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Early Th1 Cell Differentiation Is Marked by a Tfh Cell-like Transition

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SUMMARY

Follicular helper T (Tfh) cells comprise an important subset of helper T cells; however, their relationship with other helper lineages is incompletely understood. Herein, we showed interleukin-12 acting via the transcription factor STAT4 induced both *Il21* and *Bcl6* genes, generating cells with features of both Tfh and Th1 cells. However, STAT4 also induced the transcription factor T-bet. With ChIP-seq, we defined the genome-wide targets of T-bet and found that it repressed *Bcl6* and other markers of Tfh cells, thereby attenuating the nascent Tfh cell-like phenotype in the late phase of Th1 cell specification. Tfh-like cells were rapidly generated after *Toxoplasma gondii* infection in mice, but T-bet constrained Tfh cell expansion and consequent germinal center formation and antibody production. Our data argue that Tfh and Th1 cells share a transitional stage through the signal mediated by STAT4, which promotes both phenotypes. However, T-bet represses Tfh cell functionalities, promoting full Th1 cell differentiation.

INTRODUCTION

Differentiation of naive $CD4^+$ T cells into functionally distinct T helper subsets is essential for effective immunity toward diverse microbial pathogens (Abbas et al., 1996; Murphy and Reiner, 2002). These subsets are specified by extrinsic and intrinsic cues, and the resultant cell populations acquire seemingly stable phenotypes that are reinforced by epigenetic modifications (Ansel et al., 2003; Aune et al., 2009). The cytokine environment during priming with consequent activation of signal transducer and activator of transcription (STAT) family DNA binding proteins are key initiators of this process. That is, the cytokines IL-12 and IFN-γ acting via STAT4 and STAT1 are important for specification and cell fate commitment for T helper 1 (Th1) cells (Szabo et al., 2003; Wilson et al., 2009). Activation of STAT1 and STAT4 promote expression of *Tbx21* (T-bet), a master regulator that promotes Th1 cell differentiation (Afkarian et al., 2002; Lighvani et al., 2001).

Follicular helper T (Tfh) cells have recently been proposed to be another distinct subset of helper T cells with specialized function in enhancing germinal center (GC) formation and regulating B cell function (Crotty, 2011; Fazilleau et al., 2009; King et al., 2008; Nurieva and Chung, 2010; Yu and Vinuesa, 2010). Tfh cells are characterized by selective expression of a variety of molecules including the surface markers such as CXC chemokine receptor 5 (CXCR5), programmed death 1 (PD-1), and inducible costimulator (ICOS), and their signature cytokine has been suggested to be IL-21 (Crotty, 2011). As with other lineages, extrinsic cytokine cues, namely IL-6 and IL-21, acting via STAT3, have been proposed to be drivers of Tfh cell differentiation (Fazilleau et al., 2009; King et al., 2008). These factors induce expression of Bcl6, which has come to be viewed as the critical regulator of Tfh cells (King et al., 2008; Nurieva et al., 2009). Bcl6, in turn, promotes CXCR5 expression and GC formation while functioning as a transcriptional repressor for alternative lineages by suppressing cytokines, transcription factors, and microRNAs essential for other cell fates (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009). These findings have been invoked to support the lineage sovereignty of Tfh cells.

However, there are other seemingly conflicting findings with regard to the origin and generation of Tfh cell and their relationship to other lineages. First, $CXCR5^+$ Tfh cells are present in the setting of STAT3 deficiency, suggesting alternative, STAT3-independent pathways for Tfh cell development (Eddahri et al., 2009). Recent results support this by demonstrating that IL-6 and IL-21 are insufficient for Tfh cell generation in vivo (Eto et al., 2011; Poholek et al., 2010). Second, activated $CD4^+$ T cells are not devoid of Bcl6 and IL-21; these factors are not exclusively expressed by Tfh cells (Lund et al., 2005; Mehta et al., 2005; Suto et al., 2008; Wei et al., 2007). Third, it has been reported that IL-12 is capable of driving IL-21 production in human T cells, with the generation of Tfh cell-like (Ma et al., 2009; Schmitt et al., 2009), although it has also been reported that IL-12 does not induce IL-21 production in murine T cells and that T-bet negatively regulates IL-21 (Mehta et al., 2005; Suto et al., 2008). $CXCR5^+CD4^+$ human memory T cells have been reported to share functional properties with Tfh cells, but

comprise Th1, Th2, and Th17 cells. However, Th2 and Th17, but not Th1, cells have the capacity to help B cells (Morita et al., 2011). Thus, it remains to be determined whether there is a species-specific difference in the regulation of IL-21. Fourth, whether Tfh cells can become differentiated Th1, Th2, Th17, and regulatory T (Treg) cells or vice versa is also unclear; however, Treg cells can differentiate into Tfh cells in Peyer's patches in the gut (Tsuji et al., 2009). These results raise several important questions regarding the relationship between Tfh and Th1 cells. Does STAT4 directly drive markers of Tfh cells in mouse and man, and if so, how does this relate to the Th1 cell differentiation program? Specifically, what are the respective roles of STAT4 and T-bet on Tfh cell differentiation and the induction of the master regulator, Bcl6, and what are the molecular mechanisms that determine Tfh versus Th1 cell specification?

In this study, we report that IL-12 can induce IL-21- and IFN-γ-producing cells, which share features of both Tfh and Th1 cells. We found that STAT4 regulates multiple genes involved in Tfh cell development. Early on, these cells expressed both Bcl6 and T-bet. However, Tfh cell-like features were not sustained during later stage of Th1 cell differentiation because of expression of T-bet and IFN-γ, which in concert antagonized expression of Bcl6 and other Tfh cell genes and promote Th1 cell specification. Thus, the differentiation of Th1 cells represents a dynamic balance of signals mediated by STAT4 and T-bet, whose actions are distinct in terms of effects on Tfh versus Th1 cell phenotype.

RESULTS

IL-12 Induces Genes that Contribute to Both Tfh and Th1 Cell Phenotypes

To begin to understand the relationship between Th1 and Tfh cells, we polarized naive $CD4^+$ T cells under conditions known to promote production of IL-21 versus standard Th1 cell conditions. Although IL-6 generated cells that express IL-21 and not IFN-γ, IL-12 efficiently produced cells that express IFN-γ. However, a quarter of the cells induced by IL-12 produced both IFN-γ and IL-21 (Figure 1A). These results were confirmed at the level of mRNA and by ELISA and by the kinetics of induction (Figures S1A–S1C available online).

We next sought to determine whether the cells generated in response to IL-12 exhibited other Tfh cell-like features. We found that IL-12 induced expression of phenotype-associated cell surface molecules; in fact, the proportion of $CXCR5^+PD\text{-}1^+$ Tfh-like cells and expression of ICOS were greater than when IL-6 was used as a stimulus (Figures 1B and 1C). In addition, IL-12 and IL-6 induced comparable expression of Bcl6 mRNA and protein (Figures 1D and 1E).

Next, we compared the phenotypic differences between subpopulations of Th1 cells that expressed high and low levels of IL-21 or IFN-γ. Surprisingly, $IL\text{-}21^+$ cells also expressed significantly higher amount of IFN-γ, T-bet, CXCR5, PD-1, and ICOS than did $IL\text{-}21^-$ cells (Figures 1F–1H). Likewise, $IFN\text{-}\gamma^+$ cells expressed significantly higher levels of IL-21 and T-bet than did $IFN\text{-}\gamma^-$ cells (Figure 1I). Thus, our data support the contention that IL-12 has the capacity to induce IL-21-producing cells that exhibit fundamental features of both Th1 and Tfh cells.

STAT4 Positively Regulates Genes that Contribute to Both Tfh and Th1 Cell Phenotypes

IL-12 is known to activate STAT4, but it also activates other STATs including STAT3 (Bacon et al., 1995). To compare the contribution of STAT3 versus STAT4, we assessed IL-21 production in the appropriate Stat-deficient cells. IL-6 effectively induced IL-21, which was abrogated in *Stat3*-deficient ($Stat3^{-/-}$) cells (Figure 2A). IL-12-mediated IL-21 expression was also inhibited in $Stat3^{-/-}$ cells; however, *Stat4* deficiency abrogated both IL-21 and IFN-γ (Figure 2A). We considered the possibility that once produced, IL-21 could act to further increase its production via activation of STAT3 (Zeng et al., 2007) and deletion of *Stat3* would interrupt such an autocrine loop. In fact, we found that IL-12-induced IL-21 production was partially inhibited by blocking IL-21 (see below). Thus, we conclude that IL-12-dependent activation of STAT4 directly promotes IL-21 production, which in turn acts through STAT3 in a positive feedback loop to maximize induction of IL-21.

