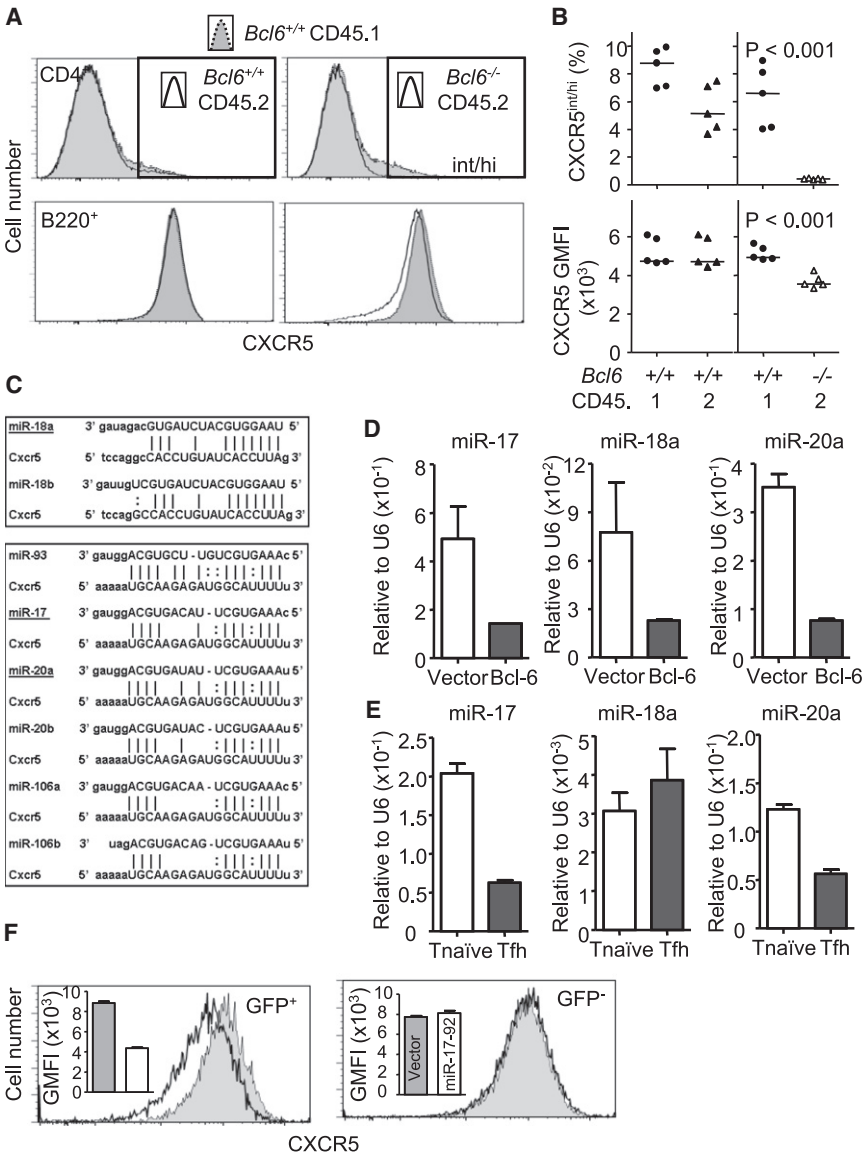

Figure 7. The miRNA Cluster 17-92 Represses CXCR5 Expression

(A) CXCR5 expression on splenic CD4$^+$ (Hutloff et al., 1999) and B220$^+$ cells (bottom) from *Bcl6*$^{+/+}$ CD45.1 : *Bcl6*$^{+/+}$ CD45.2 or *Bcl6*$^{+/+}$ CD45.1 : *Bcl6*$^{-/-}$ CD45.2 fetal liver chimeras 1 week after SRBC immunization (left).

(B) Graphs showing the percentage of CXCR5$^{int\ or\ hi}$ CD4$^+$ cells (Hutloff et al., 1999) or the Geo MFI of CXCR5 on B220$^+$ cells (bottom) among CD45.1 or CD45.2 cells from the same mice. Each symbol represents one mouse and the lines are drawn through the median values in each group. The p values were calculated by Student's t test.

(C) Predicted binding sites within the 3′ untranslated region (3′ UTR) of mouse CXCR5 by miRNAs downregulated over 50% by Bcl-6 overexpression (Table S2). Underlined miRNAs are expressed within the miR-17-92 cluster.

