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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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The transcription factors T-bet and Bcl-6 are required for the establishment of a T helper type 1 cell (T_H1 cell) and follicular helper T cell (T_{FH} cell) gene-expression profile, respectively. Here we found that high concentrations of interleukin 2 (IL-2) inhibited Bcl-6 expression in polarized T_H1 cells. Mechanistically, the low concentrations of Bcl-6 normally found in effector T_H1 cells did 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, which allowed excess Bcl-6 to repress its direct target *Prdm1* (which encodes the transcriptional repressor 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*. Thus, IL-2-signaling regulates the Bcl-6–Blimp-1 axis in T_H1 cells to maintain flexibility with a T_{FH} cell–like gene profile.

$CD4^+$ helper T cells can develop into a variety of functionally distinct subtypes after their initial encounter with foreign antigen. These subtypes include T helper type 1 cells (T_H1 cells), T_H2 cells, interleukin 17 (IL-17)-producing helper T cells (T_H17 cells) and follicular helper T cells (T_{FH} cells)[1–6]. The proper development and maintenance of helper T cell subsets is required for the clearance of specific pathogens without self damage. For example, T_H1 cells coordinate the immune response to intracellular pathogens, but their inappropriate activation results in autoimmunity[7,8].

Unique, signature gene-expression programs define each specialized helper T cell subtype. Helper T 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, naive helper T cells exposed to IL-12 and/or interferon-γ upregulate the T-box transcription factor T-bet, which is required for establishment of the T_H1 gene-expression profile[12]. In contrast, IL-4 induces the transcription factor GATA-3 to create a T_H2 gene program, whereas a combination of IL-6 and transforming growth factor-β upregulate the transcription factor 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]. So far, the signaling pathways that initially induce helper T cell lineage-defining transcription factors have been well characterized, but it is unclear whether changing environmental conditions can alter their composition after the primary commitment 'decision'.

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

Bcl-6 is a member of the BTB-POZ (bric-a-bric, tramtrack, broad complex–poxvirus zinc finger) family of transcriptional repressors. This family represses transcription by directly binding to specific DNA sequences through their zinc-finger DNA-binding domains, with the BTB-POZ domain mediating transcriptional repression[25]. It is unclear at present how Bcl-6 functionally activates T_{FH} signature genes. In T_{FH} cells, Bcl-6 represses a microRNA gene cluster to effectively stabilize the expression of several T_{FH} signature genes[13], but this does not explain the initial activation of the transcription of T_{FH} signature genes 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. That 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 the transcription factor STAT5, inhibited *Bcl6* expression in effector T_H1 cells, but when IL-2 was limiting, Foxo transcription factors were able to activate *Bcl6* transcription. Enhanced Bcl-6 expression in polarized T_H1

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cells resulted in the induction of *Cxcr5* (which encodes the chemokine receptor CXCR5) and a subset of T_{FH} genes. Mechanistically, altering the ratio of Bcl-6 to T-bet in T_H1 cells allowed Bcl-6 to repress its direct target gene *Prdm1* (which encodes the transcriptional repressor 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 induction potential for a subset of T_{FH} genes.

RESULTS

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

We found that T-bet physically interacted with Bcl-6 in T_H1 cells[24] (**Fig. 1a**), an interaction that targets T-bet–Bcl-6 complexes to a subset of T-bet DNA-binding elements[24]. That finding raised the question of why T-bet–Bcl-6 complexes are 'preferentially' targeted to the DNA-binding elements of T-bet rather than those of Bcl-6. To begin to address this question, we used coimmunoprecipitation analysis to define the domains in Bcl-6 and T-bet required for their interaction. A Bcl-6 truncation construct with deletion of its entire carboxy-terminal zinc-finger domain did not associate with T-bet[24] (**Fig. 1b** and **Supplementary Fig. 1a**). This domain contains six zinc fingers, of which the four most carboxy-terminal zinc fingers are required for DNA binding[26]. More detailed Bcl-6 truncation analysis demonstrated that the zinc fingers known to mediate the DNA-binding activity of Bcl-6 were also those required for its interaction with T-bet (**Fig. 1b**).

Next we located the domain in T-bet required for its association with Bcl-6. T-bet is composed of a central T-box DNA-binding domain as well as amino- and carboxy-terminal domains that mediate protein-protein interactions and transactivation events (**Supplementary Fig. 1a**). Truncation of the amino-terminal domain of T-bet did not impair its ability to interact with Bcl-6, whereas a T-bet C-terminal truncation construct did not associate with Bcl-6 in the coimmunoprecipitation analysis (**Fig. 1c**). Collectively, these data suggested that the interaction between T-bet and Bcl-6 has the potential to inhibit the DNA-binding activity of Bcl-6 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 the DNA-binding activity of Bcl-6, we transfected cells with luciferase reporter constructs containing either the *Prdm1* promoter alone or sequence encoding multimers of Bcl-6 DNA-binding elements upstream of the minimal SV40 promoter (3x-Bcl-6 promoter reporter). The 3x-Bcl-6 promoter reporter construct represents a simplified scenario in which the repression of a minimal promoter is solely dependent on 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 used EL4 mouse lymphoma T cells for these experiments because they endogenously express 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-Bcl-6 and *Prdm1* promoter reporters (**Supplementary Fig. 1b,c**).

If the interaction between T-bet and Bcl-6 inhibits the binding of Bcl-6 to DNA, 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 assessed whether increasing T-bet expression inhibited Bcl-6 from repressing the 3x-Bcl-6 and *Prdm1* promoter reporter constructs. For these experiments, we used a DNA-binding-mutant construct of T-bet to exclude the possibility that T-bet directly