We next evaluated the contribution of STAT4 on Tfh cell-related genes and found that the proportion of $CXCR5^+PD\text{-}1^+$ cells and ICOS expression upregulated by IL-12 were markedly decreased in $Stat4^{-/-}$ cells (Figures 2B and 2C). Although IL-6-mediated Bcl6 induction was markedly impaired, IL-12-dependent upregulation of Bcl6 was not affected by STAT3 deficiency (Figure 2D). In contrast, expression of Bcl6 by IL-12 was entirely inhibited by STAT4 deficiency (Figure 2E). Thus, IL-12-dependent Bcl6 expression is mediated by STAT4, not by STAT3. To evaluate the relevance of these findings with respect to in vivo humoral responses, we immunized wild-type and $Stat4^{-/-}$ mice with ovalbumin and assessed Tfh cell generation, GC formation, and antibody production. We found that immunized $Stat4^{-/-}$ mice generated significantly fewer Tfh and GC B cells and reduced amounts of IgG2b at day 4 postimmunization. However, no deficit was noted by day 8 (Figures S2A–S2C). Although it is possible that the reduction in number of GC B cells could be a consequence of intrinsic STAT4 deficiency in B cells themselves, the data indicated that STAT4 contributes to early induction of Tfh cells. However, the subsequent in vivo humoral response is normal, presumably because STAT3 can compensate. Taken together, these findings show that STAT4 positively regulates both Tfh and Th1 cell phenotypes.

Transient Nature of Tfh Cell-like Phenotype during Th1 Cell Differentiation

We examined the time course of the relationship between Th1 and Tfh cell phenotypes. After the initiation of Th1 cell polarization, IL-21 induction closely mirrored that of IFN-γ, at least during the initial phase up to day 5; however, the proportion of IL-21-producing cells declined thereafter (Figure 3A). This was not the case for IFN-γ-producing cells, the percentage of which continued to increase over time. Similarly, both T-bet and Bcl6 were upregulated during the initial phase. However, Bcl6 expression gradually declined, whereas T-bet expression remained elevated (Figure 3B). We evaluated whether the relative expression of T-bet and Bcl6 correlated with changes in phenotype at day 5 of developing cells. We found that T-bet expression correlated positively with IFN-γ production, whereas Bcl6 negatively correlated with IFN-γ (Figure 3C). In contrast, expression of

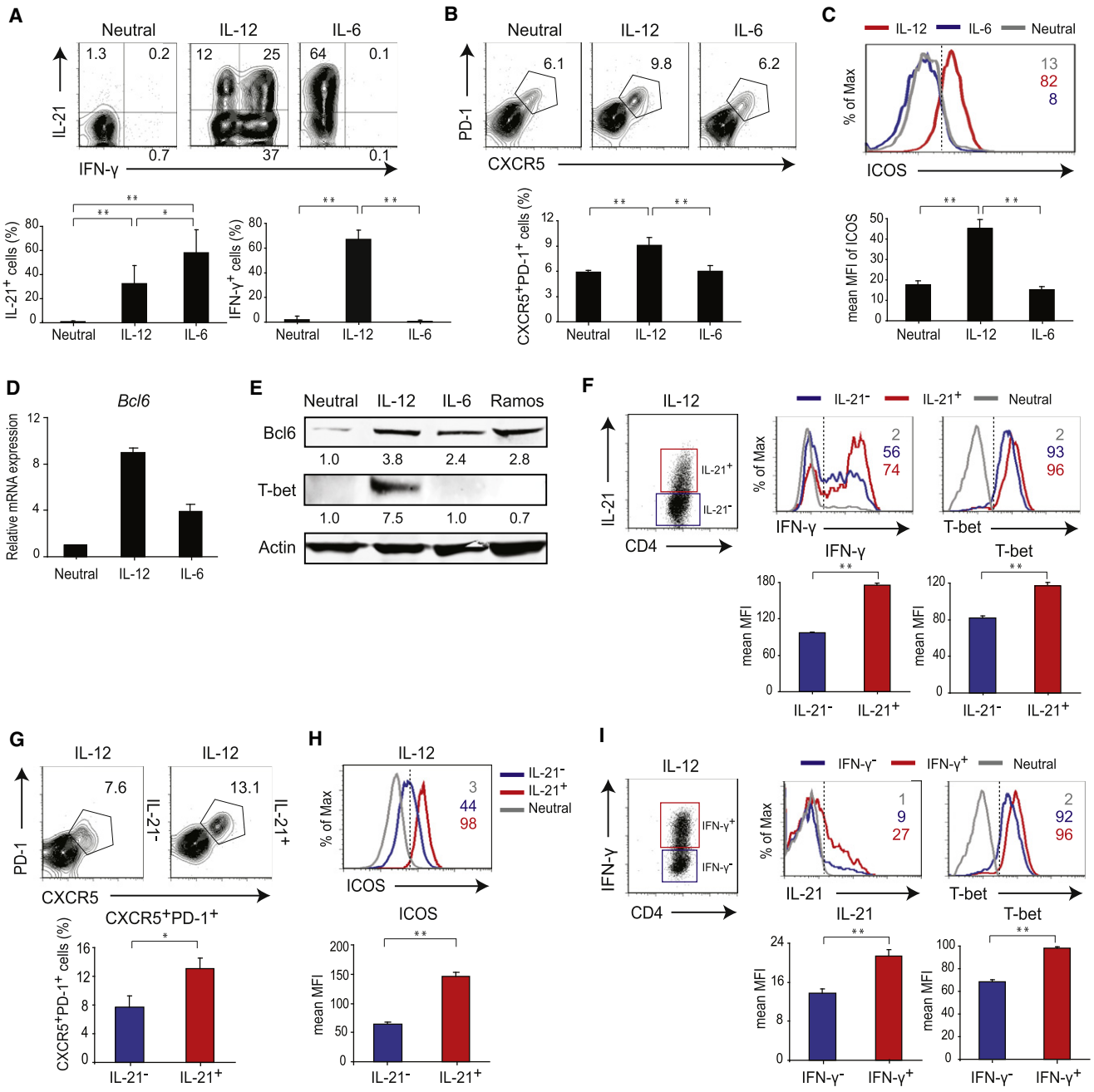

Figure 1. IL-12 Induces Genes that Contribute to Tfh and Th1 Cell Phenotypes

Sorted naive CD4$^+$ T cells were cultured under neutral, IL-12-, and IL-6-stimulated conditions for 5 days.

(A–C, F–I) FACS plots gated on CD4$^+$ cells and percentage or mean MFI of CD4$^+$ cells positive for intracellular IL-21, IFN-γ, and T-bet (A, F, I) and cell surface expression of CXCR5, PD-1 (B, G), and ICOS (C, H). Staining with an isotype control antibody was used to set gates (dotted lines).

(D) Relative mRNA expression of *Bcl6* was evaluated by qPCR.

(E) Bcl6 and T-bet levels in whole cell lysates were analyzed by immunoblotting. A human Burkitt's lymphoma cell line, Ramos, was used as a reference control.

(F–I) The percentages or MFI levels of the indicated markers gated on IL-21- or IFN-γ-positive cells (red) are compared to that of IL-21- or IFN-γ-negative cells (blue) or CD4$^+$ T cells cultured under neutral conditions (gray).

Representative data of three or more independent experiments are shown (mean ± SD). *$p < 0.05$, **$p < 0.01$. See also Figure S1.

both T-bet and Bcl6 correlated positively with IL-21 production, indicating differential modes of regulation of two cytokines. Bcl6 was necessary for CXCR5 expression and T-bet moderately downregulated its expression (Figure 3C). Collectively, a more fully differentiated Th1 cell state is associated with the elevated T-bet and eventual loss of Bcl6 expression.

