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Molecular mechanisms that control the expression and activity of Bcl-6 in T_H1 cells to regulate flexibility with a T_{FH}-like gene profile

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SUMMARY

T-bet and Bcl-6 are required to establish T_H1 or T_{FH} gene expression profiles, respectively. Here, we demonstrated that high interleukin 2 (IL-2) concentrations inhibited Bcl-6 expression in polarized T_H1 cells. Mechanistically, the low amounts of Bcl-6 normally found in effector T_H1 cells could not repress its target genes because a T-bet-Bcl-6 complex masked the Bcl-6 DNA-binding domain. T_H1 cells increased their Bcl-6/T-bet ratio in response to limiting IL-2 conditions, allowing excess Bcl-6 to repress its direct target *Prdm1* (which encodes Blimp-1). The Bcl-6-dependent repression of Blimp-1 effectively induced a partial T_{FH}-profile because Blimp-1 directly repressed a subset of T_{FH}-signature genes, including *Cxcr5*. Taken together, IL-2-signaling regulates the Bcl-6-Blimp-1 axis in T_H1 cells to maintain flexibility with a T_{FH}-like gene profile.

INTRODUCTION

$CD4^+$ T helper cells can develop into a variety of functionally distinct subtypes after their initial encounter with foreign antigen. These subtypes include T helper 1 (T_H1), T_H2, T_H17, and T follicular helper (T_{FH}) cells[1–6]. The proper development and maintenance of T helper cell subsets is required to clear specific pathogens without causing self-damage. For example, T_H1 cells coordinate the immune response to intracellular pathogens, but inappropriate activation results in autoimmunity[7, 8].

Unique, signature gene expression programs define each specialized T helper cell subtype. T helper cell specific gene profiles are created by the induction of key lineage-defining transcription factors in response to cytokine signaling events at the time of initial antigen encounter[9–14]. For example, naïve helper T cells exposed to interleukin 12 (IL-12) and/or

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AUTHOR CONTRIBUTIONS

K.J.O and A.S.W. designed, performed experiments, analyzed data and wrote the manuscript; S.E.M contributed to performing the experiments for Fig. 1c, 2b, e, Supplementary Fig. 4a, b.

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interferon-γ (IFNγ) upregulate the T-box transcription factor T-bet, which is required to establish the T_H1 gene expression profile[12]. In contrast, IL-4 induces GATA3 to create a T_H2 gene program, while a combination of IL-6 and transforming growth factor-β (TGFβ) upregulate RORγt to activate the T_H17 profile[9, 14]. Additionally, IL-6 and IL-21 can induce the transcriptional repressor Bcl-6 to functionally regulate the T_{FH}-gene program[10, 11, 13]. To date, the signaling pathways that initially induce T helper cell lineage-defining transcription factors have been well characterized, but it is currently unclear whether changing environmental conditions can alter their composition after the primary commitment decision.

Much research has been performed to examine the mechanisms by which T-bet activates T_H1-signature genes[15–20]. Recently, several studies have uncovered diverse ways in which T-bet antagonizes alternative T helper cell fates[16, 21–23]. One mechanism T-bet utilizes to directly repress transcription is by physically recruiting the transcriptional repressor Bcl-6 to a subset of target genes in committed T_H1 cells[24]. Thus, low amounts of Bcl-6 are necessary for T-bet to effectively repress alternative T helper cell gene programs[24]. These findings raised the question of how are Bcl-6 activity and expression tightly controlled in T_H1 cells to prevent it from tipping the balance towards a T_{FH}-gene profile.

Bcl-6 is a member of the BTB/POZ transcriptional repressor family. This family represses transcription by directly binding to specific DNA sequences through its zinc finger DNA binding domain, with the BTB/POZ domain mediating transcriptional repression[25]. It is currently unclear how Bcl-6 functionally activates T_{FH}-signature genes. In T_{FH} cells, Bcl-6 represses a miRNA gene cluster to effectively stabilize the expression of several T_{FH}-signature genes[13], but this does not explain the initial activation of T_{FH}-signature gene transcription in response to Bcl-6.

In this study, we found that a T-bet-Bcl-6 complex masked the Bcl-6 DNA binding domain, which blocked Bcl-6 from repressing its target genes. This finding raised the possibility that there may be flexibility between T_H1 and T_{FH}-like gene expression patterns if there are environmental conditions that change the ratio of Bcl-6 to T-bet in T_H1 cells. We demonstrated that strong IL-2-signaling, acting through STAT5, inhibited *Bcl6* expression in effector T_H1 cells, but when IL-2 was limiting, Foxo factors were able to activate *Bcl6* transcription. Enhanced Bcl-6 expression in polarized T_H1 cells resulted in the induction of *Cxcr5* and a subset of T_{FH}-genes. Mechanistically, altering the Bcl-6 to T-bet ratio in T_H1 cells allowed Bcl-6 to repress its direct target gene *Prdm1* (the gene that encodes Blimp-1). Notably, Blimp-1 was directly responsible for the repression of a subset of T_{FH}-signature genes in effector T_H1 cells. Therefore, the Bcl-6-dependent repression of Blimp-1 translated the repressive activity of Bcl-6 into the induction potential for a subset of T_{FH}-genes.

RESULTS

T-bet interacts with the Bcl-6 DNA binding domain

We have previously shown that T-bet physically interacts with Bcl-6 in T_H1 cells (Fig. 1a and [24]), which targets T-bet-Bcl-6 complexes to a subset of T-bet DNA binding elements[24]. These findings raised the question of why are T-bet-Bcl-6 complexes preferentially targeted

to the T-bet, rather than Bcl-6, DNA binding elements. To begin to address this question, we performed co-immunoprecipitation (co-IP) experiments to define the domains within Bcl-6 and T-bet that were required for their interaction. A Bcl-6 truncation construct deleting its entire C-terminal zinc finger domain (Bcl-6ΔZF) failed to associate with T-bet (Fig. 1b, Supplementary Fig. 1a and [24]). This domain contains six zinc fingers, with the four most C-terminal zinc fingers required for DNA binding[26]. A more detailed Bcl-6 truncation analysis demonstrated that the zinc fingers known to mediate Bcl-6 DNA-binding activity were also the ones required for its interaction with T-bet (Fig. 1b; Bcl-6ΔZF^{DB}).

Next, we localized the domain within T-bet that was required for its association with Bcl-6. T-bet is composed of a central T-box DNA binding domain as well as N- and C-terminal domains that mediate protein-protein interactions and transactivation events (Supplementary Fig. 1a). Truncating the N-terminal domain of T-bet (T-betΔN) did not impair its ability to interact with Bcl-6, while a T-bet C-terminal truncation construct (T-betΔC) failed to associate with Bcl-6 in co-IP experiments (Fig. 1c). Collectively, these data suggest that the interaction between T-bet and Bcl-6 has the potential to inhibit Bcl-6 DNA binding activity, while leaving the T-box DNA binding domain exposed.