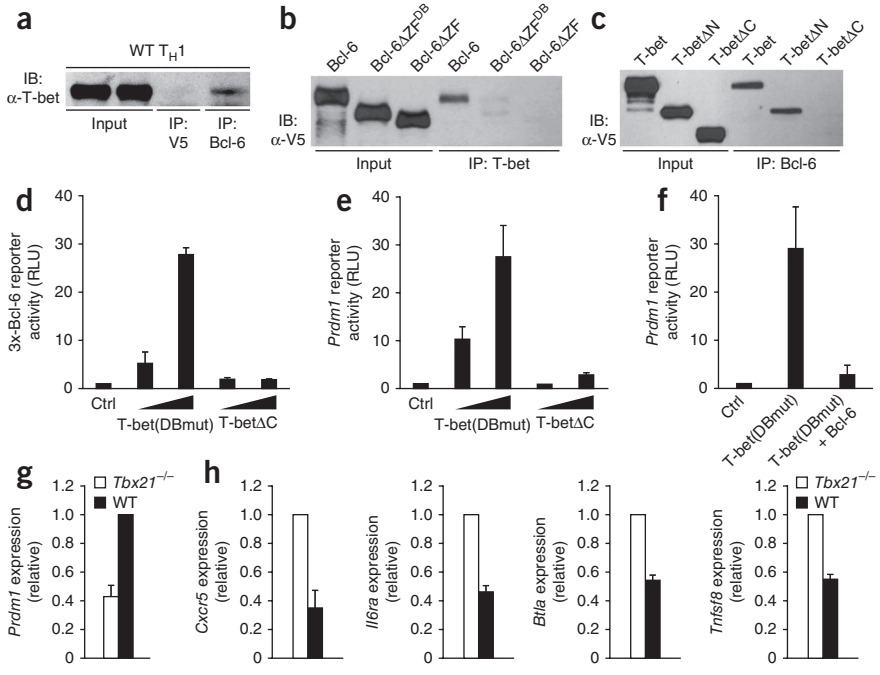

Figure 1 A T-bet–Bcl-6 complex inhibits Bcl-6-dependent repression. (**a**) Coimmunoprecipitation (IP) of endogenously expressed proteins from wild-type (WT) T_H1 cells with control antibody (to the V5 epitope tag) or anti-Bcl-6, followed by immunoblot analysis (IB) with anti-T-bet (α-T-bet). Input, immunoblot analysis without immunoprecipitation. (**b**,**c**) Coimmunoprecipitation of proteins from EL4 T cells transfected with an untagged T-bet expression construct in combination with V5-tagged wild-type Bcl-6 or Bcl-6 with deletion of zinc fingers known to mediate its DNA-binding activity (Bcl-6ΔZF^{DB}) or its entire carboxy-terminal zinc-finger domain (Bcl-6ΔZF; **b**) or with untagged Bcl-6 in combination with wild-type V5-tagged T-bet or T-bet with truncation of the amino-terminal domain (T-betΔN) or carboxy-terminal region (T-betΔC; **c**), followed by immunoprecipitation of proteins from lysates with anti-T-bet (**b**) or anti-Bcl-6 (**c**) and immunoblot analysis with anti-V5. (**d**,**e**) Luciferase activity in EL4 T cells cotransfected with a 3x-Bcl-6 promoter (**d**) or *Prdm1* promoter (**e**) luciferase reporter and either empty expression plasmid (control (Ctrl)) or increasing concentrations of expression plasmid for a T-bet DNA-binding mutant (T-bet(DBmut)) or T-bet with truncation of the carboxy-terminal region (T-betΔC); results are normalized to a renilla luciferase control and are presented in relative light units (RLU) relative to activity in the control condition. (**f**) Luciferase activity in EL4 T cells cotransfected with the *Prdm1* promoter luciferase reporter and empty vector or vector for the T-bet DNA-binding mutant alone or in combination with Bcl-6 (presented as in **d**,**e**). (**g**,**h**) Quantitative RT-PCR analysis of RNA isolated from wild-type or *Tbx21*$^{-/-}$ primary CD4$^+$ T cells polarized in T_H1 conditions; expression was normalized to expression of the control gene *Rps18* (encoding ribosomal protein S18) and results are presented relative to those of wild-type T_H1 cells (**g**) or *Tbx21*$^{-/-}$ cells (**h**). Data are representative of at least three (**a**–**f**,**h**) or five (**g**) independent experiments (mean and s.e.m. in **d**–**h**).

bound to and activated the 3x-Bcl-6 or *Prdm1* promoter reporter. Notably, overexpression of the T-bet DNA-binding-mutant construct alone substantially enhanced 3x-Bcl-6 and *Prdm1* promoter reporter activity but did not activate a control reporter containing the promoter of *Ifng* (which encodes the T_H1 cytokine interferon-γ; **Fig. 1d**,**e** and **Supplementary Fig. 1d**,**e**). We also did the promoter-reporter experiments with the T-bet C-terminal-truncation construct that did not interact with Bcl-6 (**Fig. 1c**). This construct did not inhibit Bcl-6-dependent repression (**Fig. 1d**,**e** and **Supplementary Fig. 1d**), which suggested that the interaction between T-bet and Bcl-6 was required for the ability of T-bet to alleviate the Bcl-6-dependent repression of the 3x-Bcl-6 and *Prdm1* promoter reporters.

We next hypothesized that the relative expression of T-bet and Bcl-6 defines the functional activity of Bcl-6 when both are expressed in the same cell. That is, in the presence of excess T-bet, formation of the T-bet–Bcl-6 complex inhibited most Bcl-6 from localizing to its own DNA-binding elements (**Fig. 1d**,**e**). However, an increase in the abundance of Bcl-6 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 the T-bet DNA-binding mutant and examined the functional consequences on Bcl-6-dependent repression. Bcl-6 overexpression restored repression of the 3x-Bcl-6 and *Prdm1* promoter reporters despite the presence of the T-bet DNA-binding mutant (**Fig. 1f** and **Supplementary Fig. 1f**,**g**). These data suggested that the ratio of T-bet to Bcl-6 determines whether Bcl-6 can repress its direct target genes.

Expression of T_{FH} signature genes in T-bet-deficient cells

Although the T-bet–Bcl-6 complex is functionally important for repressing a subset of T-bet target genes, it remains unclear 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* expression in primary $CD4^+$ T cells isolated from wild-type or T-bet-deficient (*Tbx21*$^{-/-}$) mice and then polarized in T_H1 conditions. In this experimental setting, both wild-type and *Tbx21*$^{-/-}$ cells have constant, low Bcl-6 expression[24], but because T-bet is not present to form a complex with Bcl-6 in the *Tbx21*$^{-/-}$ cells, this may allow 'free' Bcl-6 to repress its direct target genes. Consistent with that hypothesis, *Prdm1* expression was lower in *Tbx21*$^{-/-}$ T_H1 cells than in wild-type 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} cell–like gene-expression profile. A subset of T_{FH} signature genes[1,27], including *Cxcr5*, *Il6ra* (which encodes the IL-6 receptor α-chain), *Btla* (which encodes the inhibitory receptor CD272) and *Tnfsf8* (which encodes the ligand for the immunoregulatory receptor CD30), had higher expression in *Tbx21*$^{-/-}$ T_H1-polarized cells than in wild-type T_H1-polarized cells (**Fig. 1h**). To further explore those results, we also knocked down *Tbx21* in wild-type T_H1-polarized cells through the use of small interfering RNA (siRNA). 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 lower T-bet expression in a natural T_H1 setting. Similar to the data obtained with T-bet-deficient cells, knockdown of *Tbx21* in wild-type T_H1 cells resulted in the induction of a subset of T_{FH} signature genes (**Supplementary Fig. 2**). Collectively, these experiments suggested that the interaction between T-bet and Bcl-6 functionally regulated the activity of both T-bet and Bcl-6 in T_H1 cells[24] (**Fig. 1** and **Supplementary Figs. 1** and **2**).