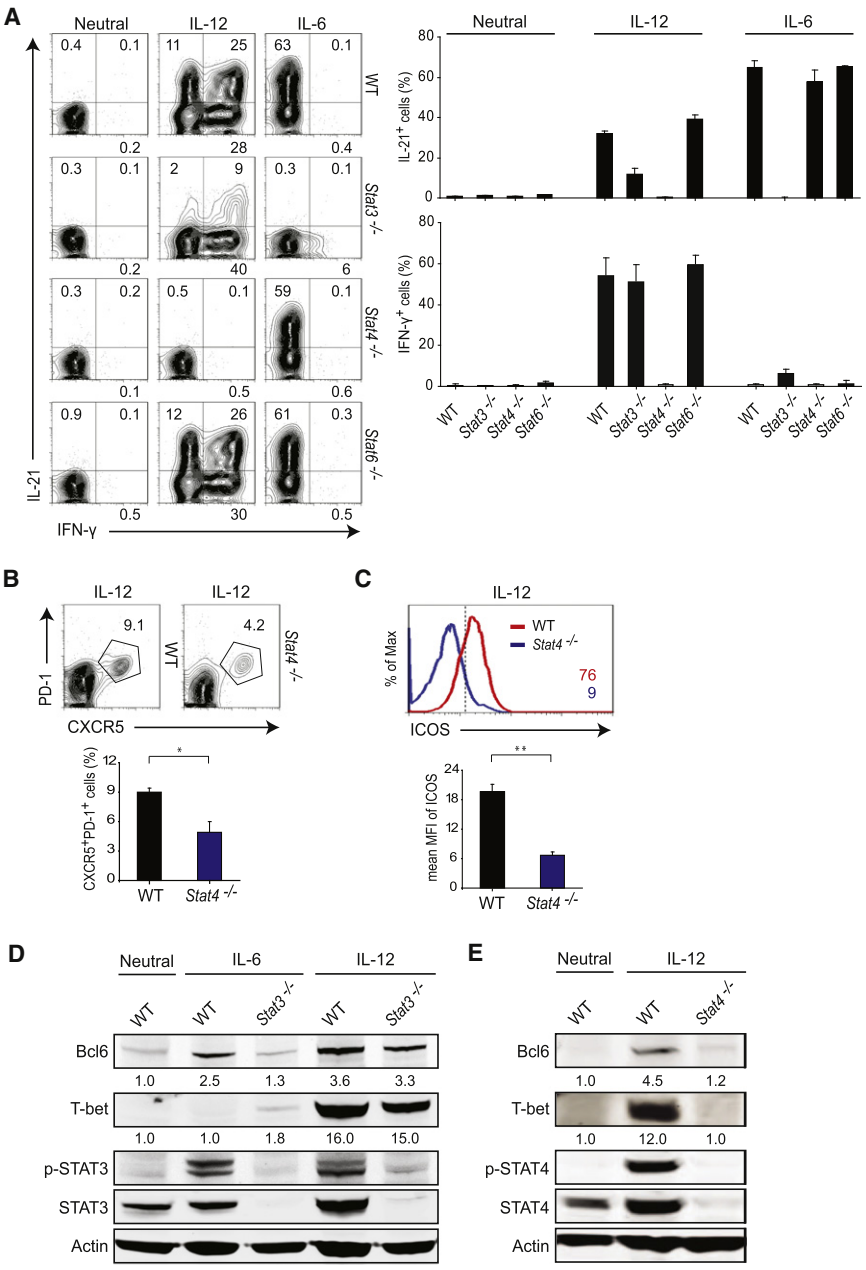

Figure 2. Induction of Tfh and Th1 Cell Phenotypes by IL-12 Is Mediated by STAT4

Sorted naive CD4$^+$ T cells from WT, *Stat3*$^{-/-}$, *Stat4*$^{-/-}$, or *Stat6*$^{-/-}$ mice were cultured under neutral, IL-12, and IL-6 conditions for 5 days.

(A–C) Shown are flow cytometry plots gated on CD4$^+$ cells and percentage or mean MFI of CD4$^+$ cells positive for expression of IL-21 and IFN-γ (A), CXCR5 and PD-1 (B), and ICOS (C).

(D and E) Levels of Bcl6, T-bet, phosho-STAT3, total STAT3, phospho-STAT4, or total STAT4 in whole cell lysates were analyzed by immunoblotting.

Representative data of three or more independent experiments are shown (mean ± SD). *p < 0.05, **p < 0.01. See also Figure S2.

T-bet Negatively Regulates Tfh Cell-like Phenotype in Th1 Cells

Previous work has indicated that T-bet negatively regulates IL-21 (Mehta et al., 2005). We therefore compared wild-type and T-bet-deficient (*Tbx21*$^{-/-}$) cells for their cytokine production after 5 days of in vitro Th1 cell polarization. Although the proportion of IFN-γ$^+$ cells was reduced in *Tbx21*$^{-/-}$ cells, as expected the proportion of IL-21$^+$ cells was significantly increased in *Tbx21*$^{-/-}$ cells (Figure 4A).

We sought to determine whether T-bet repressed other Tfh cell characteristics and found that in *Tbx21*$^{-/-}$ cells, the proportion of CXCR5$^+$PD-1$^+$ cells induced by IL-12 were enhanced (Figure 4B), as was the expression of Bcl6 (Figure 4C). To confirm the apparent repressive effect of T-bet, we expressed T-bet by retroviral transduction in cells polarized under Th1 cell conditions. As expected, overexpression of T-bet enhanced IFN-γ production but suppressed IL-21 production (Figures S3A–S3C). Importantly, we also found that T-bet inhibited IL-12-dependent Bcl6 induction (Figure 4D). Of note, T-bet-dependent inhibition of Bcl6 seemed not to be mediated via induction of Blimp-1, an important repressor for Bcl6 (Johnston et al., 2009), because Blimp1 expression was not different in the presence or absence of T-bet (Figures S3D and S3E). These data argue that T-bet is necessary for limiting other aspects of the Tfh cell phenotype in Th1 cells.

Direct, Opposing Effects of STAT4 and T-bet on the *Bcl6* Locus

To better understand how STAT4 and T-bet influence Tfh cell genes, we employed genome-wide DNase hypersensitivity (DHS) assay, along with histone- and transcription factor-ChIP-seq analysis. Consistent with previous reports (Wei et al., 2010; Wilson et al., 2009), we found that the *Ifng* locus contained multiple DHS peaks, STAT4 and T-bet binding sites, as well as permissive H3K4me3 and H3K36me3 modifications in wild-type Th1 cells (Figure 5A). These permissive histone marks were absent in *Stat4*$^{-/-}$ cells and reduced in *Tbx21*$^{-/-}$ cells, suggesting that both STAT4 and T-bet contributed to positively regulate transcription in the *Ifng* locus.

With respect to the *Il21* locus, we found that the locus was bound by STAT4 but not by T-bet (Figure 5B). Multiple DHS peaks and permissive marks were present in Th1 cell (Figure 5B), with no repressive H3K27me3 marks (data not shown). Furthermore, permissive histone marks of *Il21* locus were absent in *Stat4*$^{-/-}$ cells and increased in *Tbx21*$^{-/-}$ cells (Figure 5B). Interestingly, the *Bcl6* locus exhibited an open and active chromatin configuration, indicated by DHS peaks and permissive marks in

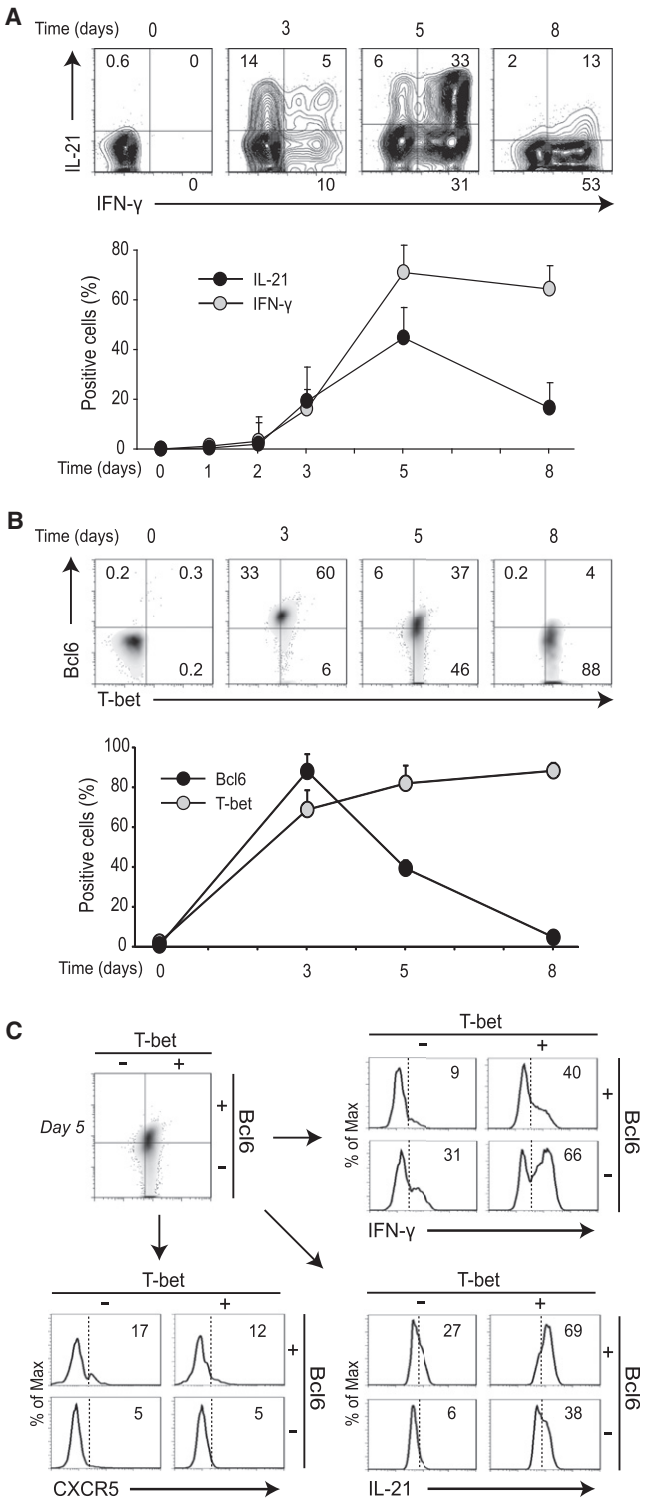

Figure 3. Transient Nature of Tfh Cell-like Phenotype during Th1 Cell Differentiation

Sorted naive CD4$^+$ T cells were cultured under IL-12 conditions for up to 8 days. Cells were analyzed by flow cytometry for expression of IL-21 and IFN-γ (A, C), Bcl6 and T-bet (B, C), and CXCR5 (C) at the indicated days. (A and B) Time course of IL-21, IFN-γ, Bcl6, and T-bet expression. The figure shows representative FACS plots gated on CD4$^+$ cells (top) and percentage of CD4$^+$ cells positive for the indicated molecules (bottom). (C) Cells were gated based on T-bet and Bcl6 expression, and expression of IFN-γ, IL-21, and CXCR5 at day 5 in developing Th1 cells were compared. Representative data from one of three independent experiments are shown (mean ± SD).