A T-bet-Bcl-6 complex inhibits Bcl-6-dependent repression

To begin to address whether the interaction between T-bet and Bcl-6 interferes with Bcl-6 DNA binding activity, we performed transfection experiments with luciferase-reporter constructs containing either the *Prdm1*-promoter alone or multimerized Bcl-6 DNA binding elements upstream of the minimal SV40 promoter (3x-Bcl6-promoter reporter). The 3x-Bcl6-promoter reporter construct represents a simplified scenario where the repression of a minimal promoter is solely dependent upon Bcl-6 DNA binding elements. The *Prdm1*-promoter reporter represents a direct Bcl-6 target gene in the context of a physiologically relevant, and thus more complex, promoter setting. We utilized EL4 T cells for these experiments because they express endogenous Bcl-6, but do not express T-bet[15, 24]. As a control, we first confirmed that Bcl-6 repressed the activity of the 3x-Bcl6- and *Prdm1*-promoter reporters (Supplementary Fig. 1b, c).

If the interaction between T-bet and Bcl-6 inhibits Bcl-6 DNA binding, then a T-bet-Bcl-6 complex would prevent Bcl-6 from targeting to its own binding sites. In this scenario, increasing T-bet expression would enhance the formation of T-bet-Bcl-6 complexes and effectively block Bcl-6 from repressing its own target genes. To test this possibility, we examined whether increasing T-bet expression inhibited Bcl-6 from repressing the 3x-Bcl6- and *Prdm1*-promoter reporter constructs. For these experiments, we utilized a T-bet DNA binding mutant construct [T-bet(DBmut)] to exclude the possibility that T-bet may directly bind to and activate the 3x-Bcl6- or *Prdm1*-promoter reporters. Importantly, overexpression of T-bet(DBmut) alone substantially enhanced 3x-Bcl6- and *Prdm1*-promoter reporter activity, but did not activate an *Ifng*-promoter reporter as a control (Fig. 1d, e, Supplementary Fig. 1d, e). We also performed the promoter reporter experiments with a T-bet construct that cannot interact with Bcl-6 (T-betΔC; see Fig. 1c). T-betΔC did not inhibit Bcl-6-dependent repression, suggesting that the interaction between T-bet and Bcl-6 was

required for T-bet's ability to alleviate the Bcl-6-dependent repression of the 3x-Bcl6- and *Prdm1*-promoter reporters (Fig. 1d, e, Supplementary Fig. 1d).

We next hypothesized that the relative expression between T-bet and Bcl-6 defines the functional capability of Bcl-6 when both are expressed in the same cell. That is, if there is excess T-bet, T-bet-Bcl-6 complex formation will inhibit the majority of Bcl-6 from localizing to its own DNA binding elements (Fig. 1d, e). However, increasing Bcl-6 abundance in the presence of constant T-bet expression would allow excess Bcl-6 to interact with its own DNA binding elements. To test this hypothesis, we overexpressed Bcl-6 in conjunction with T-bet(DBmut) and examined the functional consequence on Bcl-6-dependent repression. Bcl-6 overexpression rescued the repression of the 3x-Bcl6- and *Prdm1*-promoter reporters despite the presence of T-bet(DBmut) (Fig. 1f, Supplementary Fig. 1f, g). These data suggest that the relative ratio of T-bet to Bcl-6 determines whether Bcl-6 can repress its direct target genes.

Enhanced T_{FH}-signature gene expression in *Tbx21*$^{-/-}$ cells

Our previous study demonstrating that a T-bet-Bcl-6 complex is functionally important for repressing a subset of T-bet target genes did not address whether the T-bet-Bcl-6 complex prevents Bcl-6 from repressing its direct target genes in T_H1 cells[24]. To begin to explore this question, we examined endogenous *Prdm1* gene expression in primary CD4$^+$ T cells isolated from either wild-type or *Tbx21* (the gene that encodes T-bet)$^{-/-}$ mice polarized in T_H1 conditions. In this experimental setting, Bcl-6 is expressed at a constant, low amount in both wild-type and *Tbx21*$^{-/-}$ cells[24], but because T-bet is not present to form a complex with Bcl-6, this may allow the "free" Bcl-6 in the *Tbx21*$^{-/-}$ cells to repress its direct target genes. Consistent with this hypothesis, *Prdm1* expression was reduced in *Tbx21*$^{-/-}$ T_H1 cells (Fig. 1g).

We next wanted to determine whether changes in the functional activity of Bcl-6 in the T-bet-deficient setting would induce a T_{FH}-like gene expression profile. A subset of T_{FH}-signature genes[1, 27], including *Cxcr5*, *Il6ra*, *Btla*, and *Tnfsf8* (the gene that encodes CD30L) had increased expression in *Tbx21*$^{-/-}$ relative to wild-type T_H1 polarized cells (Fig. 1h). To further explore these results, we also performed *Tbx21* siRNA knockdown experiments in the context of wild-type T_H1 polarized cells. In this experimental strategy, CD4$^+$ T cells commit to the T_H1 pathway in the presence of T-bet, which allowed us to examine the functional consequence of reducing T-bet expression in a natural T_H1 setting. Similar to the data from the T-bet-deficient cells, the knockdown of *Tbx21* in wildtype T_H1 cells resulted in the induction of a subset of T_{FH}-signature genes (Supplementary Fig. 2). Collectively, these experiments suggest that the interaction between T-bet and Bcl-6 functionally regulates the activities of both T-bet and Bcl-6 in T_H1 cells ([24], Fig. 1 and Supplementary Fig. 1, 2).

IL-2R-signaling inhibits Bcl-6 expression in T_H1 cells

The mechanistic findings presented thus far suggest that there may be flexibility between the T_H1 and T_{FH} gene programs if environmental signaling events can regulate Bcl-6 expression in T_H1 cells. Therefore, we wanted to determine whether there are signaling pathways in

developing T_H1 cells that modulate Bcl-6 expression. Previous research has suggested that IL-2-signaling regulates Bcl-6 expression in some circumstances. Specifically, Bcl-6 is repressed when $CD8^+$ T cells are exposed to high concentrations of IL-2, whereas Bcl-6 expression is upregulated in limiting IL-2 conditions[28]. Also, an inverse correlation exists between IL-2Rα and Bcl-6 expression in $CD4^+$ T_{FH} cells[29]. Thus, we hypothesized that IL-2-receptor (IL-2R)-signaling may regulate Bcl-6 expression in T_H1 cells.

To test this possibility, we monitored Bcl-6 expression in $CD4^+$ T cells cultured in T_H1 polarizing conditions with a range of IL-2 concentrations. For these experiments, $CD4^+$ T cells were stimulated with plate bound αCD3 and αCD28 for three days in the presence of T_H1 polarizing conditions and IL-2. The cells were then split and maintained in T_H1 polarizing conditions in the presence of variable IL-2 concentrations for an additional three days. In developing T_H1 cells, Bcl-6 transcript and protein expression inversely correlated with the concentration of IL-2 (Fig. 2a, b, Supplementary Fig. 3a; and see Supplementary Fig. 3b to compare expression in high versus low IL-2). In contrast, T-bet was similarly expressed in all IL-2 conditions, indicating that T_H1 polarizing conditions are dominant over IL-2 concentrations for regulating T-bet expression (Fig. 2b, c, and Supplementary Fig. 3b, c). Together these data suggest that the environmental concentration of IL-2 regulates Bcl-6, but not T-bet, expression in developing T_H1 cells.