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

The mechanistic findings presented thus far suggested that there may be flexibility between the T_H1 and T_{FH} gene programs if environmental signaling events regulate Bcl-6 expression in T_H1 cells. Therefore, we wanted to determine whether signaling pathways in developing T_H1 cells modulate Bcl-6 expression. Published 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 the expression of IL-2 receptor-α (IL-2Rα) and the expression of Bcl-6 in $CD4^+$ T_{FH} cells[29]. Thus, we hypothesized that IL-2R signaling may regulate Bcl-6 expression in T_H1 cells.

To test that 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, we stimulated $CD4^+$ T cells for 3 d with plate-bound antibody to CD3 (anti-CD3) and anti-CD28 in the presence of T_H1-polarizing conditions and IL-2. We then split the cells and maintained them in T_H1-polarizing conditions in the presence of variable concentrations of IL-2 for an additional 3 d. In developing T_H1 cells, the expression of *Bcl6* transcripts and Bcl-6 protein inversely correlated with the concentration of IL-2 (**Fig. 2a**,**b** and **Supplementary Fig. 3a**,**b**). In contrast, T-bet had similar expression in all IL-2 conditions (**Fig. 2b**,**c** and **Supplementary Fig. 3b**,**c**), which indicated that T_H1-polarizing conditions were dominant over IL-2 concentrations in the regulation of T-bet expression. Together these data suggested that the environmental concentration of IL-2 regulated Bcl-6 expression, but not T-bet expression, in developing T_H1 cells.

To further explore whether signaling through IL-2R inhibits Bcl-6 expression in T_H1 cells, we incubated developing T_H1 cells with blocking

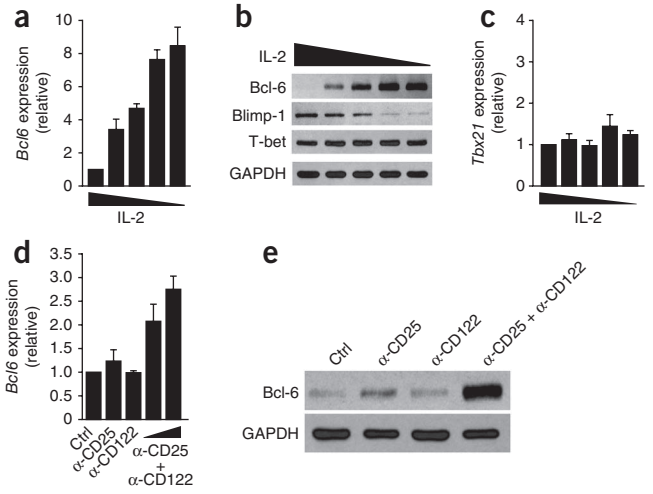

Figure 2 IL-2-signaling inhibits Bcl-6 expression in T_H1 cells. (**a**) Quantitative RT-PCR analysis of *Bcl6* expression in primary $CD4^+$ T cells cultured continuously for 6 d in T_H1-polarizing conditions, initially stimulated with plate-bound anti-CD3 and anti-CD28 and IL-2, then split at day 3 and cultured for an additional 3 d in the presence of decreasing concentrations of IL-2 (wedges); expression was normalized to that of *Rps18* and is presented relative to expression at the highest concentration of IL-2. (**b**) Immunoblot analysis of cells treated as in **a**; GAPDH (glyceraldehyde phosphate dehydrogenase) serves as a loading control throughout. (**c**) Quantitative RT-PCR analysis of *Tbx21* expression as in **a**. (**d**,**e**) Quantitative RT-PCR analysis (as in **a**) of *Bcl6* expression (**d**) and immunoblot analysis of Bcl-6 (**e**) in primary wild-type $CD4^+$ T cells cultured for 36 h in T_H1 conditions without antibody (Ctrl) or with anti-CD25 or anti-CD122 or both in combination. Data represent four (**a**,**c**) or three (**d**) independent experiments (mean and s.e.m.) or are representative of at least two (**b**) or three (**e**) independent experiments.

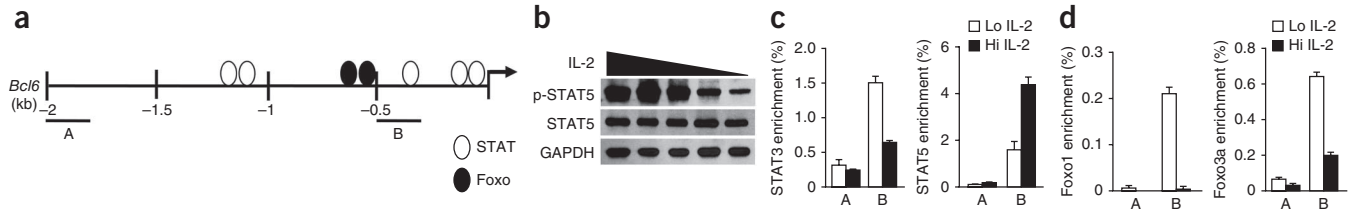

Figure 3 STAT and Foxo transcription factors regulate *Bcl6*. (**a**) STAT- and Foxo-binding elements in the *Bcl6* promoter; A and B indicate regions monitored by ChIP analysis. kb, kilobases. (**b**) Immunoblot analysis of phosphorylated (p-) and total STAT5 in wild-type T_H1 cells exposed to decreasing concentrations of IL-2 (wedge). (**c**,**d**) ChIP analysis of wild-type T_H1 polarized cells maintained in either a high (Hi IL-2) or low (Lo IL-2) concentration of IL-2, followed by immunoprecipitation of chromatin with immunoglobulin G (IgG; control) or anti-STAT3 or anti-STAT5 (**c**) or anti-Foxo1 or anti-Foxo3a (**d**) and quantitative PCR analysis of binding at the binding element (B) or a negative control region (A) in the *Bcl6* promoter (as in **a**); results were normalized to those of a standardized aliquot of input chromatin, followed by subtraction of results obtained with IgG (nonspecific background). Data are representative of three independent experiments (mean and s.e.m. in **c**,**d**).