Th1 cell (Figure 5C), with no repressive H3K27me3 marks (data not shown). H3K36me3 marks were lost in *Stat4*$^{-/-}$ cells but increased in *Tbx21*$^{-/-}$ cells compared to the wild-type cells (Figure 5C). These changes in chromatin configuration were functionally relevant as shown by the fact that they are correlated with changes in both the *Il21* and *Bcl6* gene expression (Figures 5D and 5E).

Of note, the *Bcl6* locus was accessible in naive CD4$^+$ T cells and H3K4me3 marks were consistently present in all genetic backgrounds of Th1 cells (Figure 5C). The consistent presence of DHS and H3K4me3 around the *Bcl6* locus suggests that although Bcl6 expression can vary with different states of differentiation, this locus remains poised for transcription even in the absence of active transcription, consistent with a flexible view of the Tfh cell phenotype (Lu et al., 2011).

The finding that T-bet negatively regulated Bcl6 expression suggested to us that induction of T-bet in developing Th1 cells should eventually contribute to the loss of Tfh cell characteristics over time. As shown in Figure 5F, expression of Bcl6 was minimal in naive CD4$^+$ T cells but was induced by day 3 of in vitro Th1 cell polarization to levels comparable to those found in isolated GC B cells and Tfh cells (Figures 5F and S4A–S4D). Bcl6 was induced in *Stat4*$^{-/-}$ cells at early time points (day 3), because TCR and costimulatory signals contribute to Bcl6 induction (Figures 5F and S4C). However, Bcl6 levels were not sustained in *Stat4*$^{-/-}$ cells, but were maintained in *Tbx21*$^{-/-}$ cells for up to 8 days (Figure 5F). Consistent with previous work (Nurieva et al., 2009; Yu et al., 2009), overexpression of Bcl6 suppressed both IFN-γ and T-bet expression in Th1 cells (Figures S4E–S4G). We also found that in Th1 cells, both STAT4 and T-bet bound the *Cxcr5*, *Pdcd1*, and *Icos* genes, and the chromatin structure of these genes was in an open configuration (data not shown). H3K4me3 marks around *Cxcr5* locus were increased in *Tbx21*$^{-/-}$ cells, supporting the direct repression by T-bet (data not shown). By contrast, there was little change in histone marks of the *Pdcd1* and *Icos* loci regardless of the genetic background, suggesting indirect effects of T-bet on these genes. Functionally, we found that the proportion of CXCR5$^+$PD-1$^+$ cells in developing Th1 cells was regulated in parallel with IL-21 expression. The proportions increased initially but declined with the presence of T-bet (Figure 5F). Thus, T-bet represses multiple Tfh cell-defining genes during later course of Th1 cell differentiation.

IL-12-Induced IFN-γ Suppresses Tfh Cell Phenotype

Because both IFN-γ and IL-21 were simultaneously produced in Th1 cell, we investigated the influence of these cytokines on Th1 and Tfh cell phenotypes. To address this, we cultured naive CD4$^+$ T cells under Th1 cell-inducing conditions with exogenous cytokines or neutralizing antibody. We observed that the effect of IFN-γ on cytokine production varied at different time points during Th1 cell-inducing culture. At day 3, both IFN-γ and IL-21 production were increased by addition of excess exogenous IFN-γ and decreased by addition of neutralizing anti-IFN-γ (Figure 6A), arguing that IFN-γ can be a positive regulator

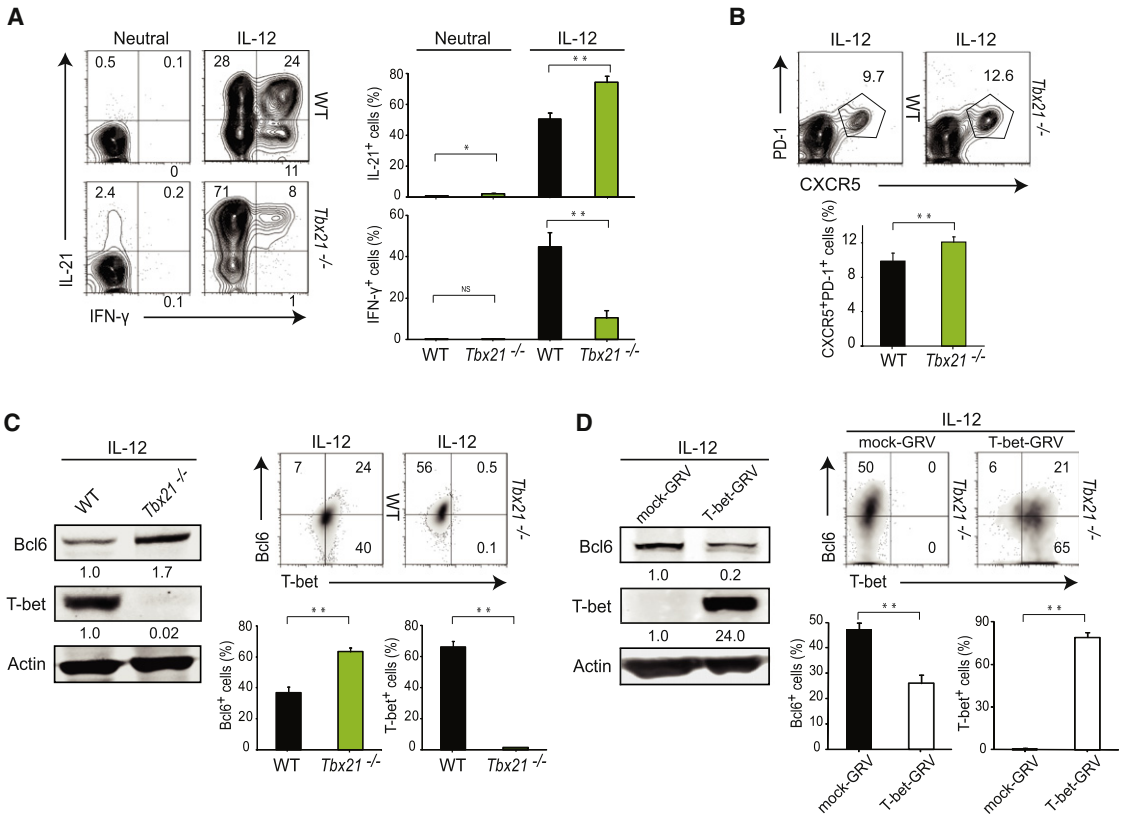

Figure 4. T-bet Represses Tfh Cell-like Phenotype in Th1 Cells

(A–C) Sorted naive CD4$^+$ T cells from WT or *Tbx21*$^{-/-}$ mice were cultured under neutral or IL-12 conditions for 5 days. Shown are FACS plots gated on CD4$^+$ cells and percentage of CD4$^+$ cells positive for expression of IL-21 and IFN-γ (A), CXCR5 and PD-1 (B), and Bcl6 and T-bet (C). Levels of Bcl6 and T-bet in whole cell lysates were also analyzed by immunoblotting (C).

(D) Sorted naive CD4$^+$ T cells from *Tbx21*$^{-/-}$ mice were infected with either control retrovirus (mock-GRV) or T-bet-expressing retrovirus (T-bet-GRV) for 3 days. GFP$^+$ cells were sorted by FACS and lysed for immunoblotting to evaluate Bcl6 and T-bet expression. Shown are FACS plots gated on GFP$^+$CD4$^+$ cells and percentage of GFP$^+$CD4$^+$ T cells positive for expression of Bcl6 and T-bet.

Representative data of three or more experiments are shown (mean ± SD). *$p < 0.05$, **$p < 0.01$. See also Figure S3.

for both IFN-γ and IL-21 in the initial phase. By contrast, at later time points, IL-21 production was significantly decreased by exogenous IFN-γ and increased by anti-IFN-γ (Figure 6A). To reconcile the seemingly contradictory effects of IFN-γ, we assessed IL-21 production in the *Ifng*-deficient cells and found that at early time points, IL-12-induced IL-21 production was reduced when *Ifng* was absent (Figure S5A). However, later on IFN-γ had the opposite effect on IL-21, Bcl6, and CXCR5 expression (Figures 6A and S5A–S5C). Thus, there seems to be a biphasic effect of IFN-γ on IL-21 production. The proportion of CXCR5$^+$PD-1$^+$ cells was significantly decreased by exogenous IFN-γ and increased by anti-IFN-γ at both time points (Figure 6B). Moreover, Bcl6 expression was suppressed and T-bet expression was increased by exogenous IFN-γ, whereas the opposite was the case by inclusion of anti-IFN-γ (Figure 6C). These effects can be attributed to induction of T-bet, as evidenced by the lack of effect in *Tbx21*$^{-/-}$ mice (data not shown; Lighvani et al., 2001).