To further explore whether signaling through the IL-2R inhibits Bcl-6 expression in T_H1 cells, we incubated developing T_H1 cells with blocking antibodies to the CD25 (IL-2Rα) and/or CD122 (IL-2Rβ) subunits of the IL-2R complex. We found that blocking both CD25 and CD122 in combination enhanced Bcl-6 transcript and protein expression (Fig. 2d, e). These data are consistent with the findings from the IL-2 titration experiments and together provide evidence that strong IL-2R-signaling inhibits Bcl-6 expression in T_H1 cells.

IL-2-signaling regulates STAT binding to the *Bcl6* promoter

We next wanted to determine the mechanism by which IL-2R-signaling regulates *Bcl6* expression in T_H1 cells. A scan of the transcription factor binding elements within the *Bcl6* promoter identified DNA binding sites for two transcription factor families, STAT and Foxo (Fig. 3a), whose activities are responsive to IL-2R-signaling in T cells[30–32]. STAT5 has been suggested to inhibit a subset of genes by either displacing activating STAT3 complexes or recruiting repressive chromatin modifying complexes to the promoter[33, 34]. Therefore, we wanted to test whether strong IL-2-signaling inhibits *Bcl6* expression by enhancing STAT5 binding and/or the relative STAT5/STAT3 ratio at the *Bcl6* promoter in T_H1 cells.

We first confirmed that STAT5 phosphorylation (p-STAT5) was enhanced with increasing IL-2 concentrations in T_H1 cells (Fig. 3b). Next, we performed a chromatin immunoprecipitation (ChIP) analysis examining STAT3 and STAT5 binding to the *Bcl6* promoter in T_H1 cells cultured in either high or low IL-2 conditions (Fig. 3c). The ChIP experiments demonstrated that the ratio of STAT3 versus STAT5 bound to the *Bcl6* promoter varied with the IL-2 concentration. Specifically, STAT5 binding to the *Bcl6* promoter increased, while STAT3 binding decreased, in T_H1 cells under high IL-2 conditions (Fig. 3c). Enhanced STAT5 binding correlated with the inhibition of *Bcl6* expression in T_H1 cells exposed to increasing concentrations of IL-2 (Fig. 2a, b). These data

are consistent with a repressive role for STAT5 in the IL-2-dependent regulation of *Bcl6* expression in T_H1 cells.

Foxo factors regulate *Bcl6* expression in T_H1 cells

Recent studies suggest that strong IL-2R-signaling inhibits Foxo transcription factor activity in T cells. IL-2 induces a miRNA that inhibits Foxo1 expression and it also prevents the nuclear translocation of Foxo family members[32, 35]. Notably, there are Foxo binding elements in the *Bcl6* promoter and Foxo transcription factors were able to activate *Bcl6* promoter activity (Fig. 3a, Supplementary Fig. 4a, b and [36]). Therefore, we wanted to determine whether *Bcl6* is a direct, IL-2-responsive Foxo transcription factor target gene in T_H1 cells.

Consistent with previous findings in non-polarized $CD4^+$ T cells[32], *Foxo1* expression was reduced when T_H1 cells were cultured in high IL-2 conditions (Supplementary Fig. 4c). We next performed ChIP experiments to assess Foxo1 and Foxo3a binding to the *Bcl6* promoter in T_H1 cells maintained in either high or low IL-2 (Fig. 3d). Both Foxo1 and Foxo3a bound to the *Bcl6* promoter when T_H1 cells were maintained in low IL-2, correlating with *Bcl6* expression in these conditions. In contrast, Foxo1 and Foxo3a binding to the *Bcl6* promoter were substantially reduced when T_H1 cells were cultured in high IL-2 (Fig. 3d). Collectively, these data suggest that IL-2 regulates the binding of the transcriptional activators Foxo1 and Foxo3a to the *Bcl6* promoter in primary T_H1 cells.

T_H1 cells upregulate a T_{FH}-like profile in low IL-2

We next wanted to determine whether enhanced Bcl-6 abundance in T_H1 cells could alter the gene expression profile of the cell (Fig. 4a–c). We first examined the expression of the direct Bcl-6 target gene *Prdm1* in T_H1 cells cultured in variable IL-2 conditions. *Prdm1* transcript and Blimp-1 protein expression were substantially reduced in T_H1 cells maintained in limiting IL-2 (Fig. 2b, 4a and Supplementary Fig. 3b). It is interesting to note that there appeared to be a threshold for the amount of Bcl-6 that was needed to effectively repress Blimp-1 expression. These data highly suggest that similar to the findings in Fig. 1, naturally increasing the Bcl-6 to T-bet ratio past a threshold point in primary T_H1 cells results in the functional repression of a prototypic Bcl-6 target gene.

We then assessed whether enhanced Bcl-6 expression in T_H1 cells was sufficient to upregulate T_{FH}-genes. *Cxcr5* was induced more than 15-fold in developing T_H1 cells cultured in low IL-2 (Fig. 4c). Three other T_{FH}-associated genes, *Il6ra*, *Btla* and *Tnfsf8*, were also upregulated in this setting (Fig. 4c). In contrast, the T_H1 cytokine gene, *Ifng*, was reduced when T_H1 cells were maintained in limiting IL-2, potentially indicating a shift in the balance of the T helper cell program (Fig. 4b). It is important to note that not all T_{FH}-signature genes were induced in T_H1 cells coincident with Bcl-6 upregulation. In particular, *Pdcd1* (the gene that encodes PD-1) and *Icos* expression were unchanged (Supplementary Fig. 5). These data suggest that enhanced Bcl-6 expression in T_H1 cells induces a partial T_{FH}-profile, but additional events are required to establish the complete program.

IL-2 regulates *Bcl6* expression in polarized T_H1 cells

We next wanted to determine whether fully polarized T_H1 cells retain the flexibility to modulate Bcl-6 expression in response to IL-2-signaling. To examine this question, $CD4^+$ T cells were continuously cultured in T_H1 polarizing conditions for nine days, with the IL-2 concentration either held constant or altered after six days of polarization. *Bcl6* expression was induced when fully polarized T_H1 cells maintained in high IL-2 were switched to low IL-2 (Fig. 5a). Notably, the upregulation of *Bcl6* in fully polarized T_H1 cells correlated with the repression of *Prdm1* and the functional induction of T_{FH}-signature genes including *Cxcr5* (Fig. 5a). In contrast, the enhanced expression of *Bcl6* and T_{FH}-genes observed in low IL-2 conditions was substantially downregulated when T_H1 polarized cells were exposed to high IL-2 conditions (Fig. 5b). Collectively, these data indicate that IL-2 regulates Bcl-6 expression in both developing and fully polarized T_H1 cells.

Blimp-1 directly represses T_{FH}-genes in T_H1 cells

Bcl-6 is a transcriptional repressor and thus it is unlikely to directly activate T_{FH}-genes. Notably, the Bcl-6-dependent repression of Blimp-1 correlated with the induction of T_{FH}-signature genes in decreasing IL-2 concentrations (Fig. 2b, 4a, c). Like Bcl-6, Blimp-1 is a transcriptional repressor[37, 38]. Mechanistically, if Blimp-1 directly represses T_{FH}-signature genes in effector T_H1 cells, then increasing the activity of Bcl-6, which directly represses Blimp-1, would effectively reduce a “Blimp-1-brake” in place on the T_{FH}-associated genes. Therefore, *Prdm1* is a good candidate for the direct Bcl-6 target gene that translates Bcl-6-mediated repression into the downstream activation potential for a subset of T_{FH}-genes.