antibodies to the IL-2Rα (CD25) and IL-2Rβ (CD122) subunits of the IL-2R complex. We found that blocking both CD25 and CD122 in combination enhanced the expression of *Bcl6* transcripts and Bcl-6 protein (**Fig. 2d**,**e**). These data were consistent with the findings from the IL-2 'titration' experiments and together provided evidence that strong IL-2R signaling inhibited 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 in the *Bcl6* promoter identified DNA-binding sites for the STAT and Foxo families of transcription factors (**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 assess whether strong IL-2 signaling inhibited *Bcl6* expression by enhancing the binding of STAT5 itself and/or the ratio of STAT5 to STAT3 at the *Bcl6* promoter in T_H1 cells.

We first confirmed that phosphorylation of STAT5 was enhanced with increasing IL-2 concentrations in T_H1 cells (**Fig. 3b**). Next we used chromatin immunoprecipitation (ChIP) to assess the binding of STAT3 and STAT5 to the *Bcl6* promoter in T_H1 cells cultured in a high or low concentration of IL-2 (**Fig. 3c**). The ChIP experiments demonstrated that the ratio of STAT3 to STAT5 bound to the *Bcl6* promoter varied with IL-2 concentration. Specifically, the binding of STAT5 to the *Bcl6* promoter was greater, whereas the binding of STAT3 was lower, in T_H1 cells cultured in a high concentration of IL-2 (**Fig. 3c**). Enhanced binding of STAT5 correlated with the inhibition of *Bcl6* expression in T_H1 cells exposed to increasing concentrations of IL-2 (**Fig. 2a**,**b**). These data were 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

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

Consistent with published findings obtained with nonpolarized $CD4^+$ T cells[32], *Foxo1* expression was lower in T_H1 cells cultured in a high concentration of IL-2 (**Supplementary Fig. 4c**). We next did ChIP experiments to assess the binding of Foxo1 and Foxo3a to the *Bcl6* promoter in T_H1 cells maintained in a high or low concentration of IL-2 (**Fig. 3d**). Both Foxo1 and Foxo3a bound to the *Bcl6* promoter when T_H1 cells were maintained in a low concentration of IL-2, which correlated with *Bcl6* expression in those conditions. In contrast, binding of Foxo1 and Foxo3a to the *Bcl6* promoter was substantially lower in T_H1 cells cultured in a high concentration of IL-2 (**Fig. 3d**). Collectively, these data suggested that IL-2 regulated binding of the transcriptional activators Foxo1 and Foxo3a to the *Bcl6* promoter in primary T_H1 cells.

T_H1 cells can upregulate a T_{FH}-like gene profile

We next wanted to determine whether the greater abundance of Bcl-6 in T_H1 cells altered the gene-expression profile of the cell (**Fig. 4**). We first examined expression of the direct Bcl-6 target *Prdm1* in T_H1 cells cultured in variable IL-2 conditions. The expression of *Prdm1* transcripts and Blimp-1 protein was substantially lower in T_H1 cells maintained in a limiting concentration of IL-2 (**Figs. 2b** and **4a** and **Supplementary Fig. 3b**). Notably, there seemed to be a threshold for the amount of Bcl-6 needed for effective repression of Blimp-1 expression. These data suggested that similar to the findings reported above (**Fig. 1**), naturally increasing the ratio of Bcl-6 to T-bet above a threshold in primary T_H1 cells resulted in the functional repression of a prototypic Bcl-6 target gene.

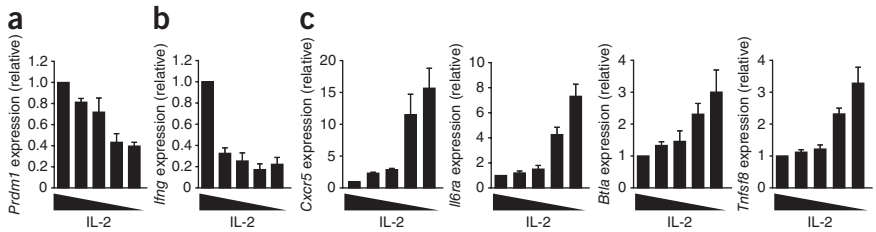

Figure 4 IL-2 regulates the expression of *Prdm1* and T_{FH} cell–associated genes in T_H1 cells. Quantitative RT-PCR analysis of the expression of *Prdm1* (**a**), *Ifng* (**b**) or T_{FH} cell–associated genes (**c**) in primary wild-type $CD4^+$ T cells cultured continuously for 6 d in T_H1-polarizing conditions, with decreasing concentrations of IL-2 (wedges) from day 3 to day 6 (as described in **Fig. 2a**; results normalized and presented as in **Fig. 2a**). Data represent four independent experiments (mean and s.e.m.).

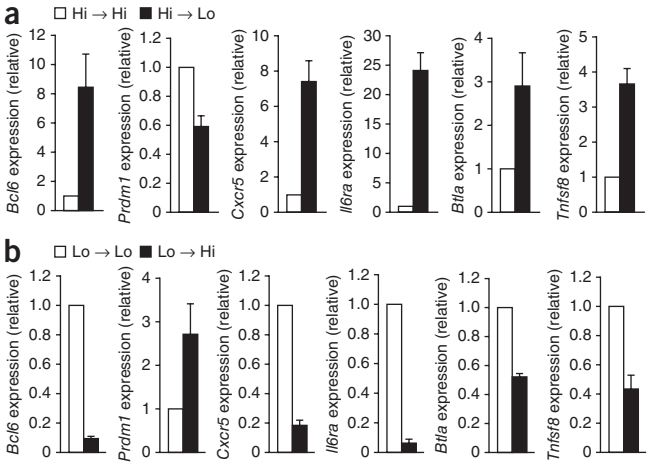

Figure 5 T_H1 cells maintain IL-2-sensitive Bcl-6 and T_{FH} gene regulation. (**a**) Quantitative RT-PCR analysis of gene expression in primary wild-type $CD4^+$ T cells cultured for 6 d in T_H1-polarizing conditions and a high concentration of IL-2, then split and maintained for an additional 3 d in T_H1-polarizing conditions with a high concentration of IL-2 (Hi→Hi) or a low concentration of IL-2 (Hi→Lo). (**b**) Quantitative RT-PCR analysis of gene expression in primary wild-type $CD4^+$ T cells cultured in T_H1-polarizing conditions for 3 d, then split and maintained in a low concentration of IL-2 for 3 more days, then split again and maintained for an additional 3 d in T_H1-polarizing conditions with a low concentration of IL-2 (Lo→Lo) or a high concentration of IL-2 (Lo→Hi). Results were normalized to the *Rps18* control and are presented relative to those of cells maintained in a high (**a**) or low (**b**) concentration of IL-2 throughout. Data represent at least three independent experiments (mean and s.e.m.).