The effects of modulating IL-21 were assessed at day 5 with exogenous IL-21 or neutralizing sIL-21R-Fc. IL-21 production was significantly increased by addition of exogenous IL-21 but decreased by addition of neutralizing sIL-21R-Fc (Figure 6D). Conversely, IFN-γ production was decreased by exogenous IL-21 and increased by sIL-21R-Fc (Figure 6D), consistent with previous data (Suto et al., 2006). The proportion of CXCR5$^+$ PD-1$^+$ cells, although not significant, showed a tendency to be increased by IL-21 and decreased by sIL-21R-Fc (Figure 6E). Bcl6 expression was increased and T-bet was decreased by exogenous IL-21, whereas the opposite was the case by sIL-21R-Fc (Figure 6F). These data suggest that IL-21 and IL-12 work in conjunction to enhance IL-21 and Bcl6 but work in opposition in regulating IFN-γ and T-bet. Thus, IFN-γ and IL-21 mimic the effects of T-bet and Bcl6 in antagonizing each other's actions, despite being expressed simultaneously in developing Th1 cells. This suggests that early on, the Tfh-Th1 cell phenotype is quite plastic and can respond dynamically to intrinsic and extrinsic factors.

T-bet Negatively Regulates Tfh Cell-Related Functionalities of CD4$^+$ T Cells during Infection with *T. gondii*

To determine whether our in vitro findings could be recapitulated in an in vivo situation, we analyzed mice infected with *T. gondii*, an obligate intracellular parasite known to induce a strong Th1 cell response (Jankovic et al., 2007). Similar to in vitro

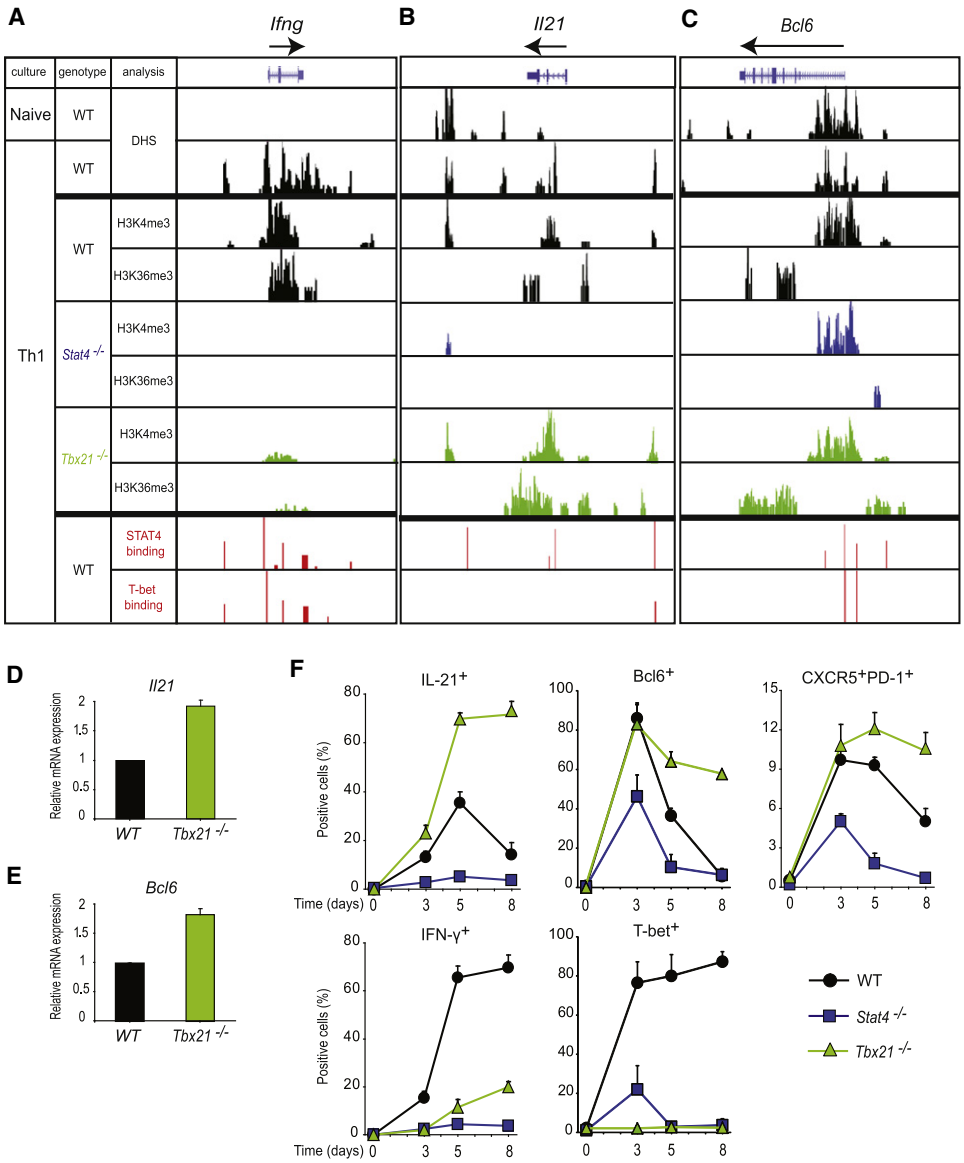

Figure 5. Opposing Effects of STAT4 and T-bet on Epigenetic Profiles of Tfh Cell Genes in Th1 Cells

Sorted naive $CD4^+$ T cells from WT, *Stat4*$^{-/-}$, or *Tbx21*$^{-/-}$ mice were cultured under Th1 cell conditions.

(A–E) Cells were restimulated with anti-CD3 and anti-CD28 and IL-12 for 2 hr.

(A–C) DNase-seq and ChIP-seq were performed with naive $CD4^+$ T cells and Th1 cells polarized for 7 days to map DHS, histone epigenetic marks, and binding of STAT4 and T-bet. The genome browser view of *Ifng* (chr10: 117,865,988-117,895,063, DHS *y* axis: 0-3, H3K4me3 *y* axis: 0-8, H3K36me3 *y* axis: 0-3, binding of STAT4 and T-bet *y* axis: 0-300), *Il21* (chr3: 37,091,490-37,164,309, DHS *y* axis: 0-2, H3K4me3 *y* axis: 0-4, H3K36me3 *y* axis: 0-2, binding of STAT4 and T-bet *y* axis: 0-150), and *Bcl6* (chr16: 23,951,786-24,014,614, DHS *y* axis: 0-3, H3K4me3 *y* axis: 0-6, H3K36me3 *y* axis: 0-2, binding of STAT4 and T-bet *y* axis: 0-60) loci are shown. *y* axis shows the normalized tag number which is the tag count per one million of total tag count (tag-per-million).

(D and E) Relative mRNA expression of *Il21* or *Bcl6* at day 5 was compared in cells from WT (black) or *Tbx21*$^{-/-}$ (green) mice.

(F) FACS analysis for IL-21, IFN-γ, Bcl6, T-bet, CXCR5, and PD-1 gated on $CD4^+$ cells were performed at day 0, 3, 5, and 8.

Representative data of two or more independent experiments are shown (mean ± SD). See also Figure S4.

experiments, IL-21$^+$CXCR5$^+$PD-1$^+$Bcl6$^+$ Tfh-like cells were generated 7 days after infection and the vast majority of IL-21$^+$ cells also expressed IFN-γ and T-bet (Figures 7A–7C). The proportions of IL-21$^+$IFN-γ$^-$ and CXCR5$^+$PD-1$^+$ cells and Bcl6 levels were further increased in *Tbx21*$^{-/-}$ mice (Figures 7A–7C). To assess the T cell-intrinsic requirement for T-bet in the course of generating transitional Tfh-Th1 cells, we adoptively transferred naive $CD4^+$ T cells from wild-type and *Tbx21*$^{-/-}$ into *Rag2* $^{-/-}$ mice and infected the recipients with *T. gondii* (Figure 7D). Wild-type and *Tbx21*$^{-/-}$ cells expanded equally in response to *T. gondii* up to day 15 (data not shown). However, we found that the Tfh cell phenotype, as evidenced by expression IL-21, Bcl6, and CXCR5, was more prominent in *Tbx21*$^{-/-}$ cells compared to wild-type cells in the same host environment