(D and E) Mature miR-17, mir-18a, and miR-20 expression quantified by real-time RT-PCR relative to U6 small nuclear RNA (U6) in mouse CD4$^+$ cells transduced with empty vector or Bcl-6 (top panel) and in mouse splenic T naive versus Tfh CD4$^+$ cells (bottom panel). Data shown represent mean values ± SD, with n = 3.

(F) Histograms showing CXCR5 fluorescence on mouse B220$^+$ cells transduced with retrovirus expressing empty vector (filled histogram) or miR-17-92 (empty histogram). The bar graphs inside the histograms show CXCR5 geometric mean fluorescence intensity in the same cells. Data shown represent mean values ± SD, with n = 3.

IL-17-producing cells in GCs is associated with autoimmunity in BXD2 mice (Hsu et al., 2008). This may explain how long-lived antibody-producing cells are generated against typical Th1, Th2, and Th17 cell-type pathogens while minimizing the potential damaging effects of excessive IFN-γ and IL-17 production in GCs. This is conceivable, in light of recent work suggesting there may be much more plasticity during T cell differentiation than was previously appreciated (Zhou et al., 2009; Tsuji et al., 2009).

Restricted expression of inflammatory cytokines by Tfh cells in GCs serves to regulate appropriate isotype switching. B cell switching to aberrant isotypes in the context of GC reactions poses a greater threat than during extrafollicular responses because the latter produce comparably low-affinity antibodies (MacLennan et al., 2003), and also the offspring of GC B cells may live for decades in an individual. Thus, Tfh cells turn down IFN-γ but do produce IL-21, which serves as a potent B cell stimulator, and favors switching to IgG1while repressing IgE (Ozaki et al., 2002). Recent data has shown that IL-21 is regulated by c-Maf, a transcription factor also expressed by Th2, Th17, and Tfh cells (Bauquet et al., 2009; Pot et al., 2009). It is still not clear whether IL-21 is also regulated by Bcl-6.

Bcl-6 also profoundly modifies chemokine receptor expression. Upon priming, CXCR5 is induced, and further upregulation of CXCR5 in addition to downregulation of CCR7 are required for the migration of CD4$^+$ T cells into follicles to become Tfh cells (Hardtke et al., 2005; Haynes et al., 2007). Bcl-6 enhanced the expression of CXCR5 and decreased CCR7 expression in activated T cells, whereas *Bcl6*$^{-/-}$ T cells were unable to upregulate CXCR5 in vivo. Interestingly, *Bcl6*$^{-/-}$ B cells also displayed lower expression of CXCR5, suggesting that Bcl-6 regulates CXCR5 in both B cells and T cells through a common mechanism, consistent with our results showing the Bcl-6-regulated miR-17-92 family reduces CXCR5 expression in mouse B cells. CXCR4 expression was also enhanced by Bcl-6. Deficiency of CXCR5 on T cells severely impairs but doesn't totally abolish their participation in the GC reaction (Arnold et al., 2007), suggesting synergetic control of multiple chemokine receptors by Bcl-6 regulates the migration of Tfh cells to the follicles.

Interestingly, enhanced Bcl-6 expression did not increase ICOS or CD40L expression (data not shown). This was not

entirely unexpected because high expression of ICOS and CD40L can also be found on extrafollicular helper T cells (Odegard et al., 2008) and other T helper cell lineages (Lohning et al., 2003). In vivo, we did not observe any apparent decrease in ICOS expression in *Bcl6*$^{-/-}$ CD4^{+} T cells (data not shown), suggesting that ICOS expression may be mainly controlled by other transcriptional regulators, cytokines, or signals from the microenvironment.

A key aspect of Bcl-6-mediated gene regulation that has emerged in this study is its ability to downregulate more than half of abundantly expressed miRNAs by 50% or more in activated T cells. Most miRNAs downregulated by Bcl-6 (31 of 34) belonged to clusters whose members are transcribed from a single transcript and therefore are coregulated. Several miRNAs within the same cluster frequently recognize similar seed sequences with 3′ UTRs and thus target the same mRNAs, which explains why a mere 50% downregulation in the amounts of individual mature miRNAs can result in substantial increases in the expression of target genes. Remarkably, not a single miRNA was upregulated (>2-fold) by Bcl-6. miRNAs are emerging as key regulators of cell fate by fine-tuning gene expression (Baek et al., 2008; Lodish et al., 2008). Tfh cells are unique among T helper cell subsets in that, despite having a very distinct cell function compared to other effector T cells, they have a phenotype that is only distinguished from non-Tfh effectors by a quantitative increase in the expression of CXCR5, CXCR4, PD-1, ICOS, IL-21 and other molecules (King et al., 2008; Vinuesa et al., 2005b). Seventeen out of thirty-four miRNAs repressed by Bcl-6 were potential regulators of these molecules. This is particularly important in light of recent findings demonstrating that miRNAs regulate proteins that typically function over a narrow range of concentrations during normal immune responses and adds to the evidence that miRNA-mediated increases or decreases in the expression of these proteins may lead to marked changes in cell function.