We then assessed whether the enhanced Bcl-6 expression in T_H1 cells was sufficient to upregulate genes associated with T_{FH} cells. *Cxcr5* was induced more than 15-fold in developing T_H1 cells cultured in a low concentration of IL-2 relative to its expression in T_H1 cells maintained in a high concentration of IL-2 (**Fig. 4c**). Three other T_{FH} cell–associated genes, *Il6ra*, *Btla* and *Tnfsf8*, were also upregulated in this setting (**Fig. 4c**). In contrast, T_H1 cells maintained in a limiting concentration of IL-2 had lower expression of *Ifng* relative to its expression in T_H1 cells cultured in a high concentration of IL-2 (**Fig. 4b**); this might indicate a shift in the balance of the helper T cell program. Notably, not all T_{FH} signature genes were induced in T_H1 cells coincident with Bcl-6 upregulation. In particular, the expression of *Pdcd1* (which encodes the inhibitory receptor PD-1) and *Icos* (which encodes the inducible T cell costimulator ICOS) was unchanged (**Supplementary Fig. 5**). These data suggested that enhanced Bcl-6 expression in T_H1 cells induced a partial T_{FH} profile, but additional events were needed 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 retained the flexibility to modulate Bcl-6 expression in response to IL-2-signaling. To investigate this, we cultured $CD4^+$ T cells continuously for 9 d in T_H1-polarizing conditions and either held the IL-2 concentration constant or altered it after 6 d of polarization. *Bcl6* expression was induced when fully polarized T_H1 cells maintained in a high concentration of IL-2 were switched to a low concentration of 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 a low concentration of IL-2 was substantially downregulated when T_H1-polarized cells were exposed to a high concentration of IL-2 (**Fig. 5b**). Collectively, these data indicated that IL-2 regulated 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 concentrations of IL-2 (**Figs. 2b** and **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 limit the 'Blimp-1 brake' in place on the T_{FH} cell–associated genes. Therefore, *Prdm1* is a good candidate as 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 repressed the endogenous expression of T_{FH} signature genes in primary T_H1 cells. We transfected wild-type T_H1 cells with control or *Prdm1*-specific siRNA and analyzed the consequences on endogenous gene expression. *Prdm1* expression was lower in T_H1 cells transfected with *Prdm1*-specific siRNA than in those transfected with the control siRNA (**Fig. 6a**). Consistent with a role for Blimp-1 in repressing T_{FH} gene expression in T_H1 cells, the expression of *Cxcr5*, *Il6ra*, *Btla* and *Tnfsf8* was substantially enhanced when Blimp-1 expression was diminished (**Fig. 6a**). These data indicated that Blimp-1 functionally repressed a subset of T_{FH} signature genes in primary effector T_H1 cells.

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

Blimp-1 binds to T_{FH} cell–associated genes in T_H1 cells

If Blimp-1 directly represses IL-2-sensitive T_{FH} signature genes in effector T_H1 cells, then binding of Blimp-1 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 a high concentration of IL-2, which coincided with the repression of these genes (**Fig. 6c** and **Supplementary Fig. 6b**). In contrast, there was substantially less binding of Blimp-1 at the *Cxcr5*, *Il6ra*, *Btla* and *Tnfsf8* promoters in response to a limiting concentration of IL-2 (**Fig. 6c** and **Supplementary Fig. 6b**). The loss of Blimp-1 binding correlated with the induction of these genes (**Figs. 4c** and **6c**). Collectively, these data suggested that Blimp-1 directly bound to and repressed 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 increase in the ratio of Bcl-6 to T-bet in T_H1 cells into the downstream activation potential for T_{FH} cell–associated genes. To address this, we examined the binding of Blimp-1 to the *Cxcr5*, *Il6ra*, *Btla* and *Tnfsf8* promoters in *Tbx21*$^{-/-}$ cells. There was less binding of Blimp-1 at these promoters in *Tbx21*$^{-/-}$ T_H1-polarized cells than in wild-type T_H1-polarized cells (**Fig. 6d** and **Supplementary Fig. 6c**), which coincided with higher gene expression (**Fig. 1h**). Thus, Blimp-1 binding inversely correlated with