We first examined whether Blimp-1 represses endogenous T_{FH}-signature gene expression in primary T_H1 cells. We transfected wild-type T_H1 cells with either a control or *Prdm1* siRNA and analyzed the consequences on endogenous gene expression. *Prdm1* expression was reduced in T_H1 cells transfected with *Prdm1*-specific versus control siRNA (Fig. 6a). Consistent with a role for Blimp-1 in repressing T_{FH}-gene expression in T_H1 cells, *Cxcr5*, *Il6ra*, *Btla*, and *Tnfsf8* expression were substantially enhanced when Blimp-1 expression was reduced (Fig. 6a). These data indicate that Blimp-1 functionally represses a subset of T_{FH}-associated genes in primary effector T_H1 cells.

To start to address whether Blimp-1 plays a direct role in repressing these genes, we cloned the promoters for *Cxcr5*, *Il6ra*, *Btla*, and *Tnfsf8* into luciferase reporter vectors to determine whether they are responsive to Blimp-1-mediated repression. Each of these promoters was repressed by the overexpression of wild-type Blimp-1, but not a DNA binding mutant Blimp-1 construct (Blimp-1ΔZF) (Fig. 6b and Supplementary Fig. 6a). Thus, the promoter reporter data support the hypothesis that Blimp-1 directly represses *Cxcr5*, *Il6ra*, *Btla*, and *Tnfsf8* transcription.

Blimp-1 binding inversely correlates with T_{FH}-expression

If Blimp-1 directly represses the IL-2-sensitive T_{FH}-associated genes in effector T_H1 cells, then Blimp-1 binding would inversely correlate with their expression. In ChIP experiments, Blimp-1 was associated with the *Cxcr5*, *Il6ra*, *Btla*, and *Tnfsf8* promoters in T_H1 cells maintained in high IL-2, coinciding with the repression of these genes (Fig. 6c and

Supplementary Fig. 6b). In contrast, Blimp-1 binding was substantially reduced at the *Cxcr5*, *Il6ra*, *Btla* and *Tnfsf8* promoters in response to limiting IL-2 (Fig. 6c and Supplementary Fig. 6b). The reduction in Blimp-1 binding correlated with the induction of these genes (Fig. 4c and 6c). Collectively, these data suggest that Blimp-1 directly binds to and represses the IL-2-sensitive T_{FH}-signature genes in effector T_H1 cells.

Finally, we wanted to explore whether Blimp-1 is a key regulatory factor that translates an increased Bcl-6/T-bet ratio in T_H1 cells into the downstream activation potential for T_{FH}-associated genes. To test this question, we examined Blimp-1 binding to the *Cxcr5*, *Il6ra*, *Btla*, and *Tnfsf8* promoters in the setting of *Tbx21*$^{-/-}$ cells. Blimp-1 binding was diminished at these promoters in *Tbx21*$^{-/-}$ in comparison to wild-type T_H1 polarized cells (Fig. 6d and Supplementary Fig. 6c), coinciding with enhanced gene expression (Fig. 1h). Taken together, Blimp-1 binding inversely correlates with increasing the Bcl-6 to T-bet ratio in T_H1 cells by either natural environmental cues (Fig. 6c) or genetic manipulation (Fig. 6d). Therefore, the IL-2-sensitive regulation of Bcl-6 expression in T_H1 cells determines the downstream potential of a subset of T_{FH}-signature genes by controlling the “Blimp-1-brake” (Supplementary Fig. 7).

DISCUSSION

This study demonstrates that variable IL-2-signaling regulates Bcl-6 expression in polarized T_H1 cells. In effector T_H1 cells, a high T-bet to Bcl-6 ratio promoted T-bet-Bcl-6 complex formation, which masked the Bcl-6 DNA binding domain. As Bcl-6 expression was enhanced in T_H1 cells maintained in low IL-2 conditions, excess Bcl-6 repressed its target gene *Prdm1*. Blimp-1 directly repressed a subset of T_{FH}-signature genes in effector T_H1 cells. Therefore, the Bcl-6-dependent repression of Blimp-1 was responsible for regulating T_{FH}-like gene expression activation potential in $CD4^+$ T cells. Collectively, these data suggest that T_H1 cells retain flexibility with a T_{FH}-like gene profile by maintaining their ability to regulate the Bcl-6-Blimp-1 axis in response to IL-2.

A long-held view in the field has been that opposing T helper cell lineage-defining transcription factors are expressed in a mutually exclusive pattern, but recent research has questioned this simplistic paradigm. There is now increasing awareness that opposing T helper cell lineage-defining transcription factors are co-expressed in many circumstances, and their co-expression is functionally important for regulating the gene expression profile of the cell[21, 24, 39–41]. This concept raises the question of how are the expression and functional activities of these factors precisely regulated during an immune response. Our study demonstrates that T-bet can dominantly control Bcl-6 activity because a T-bet-Bcl-6 complex masks the Bcl-6 DNA-binding domain, but leaves the T-bet DNA-binding domain available. This effectively lets T-bet keep Bcl-6 in check in effector T_H1 cells. However, if Bcl-6 expression increases past the threshold of T-bet control, the balance of the cell can shift towards a T_{FH}-like gene profile.

The environmental concentration of IL-2, translated through STAT and Foxo transcription factor activity, regulates Bcl-6 expression in polarized T_H1 cells. This means that T_H1 cells retain flexibility with a T_{FH}-like gene profile because they have the capacity to alter Bcl-6

expression in response to IL-2-signaling. A recent study found that IL-2-signaling is critical for the formation of multiple T helper cell lineages[42]. Our findings add to this concept and suggest that IL-2-signaling can change the phenotype of polarized T_H1 cells. Intriguingly, new research suggests that IL-2Rα expression inversely correlates with Bcl-6 expression to create a continuum of T_{CM}, T_{FH}, or effector T_H1 characteristics [29, 43]. It is possible that the IL-2R subunit expression pattern on a $CD4^+$ T cell will allow individual cells in a population to respond differently to the same environmental IL-2 conditions.

Our study provides new insight into how the transcriptional repressor Bcl-6 serves to promote T_{FH}-signature gene expression. We found that the Bcl-6-dependent repression of *Prdm1* was directly responsible for regulating the transcriptional potential for some T_{FH}-genes. Interestingly, Blimp-1 is expressed in multiple effector T helper cell subtypes, but is repressed during T_{FH} cell differentiation in vivo[10, 37]. It is possible that Blimp-1 commonly represses T_{FH}-signature genes in other effector T helper cell subtypes as well.