We found that overexpression of the miR-17-92 cluster comprising seven miRNAs that are all downregulated in Bcl-6-overexpressing cells leads to a reduction of CXCR5 in mouse primary cells. Given that at least three miRNAs in this cluster are predicted to target CXCR5, the net reduction effect of CXCR5 protein is likely to be a consequence of the cumulative effects of these different miRNAs. It is interesting to note that mice transgenic for miR-17-92 display abnormal compartmentalization and segregation of B cells and T cells in secondary lymphoid organs and paucity of B cells within follicular structures (Xiao et al., 2008). Our results suggest this is due, at least in part, to lower CXCR5 expression in both B cells and T cells.

Identifying Bcl-6 as the transcription factor that specifies Tfh cell lineage commitment firmly establishes Tfh cells as a bona fide T effector subset. Bcl-6 emerges as an important arbiter of T cell lineage fate, acting through repression of Th1, Th2, and Th17 cell transcription factors, as well numerous miRNAs that are predicted to affect expression of Tfh cell signature molecules. Bcl-6 therefore connects the differentiation of an important effector T cell subset with the regulation of chemokine receptors and other molecules. This enables the proper placement of these T cells in the GC microenvironment to facilitate T cell-dependent antibody responses.

EXPERIMENTAL PROCEDURES

Mice and Immunizations

Bcl6$^{-/-}$ (Dent et al., 1997), Thy1.1 OT-II, CD45.1 mice were maintained on a C57BL/6 background and housed in SPF conditions at the Australian National University (ANU) and Garvan Institute animal facilities. All animal experiments were carried under protocols approved by the institutes' Animal Ethics Committees. For fetal liver and bone marrow reconstitution, 2 to 5 × 10^6 cells from E15-E17 fetal liver or adult bone marrow were injected intravenously (i.v.) into 8- to 12-week-old CD45.1 or *Rag1*$^{-/-}$ mice that had been sublethally irradiated. Mice were analyzed 8 weeks after reconstitution. For generating T cell-dependent antibody responses, mice were immunized i.p. with 2 × 10^9 SRBC (Veterinary Services, IMVS). For induction of T cell-dependent responses after adoptive transfer of OT-II cells, mice were i.v. injected with splenocytes containing 3 to 5 × 10^4 OVA$^{323–339}$-specific CD4^{+} T cells and immunized intraperitoneally (i.p.) with 100 μg of OVA in alum. All these procedures were approved by the Ethics Committee of The Australian National University.

Human Lymphocyte Preparations

Tonsillar cells were isolated by mechanical disruption and Ficoll-Plaque density gradient centrifugation. Human studies were approved by the institutes' human research ethics committees.

CD4^{+} T Cell Isolation, Culture, Stimulation, and Differentiation

Mouse CD4^{+} T cells were purified with CD4 positive selection (Miltenyi Biotec) and sorted into naive (CD44^{lo} CD62L^{hi} CD25^{-}) and activated (CD44^{hi} CD62L^{lo} CD25^{-}) populations on FACSAria (>97% purity). For ChIP, human naive (CD45RO^{-}CXCR5^{lo}), Tfh (CD45RO^{+}CXCR5^{hi} PD-1^{hi}), and non-Tfh effector (CD45RO^{+} CXCR5$^{int\ or\ o}$ PD-1$^{int\ or\ lo}$) CD4^{+} T cells, and an enriched B (CD4^{-} CXCR5^{hi}) cell population were sorted from isolated tonsil cells (90%–95% purity). For other purposes, human CD4^{+} T cells were purified with the Dynal CD4 Positive Cell Isolation Kit (Invitrogen) (>98% purity). For ex vivo differentiation, mouse naive T cells were cultured in completed RPMI1640 media (Yu et al., 2008) and stimulated with plate-bound anti-CD3 (5 μg/ml) plus anti-CD28 (2 μg/ml) under Th0: no cytokines and antibodies; Thn: 10 μg/ml anti-IFN-γ and 10 μg/ml anti-IL-4; Th1: 10 ng/ml IL-12, and 10 μg/ml anti-IL-4; Th2: 10 ng/ml IL-4, 10 μg/ml anti-IFN-γ, and 10 μg/ml anti-IL-12/IL-23 p40; and Th17: 2 ng/ml TGF-β, 20 ng/ml IL-6, 10 μg/ml anti-IFN-γ, and 10 μg/ml anti-IL-4. For intracellular cytokine staining, CD4^{+} T cells were restimulated for 4–6 hr with 50 ng/ml PMA (Sigma), 1 μg/ml ionomycin (Sigma), 3 μg/ml brefeldin A (eBioscience), and 2 μM monensin (eBioscience).