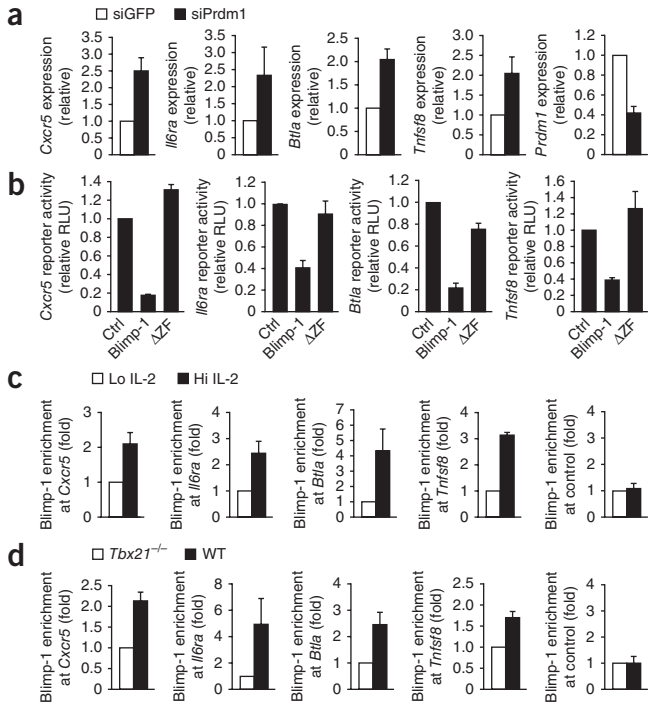

Figure 6 Blimp-1 directly represses T_{FH} genes in effector T_H1 cells. (**a**) Quantitative RT-PCR analysis (as in **Fig. 1g**) of wild-type T_H1 cells transfected with control siRNA (siGFP) or *Prdm1*-specific siRNA (siPrdm1); results are presented relative to those of cells transfected with control siRNA. (**b**) Luciferase activity of EL4 T cells transfected with a *Cxcr5*, *Il6ra*, *Btla* or *Tnfsf8* promoter luciferase reporter and empty expression vector (control) or vector for wild-type Blimp-1 or DNA-binding-mutant Blimp-1 (Blimp-1ΔZF); normalized luciferase activity is presented relative to that of cells transfected with empty expression vector. (**c**) ChIP analysis of wild-type T_H1-polarized cells maintained in a high or low concentration of IL-2, followed by immunoprecipitation of chromatin with anti-Blimp-1 or IgG and analysis of enrichment for Blimp-1 at various promoters; results were normalized (as in **Fig. 3c**) to obtain the percentage of input (**Supplementary Fig. 6b**) and are presented relative to those of cells maintained in a low concentration of IL-2. (**d**) ChIP analysis (as in **c**) of wild-type or *Tbx21*$^{-/-}$ $CD4^+$ T cells polarized in T_H1 conditions; results are presented relative to the input values for *Tbx21*$^{-/-}$ cells (**Supplementary Fig. 6c**). Data represent three independent experiments (mean and s.e.m.).

an increase in the ratio of Bcl-6 to T-bet in T_H1 cells achieved 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 determined the downstream potential of a subset of T_{FH} signature genes by controlling Blimp-1-mediated repression (**Supplementary Fig. 7**).

DISCUSSION

This study has demonstrated that variable IL-2-signaling regulated Bcl-6 expression in polarized T_H1 cells. In effector T_H1 cells, a high ratio of T-bet to Bcl-6 promoted formation of the T-bet–Bcl-6 complex, which masked the Bcl-6 DNA-binding domain. As Bcl-6 expression was enhanced in T_H1 cells maintained in a low concentration of IL-2, 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 the T_{FH} gene-expression activation potential in $CD4^+$ T cells. Collectively, these data suggested that T_H1 cells retained flexibility with a T_{FH} cell–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 helper T cell lineage–defining transcription factors are expressed in a mutually exclusive pattern, but research has questioned that simplistic paradigm. There is now increasing awareness that opposing helper T cell lineage–defining transcription factors are coexpressed in many circumstances and that their coexpression is functionally important for regulating the gene-expression profile of the cell[21,24,39–41]. This raises the question of how the expression and functional activities of these factors are precisely regulated during an immune response. Our study has demonstrated that T-bet was able to dominantly control Bcl-6 activity because a T-bet–Bcl-6 complex masked the DNA-binding domain of Bcl-6 but left the DNA-binding domain of T-bet available. This effectively allowed T-bet to keep Bcl-6 in check in effector T_H1 cells. However, when Bcl-6 expression increased past the threshold of T-bet control, the balance of the cell shifted toward a T_{FH} cell–like gene profile.

The environmental concentration of IL-2, 'translated' through the activity of STAT and Foxo transcription factors, regulated Bcl-6 expression in polarized T_H1 cells. This meant that T_H1 cells retained flexibility with a T_{FH} cell–like gene profile because they were able to alter Bcl-6 expression in response to IL-2-signaling. A published study has found that IL-2-signaling is critical for the formation of many helper T cell lineages[42]. Our findings have added to that and suggest that IL-2-signaling can change the phenotype of polarized T_H1 cells. Notably, published research suggests that IL-2Rα expression inversely correlates with Bcl-6 expression to create a continuum of central memory T cell, T_{FH} cell or effector T_H1 cell characteristics[29,43]. It is possible that the expression pattern of IL-2R subunits on a $CD4^+$ T cell will allow individual cells in a population to respond differently to the same environmental IL-2 conditions.

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

The activation of the T_{FH} gene program is a multistep process, with the Bcl-6-dependent removal of the 'Blimp-1 brake' representing a first step. Interactions between ICOS and its ligand 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 are involved in T_{FH} differentiation[44,45]. Further studies are needed to determine the complete series of molecular events that occur downstream of the removal of the Blimp-1-mediated repression required for full activation of 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 those encoding PD-1 and ICOS, were upregulated in a low concentration of IL-2, which indicated that distinct classes of T_{FH} target genes exist that require either additional or completely independent events. Notably, $CXCR5^{hi}PD\text{-}1^{lo}$ helper T cells exist outside the germinal center and represent a T_{FH}-like cell before homing to 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 for the T_{FH} gene program occur. Overexpression of Bcl-6 alone is sufficient for complete T_{FH} 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 the flexibility to adopt a T_{FH} gene program.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