We need to envision the activation of the T_{FH}-gene program as a multi-step process, with the Bcl-6-dependent removal of the “Blimp-1-brake” representing the first step. Recent studies demonstrate that Icos-IcosL interactions in the follicle are required for the full induction of a T_{FH}-gene profile[29]. Additionally, transcriptional regulators such as Batf and c-Maf play a role in T_{FH}-differentiation[44, 45]. It will be important to determine the complete series of molecular events that occur downstream of removing the “Blimp-1-brake” needed to fully activate T_{FH}-genes and whether polarized T_H1 cells can initiate all of these events. Of note, not all T_{FH}-signature genes, such as PD-1 and Icos, were upregulated in low IL-2 indicating that distinct classes of T_{FH}-target genes exist that either require additional, or completely independent, events. Interestingly, $Cxcr5^{high}/PD\text{-}1^{low}$ T helper cells exist outside of the germinal center, representing a T_{FH}-like cell prior to homing within the follicle for full differentiation[46]. We hypothesize that the $Cxcr5^+$ T_H1 cells will need to home to the follicle where the next events required in the T_{FH} gene program occur. Importantly, the overexpression of Bcl-6 alone is sufficient for complete T_{FH} cell differentiation in vivo[10, 11, 13]. Therefore, because Bcl-6 expression is regulated by IL-2-signaling in T_H1 cells, given the right circumstances in vivo, T_H1 cells may retain complete flexibility towards the T_{FH}-gene program.

METHODS

Cell Culture and Transfection

Primary T Cells—Primary $CD4^+$ T cells were isolated from the spleen and lymph nodes of either wild-type C57BL/6 or *Tbx21*$^{-/-}$ mice using the Mag Cellect kit (R&D) as previously described[15]. Following isolation, cells were grown on plate-bound αCD3/αCD28 in the presence of IL-2 and T_H1 polarizing cytokines [αIL-4 (10 μg/mL) and IL-12 (5 ng/mL)]. On day 3, cells were split and maintained in T_H1 conditions for an additional three days. During this time, the cells were cultured in a range of IL-2 concentrations (500, 100, 50, 10, or 1 IU/mL). When indicated, high IL-2 is 500 IU/mL and low IL-2 is 10 IU/mL. For the IL-2R blocking experiments, antibodies to CD25 (PC61, BD Biosciences) and CD122 (TM-β1, BD Biosciences) were used. Primary $CD4^+$ T cell transfections with the Lonza nucleofection system using mouse primary T cell solutions and program X-01 were

performed as previously described[18, 19]. The siRNA transfections were performed as previously described with an siRNA smartpool to either *Tbx21* or *Prdm1* (Dharmacon) or GFP as a control (Ambion)[18, 19]. All experiments involving mice were conducted with IACUC approval.

EL4 T Cells—Murine EL4 T cells transfections using the Lonza nucleofection system program 0–17 and solution V were performed as previously described[15]. Immunoblot analysis was conducted to determine the expression of the transfected proteins.

Promoter Reporter Assay

The 3x-Bcl6-promoter reporter construct was made by cloning three Bcl-6 DNA binding elements upstream of the minimal SV40 promoter in the pGL3-promoter reporter vector (Promega). *Bcl6* (+1998 to +1 bp), *Prdm1* (+1991 to −222 bp), *Cxcr5* (+1841 to −12 bp), *Btla* (+913 to −16 bp), *Tnfsf8* (+1458 to −226 bp), and *Il6ra* (+1209 to −123 bp) promoter reporter constructs were prepared by cloning each of the promoters into the pGL3-basic luciferase reporter construct (Promega). EL4 cells were co-transfected with the promoter reporter constructs in combination with either T-bet(DBmut), T-betΔC, Bcl-6, Blimp-1, or Blimp-1ΔZF V5-tagged expression vectors, or an empty vector control as indicated. The TK-renilla control plasmid was also co-transfected and used to normalize for transfection efficiency. Transfections were harvested after 16–24 hours and samples were analyzed with the Dual-Luciferase Reporter system (Promega). Expression amounts for transfected expression constructs were monitored by immunoblot analysis (Supplementary Fig. 1d, f, 6a)

Co-Immunoprecipitation (co-IP)

The co-IP assay was performed as described previously[18, 19, 24]. A T-bet specific antibody (H-210; Santa Cruz Biotechnologies) or a Bcl-6 specific antibody (C-19; Santa Cruz Biotechnologies) was used for immunoprecipitation. The co-immunoprecipitated proteins were detected using a V5-epitope tag specific antibody (R960-25; Invitrogen). For the endogenous co-IP studies in primary wild-type T_H1 cells, the samples were immunoprecipitated with either an antibody specific for Bcl-6 or a negative control V5 antibody. The immunoblots were then probed with a T-bet specific antibody (4B10; Santa Cruz).

RNA and qRT-PCR

RNA was obtained by Nucleospin RNA purification (Machery-Nagel) and cDNA was prepared with the First Strand Superscript II Synthesis System (Invitrogen). qPCR reactions using 20 nanograms of the cDNA template were performed with gene specific primers and the qPCR Sybr Green Mix (Biorad). All samples were first normalized to the *Rps18* (ribosomal protein S18) control. Graphs represent the normalized expression for the sample as a ratio to relative to the indicated comparison condition in each Figure panel.

Immunoblot Analysis

An equal number of primary wild-type T_H1 cells were harvested for each IL-2 treatment condition and subjected to an immunoblot analysis to determine protein expression. Antibodies for T-bet (4B10), STAT5 (C-17), and GAPDH were from Santa Cruz, while the antibodies to Bcl-6 (561520) and p-STAT5 (611964) were from BD Pharmingen. The antibody for Blimp-1 (A01647) was from Genscript.

Chromatin Immunoprecipitation (ChIP) Assay

The ChIP assay was performed as described previously[15, 17, 18, 24]. The STAT3 (C-20), STAT5 (C-17), Foxo1 (H-128), and Foxo3a (H-144) antibodies were from Santa Cruz Biotechnology, while the Blimp-1-specific antibody was from Genscript (A01647). Chromatin was harvested from either primary polarized T_H1 wild-type cells maintained in variable IL-2 conditions (high or low IL-2) or from *Tbx21*$^{-/-}$ CD4^{+} T cells as indicated. The precipitated DNA was analyzed by qPCR with promoter-specific primers (for primer sequences see Supplementary Table 1). Samples were normalized to a standardized total input DNA control followed by subtraction of the IgG antibody control as the nonspecific background for the precipitation. This value represented the percent input for each sample. Fold enrichment values were calculated by dividing the percent input of the samples by that of either the low IL-2 or *Tbx21*$^{-/-}$ samples from the same experiment.