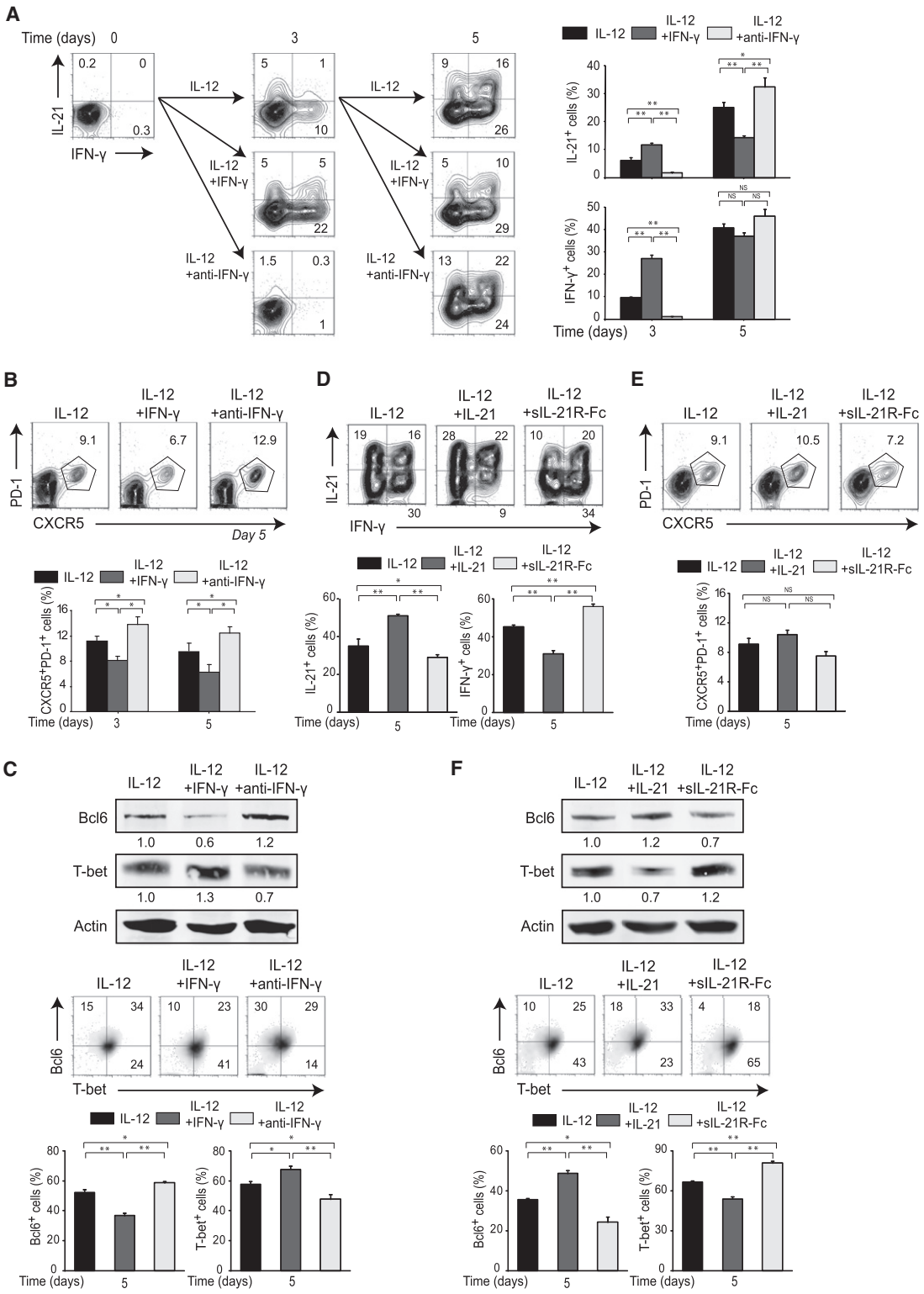

Figure 6. Effect of Modulating the Levels of IFN-γ and IL-21 during the Course of In Vitro Th1 Cell Differentiation

Sorted naive CD4$^+$ T cells were cultured under IL-12 conditions for 3–5 days. Combinations of IFN-γ, IFN-γ antibody, IL-21, or soluble IL-21 receptor-Fc were added as indicated to enhance or deplete the levels of IFN-γ or IL-21 in the culture.

(A, B, D, and E) Levels of IL-21 and IFN-γ (A, D) and CXCR5 and PD-1 (B, E) on CD4$^+$ cells were determined by FACS at day 3 and/or 5.

(C and F) Levels of Bcl6 and T-bet on CD4$^+$ cells were determined by FACS and by immunoblotting at day 5.

Representative data of three independent experiments are shown (mean ± SD). *$p < 0.05$, **$p < 0.01$. See also Figure S5.

(Figures 7E–7H). These data show the coemergence of IL-21- and IFN-γ-producing cells, both as single-positive and double-positive cells, 7 days postinfection. However, the proportion of double-positive cells declined at day 15 in wild-type and *Tbx21*$^{-/-}$ cells. In contrast, IFN-γ single-positive cells were increased in wild-type cells whereas IL-21 single-positive cells were increased in *Tbx21*$^{-/-}$ cells (Figure 7F). By day 15, there was reduction in expression of Bcl6 and the proportions of CXCR5^{+}PD-1^{+} cells in wild-type population whereas Tfh cell markers were sustained in *Tbx21*$^{-/-}$ population for up to 15 days (Figures 7E, 7G, and 7H). Collectively, the data indicate that T cell-intrinsic T-bet deficiency allows expansion of Tfh-like cells during Th1 cell responses.

We next sought to assess whether the observed expansion of Tfh-like cells in *Tbx21*$^{-/-}$ mice enhanced humoral responses. Because of the early lethality associated with *T. gondii* infection of *Tbx21*$^{-/-}$ mice, we were unable to measure B cell responses. As an alternative strategy, we compared production of antibody against soluble *T. gondii* antigen (STAg) in wild-type and *Tbx21*$^{-/-}$ mice. At 20 days postimmunization, GC formation and antibody production were remarkably enhanced in *Tbx21*$^{-/-}$ mice, highlighting the physiological relevance of T-bet in regulating Tfh cell-mediated humoral responses (Figures 7I and 7J). Thus, the emergent Tfh cell phenotype is constrained by the induction of T-bet as cells become bona fide Th1 cells.

DISCUSSION

The extent to which Tfh and other CD4^{+} T cell subsets represent distinct lineages that express lineage-defining master regulators is subject of intense ongoing research (Murphy and Stockinger, 2010; O'Shea and Paul, 2010). The present data demonstrate a previously unappreciated degree of phenotypic overlap between Tfh and Th1 cells and illustrate molecular interplay among "master" transcription factors such that dynamically regulated expression of T-bet and Bcl6 affect STAT4-mediated gene expression and ultimately define cellular phenotype.

Previous work has suggested that a species-specific effect of IL-12 inducing IL-21 and other features of Tfh cells in human but not mouse cells (Ma et al., 2009; Mehta et al., 2005; Schmitt et al., 2009; Suto et al., 2008). However, recent work has shown that murine Th1 cells can express IL-21 (Eto et al., 2011) and our results indicate that IL-12 also has the capacity to induce Bcl6 and Tfh cell surface markers. With this regard, temporal considerations, rather than species difference, may provide an explanation for the apparent discrepancy. In previous studies, cytokine production was measured in cells polarized for 5–7 days, times at which the Tfh cell-like phenotype of Th1 cells has begun to wane. In addition, we used greater IL-12 concentrations than others; however, we do not believe that our results are due to artificially high amounts of IL-12. Our results were confirmed in vivo in a model associated with vigorous IL-12 production and intense Th1 cell responses induced by *T. gondii*.

The present study also provides the evidence to explain why others have found STAT3 not to be essential for Tfh cell differentiation (Eddahri et al., 2009; Schmitt et al., 2009). IL-12 acting via STAT4 can be an early inducer of Tfh cell phenotype. Consistent with redundant functions of STAT3 and STAT4 is recent work which demonstrates that neither IL-6 nor IL-21 alone are required for induction of Bcl6^{+} Tfh cells in vivo and in vitro (Eto et al., 2011; Poholek et al., 2010). STAT4 appears to provide an alternative mechanism, as our data argue that STAT3 and STAT4 redundantly serve to induce IL-21, Bcl6, and other Tfh cell molecules; both STAT4 and STAT3 directly bind these genes and promote gene expression (Durant et al., 2010; Schmitt et al., 2009; Wei et al., 2007, 2010). What differs between STAT3 and STAT4 is the capacity to efficiently induce *Tbx21* gene. It is notable in this regard that IFN-γ acting via STAT1 is also a potent inducer of T-bet (Lighvani et al., 2001). However, unlike IL-12-STAT4, IFN-γ-STAT1 inhibits Bcl6 expression.

Although Tfh cells are a functionally critical subset of helper T cells, it remains an open question whether Tfh cells are truly a distinct lineage parallel to other subsets or rather represent a temporary "state" of differentiation. As described in this manuscript, during the course of Th1 cell differentiation, we noted the appearance of IL-12-induced IL-21^{+}IFN-γ^{+}Bcl6^{+}T-bet^{+} Tfh-Th1-like cells, which were followed by the generation of fully differentiated IL-21^{-}IFN-γ^{+}Bcl6^{-}T-bet^{+} Th1 cells. Our ChIP-seq findings do indicate that T-bet directly binds to *Bcl6* locus and multiple other Tfh cell-related genes. The present data confirm that both STAT4 and T-bet are indispensable positive regulators for full induction of IFN-γ. Although STAT4 can promote Tfh cell-like features, induction of T-bet inhibits Tfh cell genes and tips the balance toward a more Th1 cell-dominant phenotype. Our data indicate that early on after IL-12 stimulation, cells are uncommitted and complete differentiation reflects the counter-regulatory effects of STAT4 and T-bet.