K.J.O. and A.S.W. designed and did experiments, analyzed data and wrote the manuscript; and S.E.M contributed to the experiments in **Figures 1c** and **2b**,**e**, and **Supplementary Figure 4a**,**b**.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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1. Crotty, S. Follicular helper CD4 T cells (T_{FH}). *Annu. Rev. Immunol.* **29**, 621–663 (2011).
2. Murphy, K.M. & Stockinger, B. Effector T cell plasticity: flexibility in the face of changing circumstances. *Nat. Immunol.* **11**, 674–680 (2010).
3. O'Shea, J.J. & Paul, W.E. Mechanisms underlying lineage commitment and plasticity of helper $CD4^+$ T cells. *Science* **327**, 1098–1102 (2010).
4. Reinhardt, R.L., Kang, S.J., Liang, H.E. & Locksley, R.M. T helper cell effector fates–who, how and where? *Curr. Opin. Immunol.* **18**, 271–277 (2006).
5. Zhou, L., Chong, M.M. & Littman, D.R. Plasticity of $CD4^+$ T cell lineage differentiation. *Immunity* **30**, 646–655 (2009).
6. Zhu, J., Yamane, H. & Paul, W.E. Differentiation of effector CD4 T cell populations. *Annu. Rev. Immunol.* **28**, 445–489 (2010).
7. Peng, S.L. The T-box transcription factor T-bet in immunity and autoimmunity. *Cell Mol. Immunol.* **3**, 87–95 (2006).
8. Lazarevic, V. & Glimcher, L.H. T-bet in disease. *Nat. Immunol.* **12**, 597–606 (2011).
9. Ivanov, I.I. *et al.* The orphan nuclear receptor RORγt directs the differentiation program of proinflammatory IL-17^+ T helper cells. *Cell* **126**, 1121–1133 (2006).
10. Johnston, R.J. *et al.* Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* **325**, 1006–1010 (2009).
11. Nurieva, R.I. *et al.* Bcl6 mediates the development of T follicular helper cells. *Science* **325**, 1001–1005 (2009).
12. Szabo, S.J. *et al.* A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* **100**, 655–669 (2000).
13. Yu, D. *et al.* The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity* **31**, 457–468 (2009).
14. Zheng, W. & Flavell, R.A. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* **89**, 587–596 (1997).
15. Beima, K.M. *et al.* T-bet binding to newly identified target gene promoters is cell type-independent but results in variable context-dependent functional effects. *J. Biol. Chem.* **281**, 11992–12000 (2006).
16. Djuretic, I.M. *et al.* Transcription factors T-bet and Runx3 cooperate to activate *Ifng* and silence *Il4* in T helper type 1 cells. *Nat. Immunol.* **8**, 145–153 (2007).
17. Lewis, M.D., Miller, S.A., Miazgowicz, M.M., Beima, K.M. & Weinmann, A.S. T-bet's ability to regulate individual target genes requires the conserved T-box domain to recruit histone methyltransferase activity and a separate family member-specific transactivation domain. *Mol. Cell. Biol.* **27**, 8510–8521 (2007).
18. Miller, S.A., Huang, A.C., Miazgowicz, M.M., Brassil, M.M. & Weinmann, A.S. Coordinated but physically separable interaction with H3K27-demethylase and H3K4-methyltransferase activities are required for T-box protein-mediated activation of developmental gene expression. *Genes Dev.* **22**, 2980–2993 (2008).
19. Miller, S.A., Mohn, S.E. & Weinmann, A.S. Jmjd3 and UTX play a demethylase-independent role in chromatin remodeling to regulate T-box family member-dependent gene expression. *Mol. Cell* **40**, 594–605 (2010).
20. Szabo, S.J. *et al.* Distinct effects of T-bet in TH1 lineage commitment and IFN-γ production in CD4 and CD8 T cells. *Science* **295**, 338–342 (2002).
21. Hwang, E.S., Szabo, S.J., Schwartzberg, P.L. & Glimcher, L.H. T helper cell fate specified by kinase-mediated interaction of T-bet with GATA-3. *Science* **307**, 430–433 (2005).
22. Lazarevic, V. *et al.* T-bet represses T_H17 differentiation by preventing Runx1-mediated activation of the gene encoding RORγt. *Nat. Immunol.* **12**, 96–104 (2011).
23. Mehta, D.S., Wurster, A.L., Weinmann, A.S. & Grusby, M.J. NFATc2 and T-bet contribute to T-helper-cell-subset-specific regulation of IL-21 expression. *Proc. Natl. Acad. Sci. USA* **102**, 2016–2021 (2005).
24. Oestreich, K.J., Huang, A.C. & Weinmann, A.S. The lineage-defining factors T-bet and Bcl-6 collaborate to regulate Th1 gene expression patterns. *J. Exp. Med.* **208**, 1001–1013 (2011).
25. Basso, K. & Dalla-Favera, R. BCL6: master regulator of the germinal center reaction and key oncogene in B cell lymphomagenesis. *Adv. Immunol.* **105**, 193–210 (2010).
26. Mascle, X., Albagli, O. & Lemercier, C. Point mutations in BCL6 DNA-binding domain reveal distinct roles for the six zinc fingers. *Biochem. Biophys. Res. Commun.* **300**, 391–396 (2003).
27. Vinuesa, C.G., Tangye, S.G., Moser, B. & Mackay, C.R. Follicular B helper T cells in antibody responses and autoimmunity. *Nat. Rev. Immunol.* **5**, 853–865 (2005).
28. Pipkin, M.E. *et al.* Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells. *Immunity* **32**, 79–90 (2010).
29. Choi, Y.S. *et al.* ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. *Immunity* **34**, 932–946 (2011).
30. Liao, W., Lin, J.X. & Leonard, W.J. IL-2 family cytokines: new insights into the complex roles of IL-2 as a broad regulator of T helper cell differentiation. *Curr. Opin. Immunol.* **23**, 598–604 (2011).
31. Merkenschlager, M. & von Boehmer, H. PI3 kinase signalling blocks Foxp3 expression by sequestering Foxo factors. *J. Exp. Med.* **207**, 1347–1350 (2010).
32. Stittrich, A.B. *et al.* The microRNA miR-182 is induced by IL-2 and promotes clonal expansion of activated helper T lymphocytes. *Nat. Immunol.* **11**, 1057–1062 (2011).
33. Mandal, M. *et al.* Epigenetic repression of the Igk locus by STAT5-mediated recruitment of the histone methyltransferase Ezh2. *Nat. Immunol.* **12**, 1212–1220 (2011).
34. Yang, X.P. *et al.* Opposing regulation of the locus encoding IL-17 through direct, reciprocal actions of STAT3 and STAT5. *Nat. Immunol.* **12**, 247–254 (2011).
35. Riou, C. *et al.* Convergence of TCR and cytokine signaling leads to FOXO3a phosphorylation and drives the survival of $CD4^+$ central memory T cells. *J. Exp. Med.* **204**, 79–91 (2007).
36. Hurtz, C. *et al.* BCL6-mediated repression of p53 is critical for leukemia stem cell survival in chronic myeloid leukemia. *J. Exp. Med.* **208**, 2163–2174 (2011).
37. Crotty, S., Johnston, R.J. & Schoenberger, S.P. Effectors and memories: Bcl-6 and Blimp-1 in T and B lymphocyte differentiation. *Nat. Immunol.* **11**, 114–120 (2010).
38. Martins, G. & Calame, K. Regulation and functions of Blimp-1 in T and B lymphocytes. *Annu. Rev. Immunol.* **26**, 133–169 (2008).
39. Hegazy, A.N. *et al.* Interferons direct Th2 cell reprogramming to generate a stable GATA-3^+T-bet^+ cell subset with combined Th2 and Th1 cell functions. *Immunity* **32**, 116–128 (2010).
40. Koch, M.A. *et al.* The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat. Immunol.* **10**, 595–602 (2009).
41. Wang, Y., Su, M.A. & Wan, Y.Y. An essential role of the transcription factor GATA-3 for the function of regulatory T cells. *Immunity* **35**, 337–348 (2011).
42. Liao, W., Lin, J.X., Wang, L., Li, P. & Leonard, W.J. Modulation of cytokine receptors by IL-2 broadly regulates differentiation into helper T cell lineages. *Nat. Immunol.* **12**, 551–559 (2011).
43. Pepper, M., Pagan, A.J., Igyarto, B.Z., Taylor, J.J. & Jenkins, M.K. Opposing signals from the Bcl6 transcription factor and the interleukin-2 receptor generate T helper 1 central and effector memory cells. *Immunity* **35**, 583–595 (2011).
44. Bauquet, A.T. *et al.* The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. *Nat. Immunol.* **10**, 167–175 (2009).
45. Ise, W. *et al.* The transcription factor BATF controls the global regulators of class-switch recombination in both B cells and T cells. *Nat. Immunol.* **12**, 536–543 (2011).
46. Lee, S.K. *et al.* B cell priming for extrafollicular antibody responses requires Bcl-6 expression by T cells. *J. Exp. Med.* **208**, 1377–1388 (2011).