STATISTICS

The error bars for all graphs were calculated as the standard error of the mean (s.e.m).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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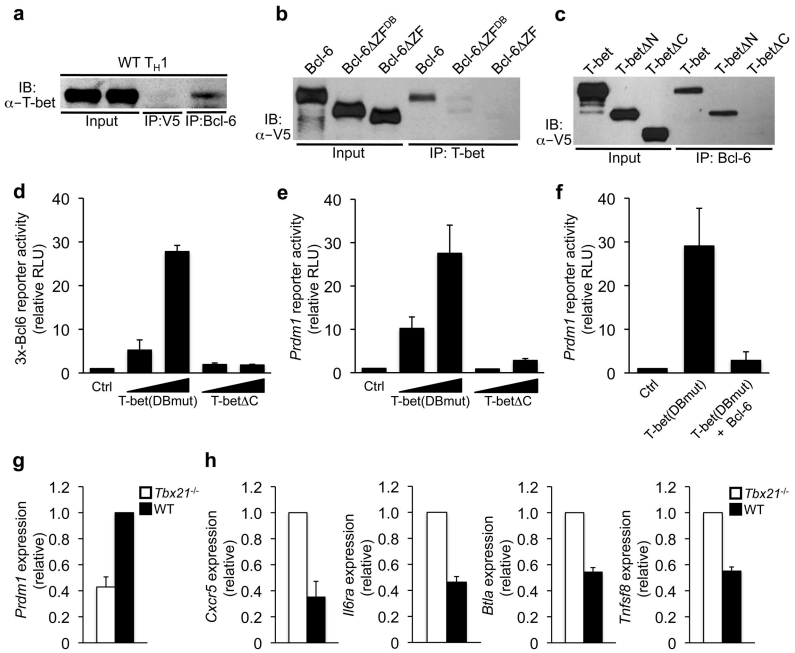

Figure 1. A T-bet-Bcl-6 complex inhibits Bcl-6-dependent repression

(**a**) A co-IP was performed from wild-type T_H1 cells with either a control or Bcl-6-specific antibody, followed by immunoblot analysis with a T-bet-specific antibody. (**b**) EL4 T cells were transfected with an untagged T-bet expression construct in combination with V5-epitope tagged wild-type Bcl-6, Bcl-6ΔZF^{DB} or Bcl-6ΔZF as indicated. (**c**) EL4 T cells were transfected with an untagged Bcl-6 expression construct in combination with V5-epitope tagged wild-type T-bet, T-betΔN or T-betΔC as indicated. Lysates were immunoprecipitated with an antibody to (**b**) T-bet or (**c**) Bcl-6. (**b, c**) Immunoblots were probed with a V5-epitope specific antibody. (**d, e**) EL4 T cells were co-transfected with (**d**) 3x-Bcl6-promoter reporter or (**e**) *Prdm1*-promoter reporter constructs and either a control empty expression plasmid or increasing concentrations of a T-bet DNA-binding mutant construct [T-bet(DBmut)] or T-betΔC. (**f**) EL4 T cells were co-transfected with a *Prdm1*-promoter reporter and either an empty vector control or T-bet(DBmut) alone or in combination with Bcl-6. (**d–f**) Luciferase values were normalized to a *renilla* control and are represented relative to the control sample (relative RLU). (**g, h**) RNA was isolated from wild-type (black bars) or *Tbx21*$^{-/-}$ (white bars) primary CD4$^+$ T cells polarized in T_H1 conditions. Quantitative RT-PCR results for the genes indicated on the y-axis were first normalized to an *Rps18* control, then represented relative to the (**g**) WT T_H1 or (**h**) *Tbx21*$^{-/-}$ sample. Data in (**a–c**) are representative of at least three independent experiments. Data in (**d–h**) represent the mean of at least three (**d–f, h**) or five (**g**) independent experiments (error bars, s.e.m.).

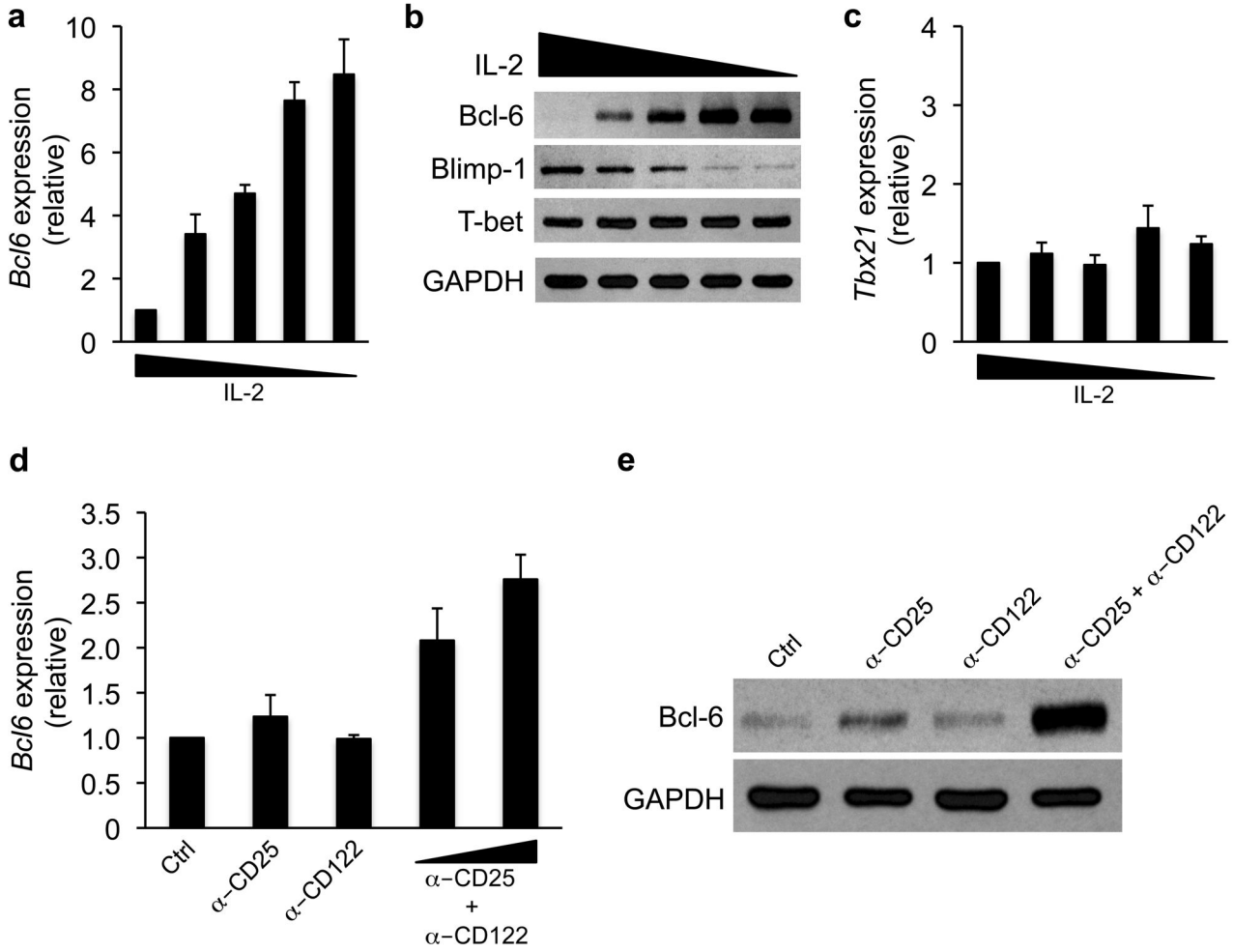

Figure 2. IL-2-signaling inhibits Bcl-6 expression in T_H1 cells

(**a–c**) Primary $CD4^+$ T cells were continuously cultured in T_H1 polarizing conditions for six days. All cells were initially stimulated with plate bound αCD3 and αCD28 and IL-2. At day three, the cells were split and cultured in the presence of decreasing concentrations of IL-2 for an additional 3 days. (**a**, **c**) RNA was then isolated and transcripts for (**a**) *Bcl6* or (**c**) *Tbx21* were determined by quantitative RT-PCR. Samples were first normalized to the *Rps18* control followed by their graphical representation as a ratio relative to the most concentrated IL-2 condition. (**b**) An immunoblot analysis was performed with antibodies to the indicated proteins and GAPDH as a control for equal protein loading. (**d, e**) Primary wild-type $CD4^+$ T cells were cultured in T_H1 conditions and incubated with antibodies to CD25, CD122, or both in combination. The cells were harvested after 36 hours and (**d**) *Bcl6* transcripts were assessed as in (**a**), or (**e**) Bcl-6 protein expression was determined by immunoblot analysis. Data represent the mean of either four (**a, c**) or three (**d**) independent experiments (error bars, s.e.m.). Data in (**b, e**) are representative of at least (**b**) two or (**e**) three independent experiments.