Previous work has shown that Bcl6 functions as a transcriptional repressor for T-bet (Nurieva et al., 2009; Yu et al., 2009); however, it also appears that T-bet can limit expression of Bcl6. Our results indicate that expression of T-bet and Bcl6 antagonized each other's function, as did IFN-γ and IL-21. The action of T-bet to suppress the generation of Tfh cells and humoral responses were confirmed in vivo dominant Th1 cell response by *T. gondii*. Thus, T-bet-mediated downregulation of Bcl6 is required to become bona fide Th1 cells. Recent work also shows that Bcl6 and T-bet cooperate and can reduce IFN-γ production during Th1 cell differentiation (Oestreich et al., 2011). Our data indicate that T-bet does not counterregulate IL-21 or Bcl6 during initial phase of Th1 cell differentiation; presumably there are threshold levels of T-bet and Bcl6 expression that need to be reached, which subsequently dictate the outcome of repression of Tfh or Th1 cell phenotypes. Interestingly, there seems to be a biphasic effect of IFN-γ: early on it promotes IL-21 but later it inhibits. This finding may also be a contributor to the seemingly contradictory effects of Bcl6 and T-bet.

Although Bcl6 has been argued to be the "master regulator" transcription factor for Tfh cells (King et al., 2008; Nurieva et al., 2009), our data put this factor in a somewhat different light. We noted that the *Bcl6* locus is accessible in naive CD4^{+} T cells, a finding that is consistent with our data and the data of others indicating that Bcl6 is readily inducible by TCR stimulation alone and further induced by STAT4 or STAT3 (Durant et al., 2010; Lund et al., 2005; Ma et al., 2009; Nurieva et al., 2009; Schmitt et al., 2009). Equally interesting is the fact that the *Bcl6* locus is not repressed in fully polarized Th1 cells. The accessibility, assessed by H3K4me3 modifications of the *Bcl6* locus, implies

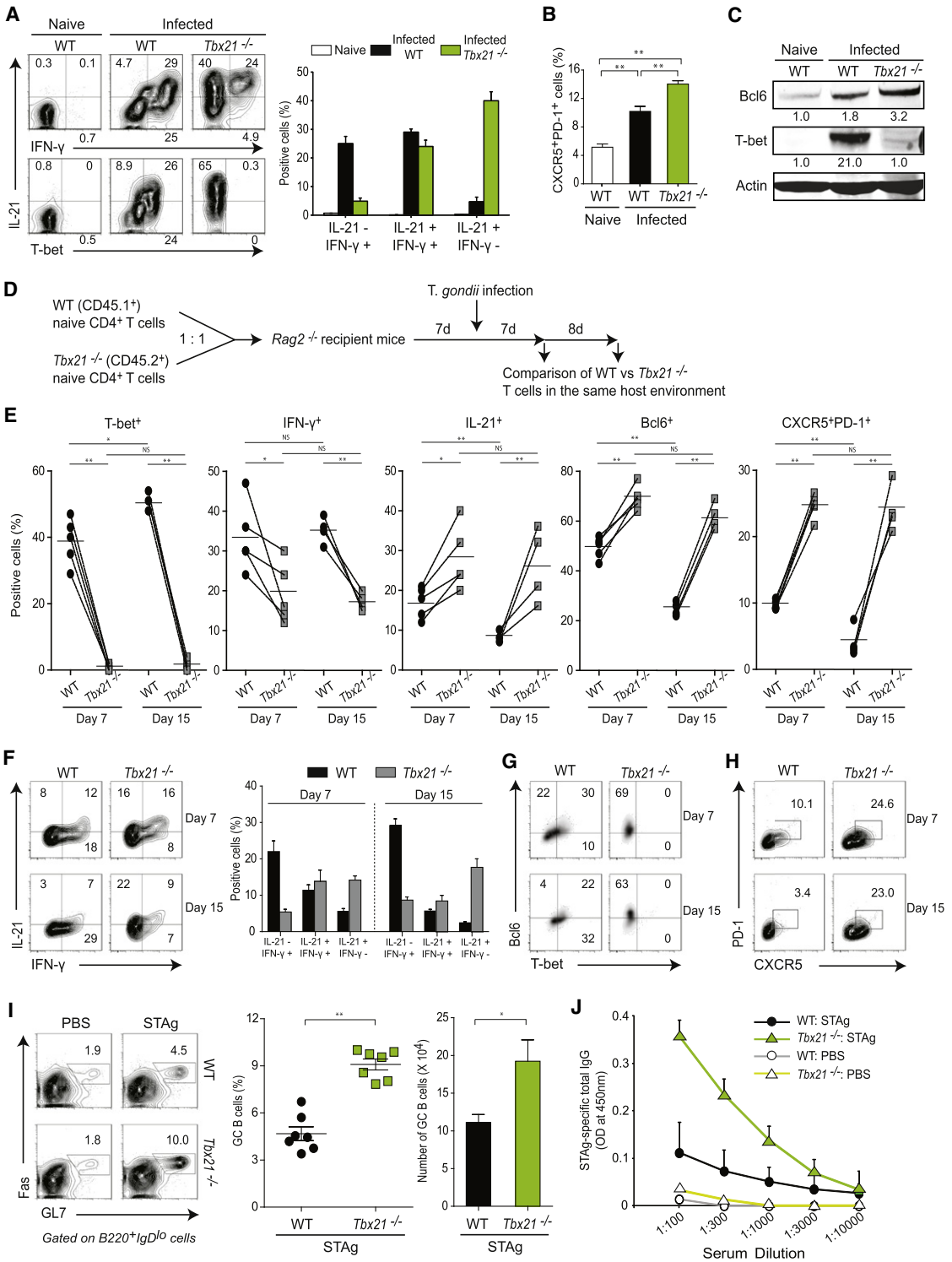

Figure 7. T-bet Negatively Regulates Tfh Cell-Related Functionalities of CD4$^+$ T Cells during Infection with *T. gondii*

(A–C) WT or *Tbx21*$^{-/-}$ mice were infected with 20 cysts of the avirulent *T. gondii* strain ME49. Splenocytes were analyzed by FACS 7 days after infection for expression of IL-21, IFN-γ, and T-bet (A) gated on CD4$^+$ cells, and CXCR5 and PD-1 gated on CD4$^+$CD44$^+$ cells (B). Data indicate mean ± SD from at least four mice per group in two separate infections. Bcl6 and T-bet levels in whole cell lysates from sorted CD4$^+$ cells were determined by immunoblotting (C).

(D–H) *Rag2* $^{-/-}$ mice were reconstituted with 1:1 mixture of WT (CD45.1$^+$) and *Tbx21*$^{-/-}$ (CD45.2$^+$) naive CD4$^+$ T cells. 7 days later, mice were infected with *T. gondii* and followed for 7–15 days (D). Expression of IL-21 and IFN-γ (E, F), Bcl6 and T-bet (E, G), and CXCR5 and PD-1 in CD4$^+$CD44$^+$ cells (E, H) was

that this factor can be induced in other lineages (Lu et al., 2011). Collectively, these results argue that it may not be appropriate to view Bcl6 simply as a master regulator of Tfh cells.

During the course of LCMV infection, it was noted that Tfh cells maintained expression of IFN-γ and T-bet; however, the levels were low compared to typical Th1 cells (Johnston et al., 2009). Other work showed that in chronic LCMV infection, the induced Th1 cells "converted" to become Tfh cells later during the immune response (Fahey et al., 2011). These data, along with ours, suggest a model in which signals associated with chronic antigenic exposure can sustain Bcl6 expression, which overrides the inhibitory function of T-bet. Over time, limitation of T-bet expression favors transition of nascent Th1 cells to become Tfh cells. Conversely, signals that drive expression of T-bet and limit Bcl6 expression favor "terminal differentiation" of Th1 cells to become effector cells and limit Tfh cell functionalities. Importantly, T-bet is not the only factor that regulates Bcl6 expression. Previous work has demonstrated that Bcl6 expression is not sustained in effector $CD4^+$ T cells and that Bcl6 and Blimp-1 antagonize each other's function (Johnston et al., 2009). Additionally, ICOS-dependent signals are also important for induction of Bcl6 (Choi et al., 2011). Thus, it is clear that many factors contribute to regulation of Bcl6 expression and that Bcl6, in turn, regulates other T cell transcription factors. Rather than invoking models of master regulators in terminally differentiated cells, it may be more accurate to think of Bcl6, T-bet, Blimp-1, and other factors as extrinsically regulated players that dynamically regulate T cell function.

The finding that IL-12 can generate uncommitted Tfh-Th1 cells may have significance to human disease. For instance, in inflammatory bowel diseases, apparently uncommitted, IL-21^+IFN-γ^+ cells have been found in the gut and have been suggested to be important for intestinal tissue damage (Monteleone et al., 2005; Sarra et al., 2010). These finding are also of interest given the genetic link between polymorphisms of the *Il21, Il2*, and *Stat4* genes and susceptibility to human autoimmune disease (Daha et al., 2009; Remmers et al., 2007). It is possible that alterations to the function of STAT4 might not only affect IFN-γ regulation but could also influence Tfh cell genes.