ONLINE METHODS

Cell culture and transfection. Primary $CD4^+$ T cells were isolated from the spleen and lymph nodes of wild-type C57BL/6 mice or *Tbx21*$^{-/-}$ mice with a Mag Cellect kit (R&D Systems) as described[15]. After isolation, cells were grown on plate-bound anti-CD3 (145-2C11; BD Biosciences) and anti-CD28 (37.51; BD Biosciences) in the presence of IL-2 (NCI) and T_H1-polarizing cytokines (anti-IL-4 (10 μg/ml; 11B.11; NCI) and IL-12 (5 ng/ml; R&D Systems)). On day 3, cells were split and maintained in T_H1 conditions for an additional 3 d. During this time, the cells were cultured in a range of IL-2 concentrations (500, 100, 50, 10 or 1 IU/ml). In this context, 500 IU/ml represents a high concentration of IL-2 (Hi) and 10 IU/ml represents a low concentration of IL-2 (Lo). For IL-2R blockade, anti-CD25 (PC61; BD Biosciences) and anti-CD122 (TM-β1; BD Biosciences) were used. Primary $CD4^+$ T cells were transfected through the use of the Lonza nucleofection system with mouse primary T cell solutions and program X-01 as described[18,19]. Cells were transfected with siRNA as described with an siRNA smartpool to either *Tbx21* or *Prdm1* (Dharmacon) or GFP as a control (Ambion)[18,19]. All experiments involving mice had approval of the Institutional Animal Care and Use Committee.

EL4 T cells were transfected through the use of the Lonza nucleofection system program 0-17 and solution V as described[15]. Immunoblot analysis was used to assess expression of the transfected proteins.

Promoter reporter assay. The 3x-Bcl-6 promoter reporter construct was made by cloning of sequence encoding three Bcl-6 DNA-binding elements upstream of the minimal SV40 promoter in the pGL3-promoter reporter vector (Promega). Promoter reporter constructs for *Bcl6* (positions +1998 to +1 (in base pairs)), *Prdm1* (positions +1991 to −222), *Cxcr5* (positions +1841 to −12), *Btla* (positions +913 to −16), *Tnfsf8* (positions +1458 to −226), and *Il6ra* (+1209 to −123 bp) were prepared by cloning of each promoter into the pGL3-basic luciferase reporter construct (Promega). EL4 cells were cotransfected with each promoter reporter construct in combination with expression vectors for V5-tagged T-bet DNA-binding mutant, T-bet with truncation of the carboxy-terminal region, Bcl-6, Blimp-1 or a Blimp-1 DNA-binding mutant or empty vector. A TK-renilla control plasmid was also cotransfected and used for normalization of transfection efficiency. Transfected cells were collected after 16–24 h and samples were analyzed with the Dual-Luciferase Reporter system (Promega). Expression of the transfected constructs was monitored by immunoblot analysis (**Supplementary Figs. 1d**,**f** and **6a**).

Coimmunoprecipitation. Coimmunoprecipitation assays were done as described[18,19,24]. Anti-T-bet (H-210; Santa Cruz Biotechnologies) or anti-Bcl-6 (C-19; Santa Cruz Biotechnologies) was used for immunoprecipitation. Coimmunoprecipitated proteins were detected with anti-V5 (R960-25; Invitrogen). Anti-Bcl-6 (C-19; Santa Cruz Biotechnologies) or anti-V5 (control antibody; ab15828; Abcam) was used for the coimmunoprecipitation of endogenous proteins from primary wild-type T_H1 cells. Immunoblots were then probed with anti-T-bet (4B10; Santa Cruz).

RNA and quantitative RT-PCR. RNA was obtained by Nucleospin RNA purification (Machery-Nagel) and cDNA was prepared with the First Strand Superscript II Synthesis System (Invitrogen). Quantitative PCR used 20 ng cDNA template, gene-specific primers (sequences, **Supplementary Table 1**) and qPCR Sybr Green Mix (Biorad). All results were first normalized to those of the *Rps18* control and are presented as normalized expression for the sample relative to the appropriate comparison condition (noted in legends).

Immunoblot analysis. An equal number of primary wild-type T_H1 cells were collected for each IL-2 treatment condition and analyzed by immunoblot for measurement of protein expression. Anti-T-bet (4B10), anti-STAT5 (C-17) and anti-GAPDH (FL-335) were from Santa Cruz; anti-Bcl-6 (561520) and antibody to phosphorylated STAT5 (611964) were from BD Pharmingen. Anti-Blimp-1 (A01647) was from Genscript.

ChIP assay. ChIP assays were done as described[15,17,18,24]. Anti-STAT3 (C-20), anti-STAT5 (C-17), anti-Foxo1 (H-128) and anti-Foxo3a (H-144) were from Santa Cruz Biotechnology; anti-Blimp-1 (A01647) was from Genscript. Chromatin was collected from primary polarized wild-type T_H1 cells maintained in a high or low concentration of IL-2 or from *Tbx21*$^{-/-}$ $CD4^+$ T cells. Precipitated DNA was analyzed by quantitative PCR with promoter-specific primers (sequences, **Supplementary Table 1**). Results were normalized to those of a standardized total input DNA control followed by subtraction of results obtained with IgG (nonspecific background) to obtain the percent input for each sample. Enrichment was calculated by division of the percent input of the samples by that of cells maintained in a low concentration of IL-2 or *Tbx21*$^{-/-}$ cells from the same experiment.

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