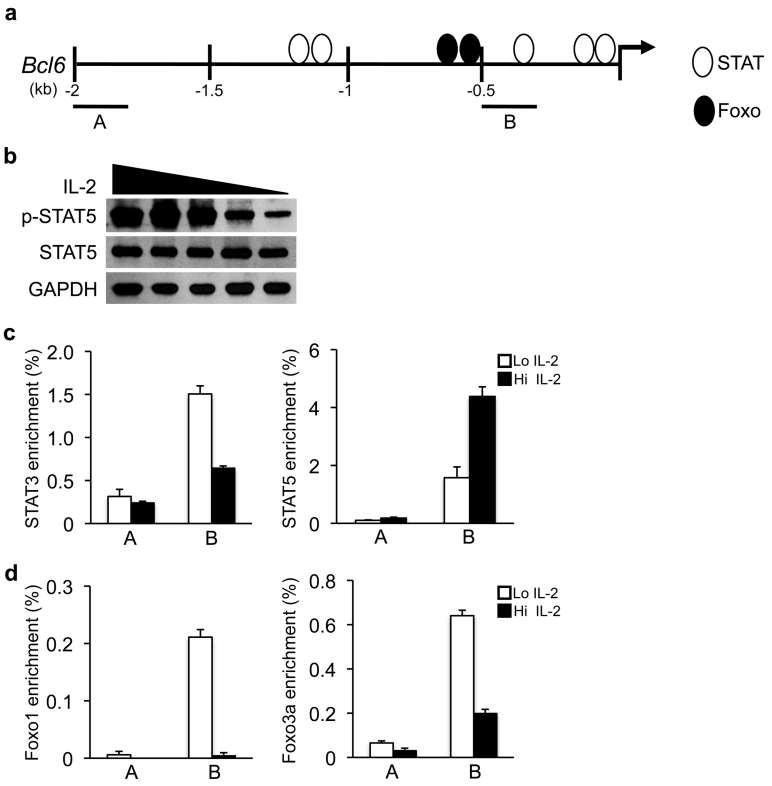

Figure 3. STAT and Foxo transcription factors regulate *Bcl6*

(**a**) Schematic representing STAT and Foxo DNA-binding element locations in the *Bcl6* promoter. Regions monitored in ChIP analysis (A or B) are shown. (**b**) Immunoblot analysis of p-STAT5 and total STAT5 protein in wild-type T_H1 cells exposed to variable IL-2 concentrations. (**c, d**) ChIP experiments were performed with wild-type T_H1 polarized cells maintained in either high (Hi; black bars) or low (Lo; white bars) IL-2 conditions. Chromatin samples were immunoprecipitated with either an antibody to (**c**) STAT3, STAT5 or (**d**) Foxo1, Foxo3a, or (**c, d**) an IgG antibody control and quantitated by qPCR to monitor the identified binding elements (B) or a negative control region (A) in the *Bcl6* promoter. To calculate the percent input, the values for the precipitated samples were first normalized to a standardized aliquot of the input chromatin for each condition, followed by subtraction of the IgG antibody control as the nonspecific background of the experiment. Data in (**b**) are representative of three independent experiments. Data in (**c, d**) represent the mean of three independent experiments (error bars, s.e.m.).

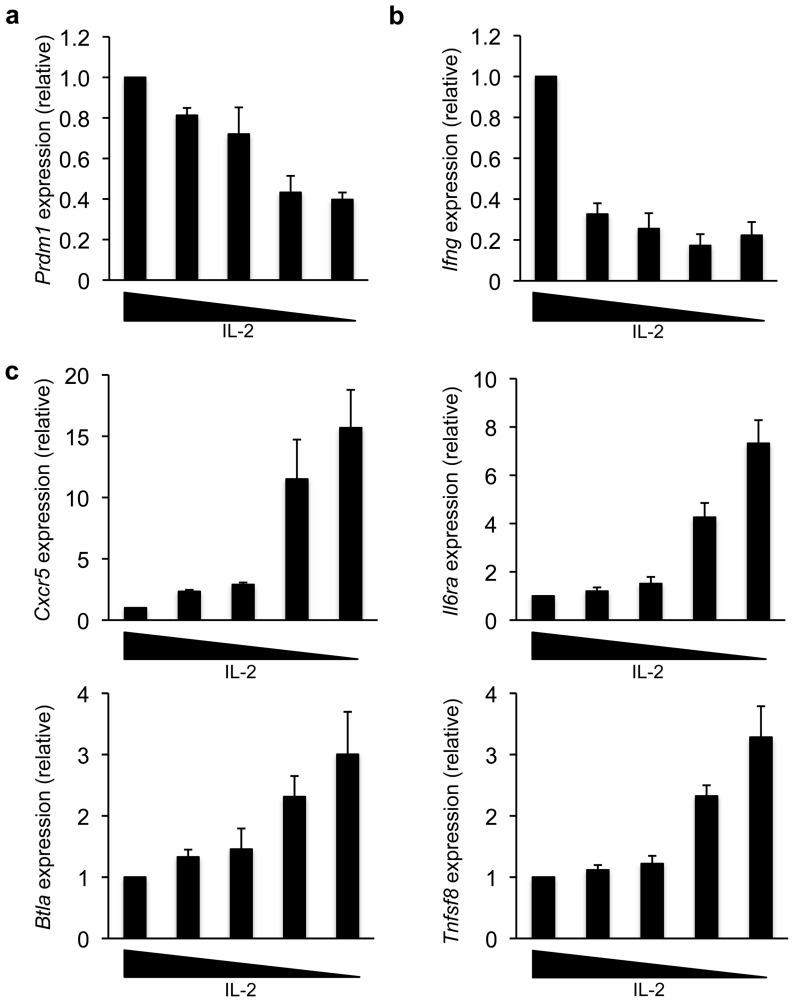

Figure 4. IL-2 regulates *Prdm1* and T_{FH}-gene expression in T_H1 cells

(**a–c**) Primary wild-type $CD4^+$ T cells were continuously cultured in T_H1 polarizing conditions for six days, with variable IL-2 concentrations from days 3–6 as described in Fig. 2a. Transcript amounts for the indicated gene were determined by quantitative RT-PCR. Values were normalized and expressed as described in Fig. 2a. Data in (**a–c**) represent the mean of four independent experiments (error bars, s.e.m.).