Flow Cytometry

Antibodies were listed in the Supplemental Data. The staining procedure has been described (Yu et al., 2008), except that anti-mouse CCR7 staining was performed at 37°C. Intracellular cytokines and transcription factors were stained with the BD Cytofix/Cytoperm kit (BD PharMingen) and Mouse Regulatory T Cell Staining Kit (eBioscience), respectively. Flow cytometric analysis was performed on LSR II or FACSCanto II and analyzed with FlowJo software (Tree Star).

Immunofluorescence

Frozen spleen sections were fixed in cold acetone, stained, mounted in VECTASHIELD (Vector Laboratories), and viewed with a Zeiss Axiovert 200M microscope. The images were acquired with AxioVision (release 4.7.1) software (Carl Zeiss).

Quantitative Real-Time RT-PCR

MiRNA expression was assessed by real-time RT-PCR with the TaqMan miRNA assay protocol (Applied Biosystems). Gene and miRNA expression were detected with the ABI 7900 Prism and fold changes in expression were determined by the $2^{-\Delta\Delta Ct}$ method, with the results normalized to RNU 6 (U6) for miRNA and β-actin for gene expression.

Retrovirus Production and Transduction

Retrovirus preparation has been described before (Yu et al., 2007). Bcl-6 cDNA was PCR amplified and cloned into the vector (Bcl-6-IRES-GFP). For retroviral

transduction, sorted mouse naive $CD4^+$ T or B cells were stimulated with plate-bound anti-CD3 (5 μg/ml) plus anti-CD28 (2 μg/ml) and 10 ng/ml of IL-2 (for T cells) or soluble LPS (10 μg/ml) (for B cells) for 2 days. Cells were spun with retroviral supernatants and 4 μg/ml of polybrene (Sigma) at 800 g-force for 1 hr at 30°C and then cultured in fresh medium before analysis at the times indicated.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed in accordance with the instructions (Upstate Biotechnology) as detailed in the Supplemental Data. Ct values from the PCR amplification plots were converted to arbitrary copy number with the formula $10^5/2^{(Ct\ -17)}$. We then normalized sample data to the corresponding total input before fold change above the average no antibody control was calculated to yield the ChIP enrichment ratio as previously described (Pokholok et al., 2006). In addition, we calculated the data to yield the percentage of total input. All ChIP experiments were performed at least in duplicate.

miRNA Microarrays

Mouse naive $CD4^+$ T cells were transduced with a Bcl-6-expressing retrovirus or the control vector and cultured in Th0 condition for 2 days. Cells expressing medium to high levels of GFP were sorted, and total RNAs were isolated with the mirVana miRNA Isolation Kit (Applied Biosystems). MiRNA expression was profiled with Agilent 8x15K mouse miRNA microarray miRNA-v1_95_May07 (miRBase Release 9.2) by Ramaciotti Centre for Gene Function Analysis (Sydney). Each sample was run in duplicate. For analysis, probes were quality-controlled by deleting any probe that had no positive signals; the average of each probe was calculated for replicates and probes with negative signals were set to 0.

Bioinformatic Promoter and miRNA Binding Analysis

Bcl-6 binding site frequency and density in promoter regions were performed with Gene2Promoter (release 4.2) and GEMS Launcher (release 4.3) within the Genomatix Suite. We used MatInspector (release 7.4.3) to identify Bcl-6 sites with optimized core similarity and matrix similarity score settings. MiRNA target sequences in 3′ UTR were predicted by miRanda (September 2008 Release, http://www.microrna.org/microrna/home.do) and Targetscan (Release 4.2, http://www.targetscan.org/vert_42/) with default settings.

ACCESSION NUMBERS

The miRNA array data for this paper have been deposited in GEO under accession number GSE17000.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, 14 figures, and 4 tables and can be found with this article online at http://www.cell.com/immunity/supplemental/S1074-7613(09)00314-8.

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