Taken together, our findings indicate that the diverse actions of STAT4 and T-bet underlie phenotypic heterogeneity between Tfh and Th1 cells. These findings help to clarify seemingly contradictory findings and argue for a coherent stepwise process of Tfh-Th1 cell differentiation. Acting through STAT4, IL-12 induces a transitional stage of Tfh-Th1 cells, which express IL-21 and Bcl6. However, STAT4 also promotes T-bet expression, which along with IFN-γ ultimately limits the Tfh cell phenotype. It is by no means inconceivable that other subsets also share transitional stages with Tfh cells. Future studies are needed to dissect these intriguing degrees of heterogeneity for Tfh cell differentiation that should offer opportunities for the treatment of autoimmune diseases.

EXPERIMENTAL PROCEDURES

Mice, Cell Isolation, and Cell Culture

C57BL/6J, *Tbx21*$^{-/-}$, *Ifng*$^{-/-}$, and *Stat6*$^{-/-}$ mice were purchased from Jackson Laboratory. *Stat4*$^{-/-}$ mice were provided by M. Kaplan. *Stat3*$^{-/-}$ (*Cd4* Cre; *Stat3*$^{fl/fl}$) mice were generated as previously described (Wei et al., 2007). *Rag2*$^{-/-}$ and B6.SJL (CD45.1) mice were from Taconic Farms. All mice were handled in accordance with the guidelines of the NIH Animal Care and Use Committee. Splenic and lymph node T cells were obtained by disrupting organs of 8- to 12-week-old mice. All cell cultures were performed in RPMI supplemented with 10% fetal calf serum, 2 mM glutamine, 100 IU/mL penicillin, 0.1 mg/mL streptomycin, 10 mM HEPES, 1 mM sodium pyruvate, and 2.5 μM β-mercaptoethanol. T cells were enriched with a $CD4^+$ T cell kit and AutoMacs isolator (Miltenyi Biotec). Naive $CD4^+$ T cells were isolated by flow cytometry, staining with CD4, CD62L, CD44, and CD25 antibodies, and were cultured in the plate-bound anti-CD3 and anti-CD28 (10 μg/mL each) under neutral (anti-IFN-γ and anti-IL-4 [10 μg/mL each]), IL-12 (IL-12 [10 ng/mL] and anti-IL-4), or IL-6 (IL-6 [20 ng/mL], anti-IFN-γ, and anti-IL-4) conditions for 3 days and then cultured further in IL-2 (50 U/mL) alone (neutral condition) or in combination with IL-12 (IL-12 condition; Th1) or IL-6 (IL-6 condition) and grown an additional 2–5 days. Where indicated, 100 ng/mL IFN-γ, 20 ng/mL IL-21, or 10 μg/mL soluble IL-21 R/Fc chimera (R&D) was added. Cytokines were from R&D Systems. Anti-CD3 and anti-CD28 were from eBioscience and other antibodies were from BD Biosciences.

Intracellular Staining and Flow Cytometry

For intracellular cytokine staining, cells were restimulated for 2 hr with 50 ng/ml PMA and 1 μg/ml ionomycin with the addition of brefeldin A (GolgiPlug; BD), then fixed and permeabilized with Cytofix/Cytoperm solution (BD). Intracellular IL-21 staining was performed with soluble IL-21 R/Fc, followed by PE-labeled affinity-purified F(ab′)2 fragment of goat anti-human Fcγ (Jackson ImmunoResearch Laboratories) (Suto et al., 2008), then further stained with PerCP-Cy5.5 anti-CD4 and APC anti-IFN-γ (BD). The following antibodies were used for cell surface or intracellular staining: FITC anti-CD44, FITC anti-Fas, PE anti-PD-1, PE anti-ICOS (BD), APC-Cy7 anti-CD4, PE-Cy7 anti-CD45.1 (Biolegend), PerCP-Cy5.5 anti-B220, APC anti-GL7, PE anti-Bcl6, and Alexa Fluor-647 anti-T-bet (eBioscience). CXCR5 staining was done with biotinylated anti-CXCR5 for 1 hr, followed by APC-labeled streptavidin (BD). Stained cells were analyzed on a flow cytometer (FACSCalibur or FACSCanto; BD). Events were collected and analyzed with FlowJo software (Tree Star). Analysis of expression of surface molecules and intracellular transcription factors were evaluated in the presence and absence of PMA/ionomycin; however, the level of expression did not change with this short stimulation.

DNase Hypersensitivity Mapping by DNase-seq

6–10 × 10^7 cells were treated with 0.01% NP-40 for 5 min to isolate 2 × 10^7 nuclei. Afterwards, DNaseI digestions (60–180 U/mL) were performed for 3 min at 37°C. DNA fragments of 100–500 bp were isolated by sucrose gradient centrifugation. Purified DNA was end repaired, ligated to the Illumina single read adaptors, amplified, and further purified to the fraction of 220–250 bp in size. The resulted libraries are sequenced with the Illumina Genome Analyzer Iix (Illumina). Only unique reads of 36 bp were mapped to the mouse genome (mm9) with SICER peak calling program.

ChIP-seq Analysis

ChIP-seq experiments and data processing were performed as described previously (Wei et al., 2009, 2010). The following antibodies were used: anti-H3K4me3 (ab8580); anti-H3K36me3 (ab9050) from Abcam; anti-T-bet (sc-21003) from Santa Cruz Biotechnology. The genome browser views for STAT4 binding in wild-type cells and for histone modification profiles in

determined by FACS. The levels of expression of the indicated markers on CD45.1-positive cells (WT) are compared to that of CD45.1-negative cells (*Tbx21*$^{-/-}$). n = 5.

(I and J) WT or *Tbx21*$^{-/-}$ mice were immunized with 20 μg/mL STAg i.p. every 5 days for three times. Splenic GC B cells ($B220^+IgD^{lo}GL7^{hi}Fas^{hi}$) were analyzed by FACS 20 days postimmunization (I). Serum levels of STAg-specific total IgG were measured by ELISA (J). n = 7 each.

Representative data from one of two independent experiments are shown (mean ± SD). *p < 0.05, **p < 0.01.

Stat4$^{-/-}$ T cells are generated from the data deposited to Gene Expression Omnibus (GEO) database (Wei et al., 2010).

Immunoblotting

Equal amounts of total protein were electrophoresed on 4%–12% Bis-Tris gels (Invitrogen), transferred to nitrocellulose, and blotted with the following antibodies: anti-Bcl6, anti-phosho-STAT4, anti-phosho-STAT3, anti-STAT3 (Cell Signaling), anti-T-bet, anti-STAT4 (Santa Cruz), or anti-actin (Millipore). IRDye700- and IRDye800-labeled secondary antibodies (Rockland) were used for detection and specific bands were visualized with an Odyssey infrared imaging system (LI-COR Biosciences). Band intensity was quantified and normalized to loading controls. The relative levels of Bcl6 and T-bet were compared with each value of control conditions set as 1.0.

Quantitative Real-Time PCR

Total RNA was isolated with the use of the *mir*Vana miRNA kit (Applied Biosystems/Ambion); cDNA was synthesized with the Taqman reverse transcription Kit (Applied Biosystems). Quantitative PCR was performed with an ABI PRISM 7700 sequence detection system with site-specific primers and probes (Applied Biosystems). The comparative threshold cycle method and an internal control (β-actin) were used to normalize the expression of the target genes. List of primers and probes from Applied Biosystems: mouse ACTB, 4352341E; Il21, Mm00517640_m1; Bcl6, Mm01342164_m1.

Retroviral Transduction

T-bet-expressing retrovirus vector (T-bet-GFP-retrovirus; T-bet-GRV) was kindly provided by W. Strober. cDNA encoding murine Bcl6 was subcloned into pMY-IRES-GFP retroviral vector (Cell Biolabs). All virus constructs coexpress GFP as a selection marker for sorting. Sorted naive CD4$^+$ T cells from WT or *Tbx21*$^{-/-}$ mice were cultured in the presence of plate-bound anti-CD3, anti-CD28 with anti-IL-4 for 24 hr. After that, the cells were infected with either control or T-bet- or Bcl6-expressing retrovirus and then cultured further in IL-12 and anti-IL-4 for 3 days.

In Vivo Challenge with *T. gondii*

Age- and sex-matched WT and *Tbx21*$^{-/-}$ mice were challenged i.p. with 20 cysts of the avirulent ME49 strain of *T. gondii* as preciously described (Jankovic et al., 2007). For the measurement of humoral responses, mice were immunized with 20 μg/mL STAg i.p. every 5 days for three times in total. Splenocytes were harvested and were analyzed by FACS. Serum was also collected and STAg-specific IgG was measured by ELISA. To assess the T cell-intrinsic requirement for T-bet, we generated chimeric *Rag2*$^{-/-}$ mice, reconstituting with approximately five million WT (CD45.1) and approximately five million *Tbx21*$^{-/-}$ (CD45.2) naive CD4$^+$ T cells. Transferred cells were allowed to expand for 7 days before infection with 20 cysts of *T. gondii* strain ME49 i.p., and tissues were harvested and analyzed at days 7 and 15.

Statistical Analysis

Statistical significance was determined by the Mann-Whitney U test. A p value less than 0.05 denoted the presence of a statistically significant difference.

ACCESSION NUMBERS

The sequencing data are available in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo) under the accession number GSE33802.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at doi:10.1016/j.immuni.2011.11.012.

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