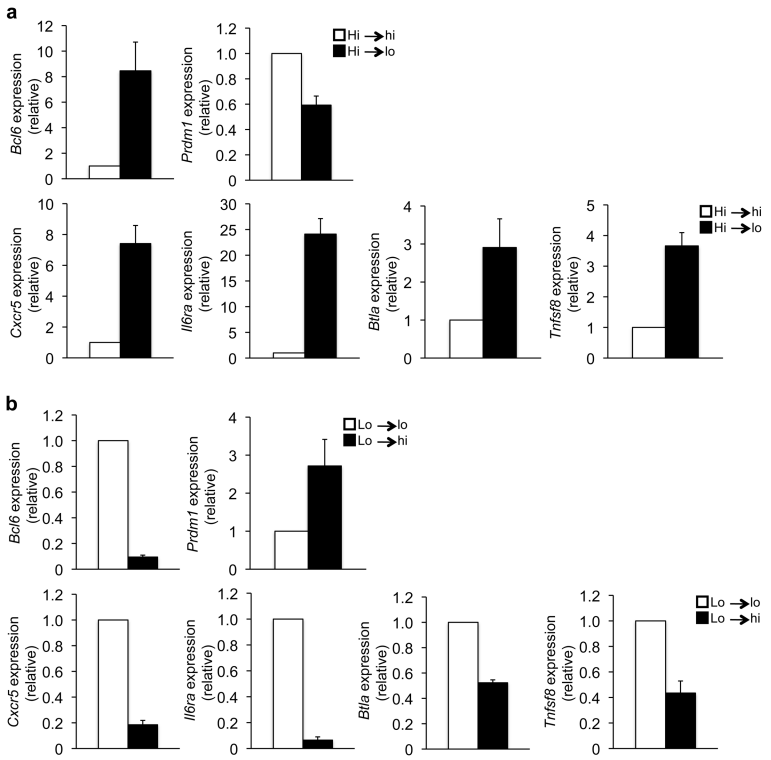

Figure 5. T_H1 cells maintain IL-2-sensitive Bcl-6 and T_{FH}-gene regulation

(**a**) Primary wild-type $CD4^+$ T cells were cultured in T_H1 conditions and high IL-2 for six days. On day 6, the polarized T_H1 cells were split and maintained in T_H1 conditions and either high IL-2 (Hi→hi; white bars) or low IL-2 (Hi→lo; black bars) for an additional 3 days. (**b**) Primary wild-type $CD4^+$ T cells were cultured in T_H1 conditions with the cells maintained in low IL-2 after the day 3 split. On day 6, the T_H1 polarized cells were split and maintained for an additional three days in T_H1 polarizing conditions with either low IL-2 (Lo→lo; white bars) or high IL-2 (Lo→hi; black bars). (**a, b**) RNA was isolated and transcript amounts for the genes indicated on the y-axis were determined by quantitative RT-PCR. Results were first normalized to the *Rps18* control and are graphically represented as the ratio relative to the sample maintained in (**a**) high IL-2 (Hi→hi) or (**b**) low IL-2 (Lo→lo). Data in (**a–b**) represent the mean of at least three independent experiments (error bars, s.e.m.).

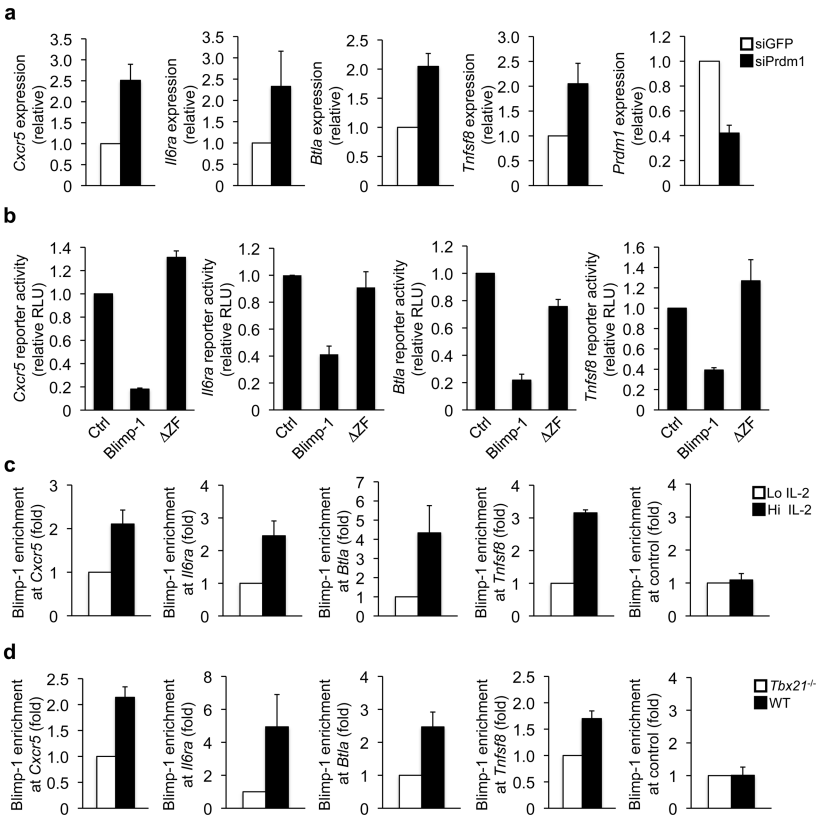

Figure 6. Blimp-1 directly represses T_{FH}-genes in effector T_H1 cells

(**a**) Wild-type T_H1 cells were transfected with either a control (siGFP; white bars) or *Prdm1* (siPrdm1; black bars) siRNA. The genes indicated on the y-axis were analyzed as in Fig. 1g and are represented relative to the siGFP sample. (**b**) EL4 T cells were transfected with either a *Cxcr5*, *Il6ra*, *Btla*, or *Tnfsf8* promoter reporter and either an empty expression vector control, wild-type Blimp-1, or Blimp-1ΔZF. Normalized luciferase values are expressed relative to the vector control. (**c**) ChIP experiments were performed with wild-type T_H1 polarized cells maintained in either high (Hi; black bars) or low (Lo; white bars) IL-2 conditions. Chromatin samples were immunoprecipitated with either an antibody to Blimp-1 or an IgG control. Blimp-1-precipitated samples were normalized as in Fig. 3c to obtain the percent input value (Supplementary Fig. 6b). The data are graphically represented as fold enrichment, which is the ratio of the percent input for each sample relative to the low IL-2 sample from the same experiment (Hi; black bars or Lo; white bars). (**d**) Chromatin was isolated from either wild-type (black bars) or *Tbx21*$^{-/-}$ (white bars) $CD4^+$ T cells polarized in T_H1 conditions. The ChIP experiments were performed and normalized as in (**c**) with the exception that the fold enrichment is relative to the *Tbx21*$^{-/-}$ percent input values (Supplementary Fig. 6c). Data in (**a–d**) represent the mean of three independent experiments (error bars, s.e.m.).