ICOS Receptor Instructs T Follicular Helper Cell versus Effector Cell Differentiation via Induction of the Transcriptional Repressor Bcl6

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SUMMARY

The nature of follicular helper CD4^{+} T (Tfh) cell differentiation remains controversial, including the minimal signals required for Tfh cell differentiation and the time at which Tfh cell differentiation occurs. Here we determine that Tfh cell development initiates immediately during dendritic cell (DC) priming in vivo. We demonstrate that inducible costimulator (ICOS) provides a critical early signal to induce the transcription factor Bcl6, and Bcl6 then induces CXCR5, the canonical feature of Tfh cells. Strikingly, a bifurcation between Tfh and effector Th cells was measurable by the second cell division of CD4^{+} T cells, at day 2 after an acute viral infection: IL2Rα^{int} cells expressed Bcl6 and CXCR5 (Tfh cell program), whereas IL2Rα^{hi} cells exhibited strong Blimp1 expression that repressed Bcl6 (effector Th cell program). Virtually complete polarization between Bcl6^{+} Tfh cells and Blimp1^{+} effector Th cell populations developed by 72 hr, even without B cells. Tfh cells were subsequently lost in the absence of B cells, demonstrating a B cell requirement for maintenance of Bcl6 and Tfh cell commitment via sequential ICOS signals.

INTRODUCTION

Naive CD4^{+} T cells differentiate into different effector cells and elicit various immunological effector functions, such as clearing viruses (Th1 cells), helminths (Th2 cells), and fungi (Th17 cells) or suppressing immune responses (iTreg cells) (Zhu et al., 2010). Follicular helper T (Tfh) cells are the CD4^{+} T cells specialized in B cell help (Crotty, 2011). Tfh cells express the B cell homing chemokine receptor, CXCR5, among other molecules important for their differentiation and function. CXCR5 surface expression not only phenotypically distinguishes Tfh cells from other effector CD4^{+} T cells but functionally drives Tfh cell migration into B cell follicles in a CXCL13-dependent manner. T cell help to B cells is a pivotal process of adaptive immune responses. Tfh cells first interact with cognate B cells at the T cell-B cell border and subsequently induce germinal center B

cell differentiation and germinal center formation within the follicle (Allen et al., 2007). Tfh cells have a gene expression profile that is distinct from Th1, Th2, Th17, and iTreg cells (Chtanova et al., 2004; Nurieva et al., 2008; Rasheed et al., 2006; Vinuesa et al., 2005; Yusuf et al., 2010) and is particularly enriched for cell surface molecules, reflecting the importance of cell-cell interactions between Tfh cells and B cells for Tfh cell functions. The Tfh cell program in both mice and humans is associated with high expression of CXCR5, PD-1, ICOS, BTLA, IL-21, SAP, and Bcl6 (Crotty, 2011).

Effector CD4^{+} T cell differentiation is controlled by specific transcription factors (Murphy and Stockinger, 2010; Zhu et al., 2010). We and others recently identified a transcriptional repressor, Bcl6, as a critical regulator of Tfh cell differentiation (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009). Bcl6 is a BTB domain containing transcriptional repressor previously known to be required in B cells for germinal center B cell differentiation, and Bcl6 is an important oncogene in germinal center B cell-derived lymphomas (Ci et al., 2008; Klein and Dalla-Favera, 2008). Bcl6-deficient CD4^{+} T cells are not able to differentiate into Tfh cells in vivo (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009), whereas constitutive Bcl6 expression induced CD4^{+} T cell differentiation into Tfh cells (Johnston et al., 2009). These results demonstrated that not only is Bcl6 expression required in B cells for germinal center development, it is also simultaneously essential in CD4^{+} T cells, and thus Bcl6 must regulate complementary gene expression programs in two different lymphocyte cell types differentiating in parallel. One of the functions of Bcl6 in Tfh cell differentiation is to regulate miRNA expression. Bcl6 downregulated miRNA clusters that were shown to partially inhibit CXCR5 in B cells (Yu et al., 2009). Bcl6 can repress expression or function of transcription factors necessary for other CD4^{+} T cell differentiation pathways, such as RORγt (Nurieva et al., 2009). Bcl6 also directly represses the transcription factor Blimp1 (encoded by *Prdm1*) (Johnston et al., 2009; Tunyaplin et al., 2004). All of these data demonstrate that Bcl6 is necessary and sufficient for Tfh cell differentiation by CD4^{+} T cells. Therefore, a deeper understanding of the regulation of Bcl6 expression is essential to understand Tfh cell differentiation.

Different models have been proposed to explain Tfh cell differentiation, which hinge on different Bcl6 expression kinetics and mechanisms of induction. One model proposed is cytokine-dependent Tfh cell differentiation. Th1 and Th2 CD4^{+} T cell

differentiation can be achieved in vitro by providing naive CD4+ T cells with activation signals through the TCR and appropriate cytokine receptors (Zhu et al., 2010). Analogously, it was proposed that Tfh cells can be differentiated in vitro by IL-6 or IL-21. Both Bcl6 mRNA and CXCR5 mRNA were upregulated in the presence of IL-6 or IL-21 (Nurieva et al., 2008, 2009). However, this simple model has been challenged by studies that found normal Tfh cell differentiation in vivo in the absence of IL-6 or IL-21 (Poholek et al., 2010; Zotos et al., 2010), though partial defects were found in other studies (Linterman et al., 2010; Vogelzang et al., 2008), possibly because of reduced maintenance of Tfh cells (Linterman et al., 2010). There have been conflicting reports regarding cytokine induction of Bcl6 and CXCR5 in vitro (Dienz et al., 2009; Eddahri et al., 2009). In our studies, limited Bcl6 and CXCR5 mRNA was induced by in vitro purified CD4+ T cells cultured in the presence of TCR stimulation plus IL-6 or IL-21, and no substantial expression of Bcl6 protein or CXCR5 protein was found (Eto et al., 2011). Cytokines alone appear to be insufficient for Tfh cell differentiation.

Other models have focused on the importance of B cell-dependent Tfh cell differentiation (Crotty et al., 2010). This model is supported by the observation that Tfh cell differentiation was severely defective in B cell-deficient mice (μMT) or cognate B cell-deficient mice (MD4-μMT) after protein immunization, viral infection, or parasite infection (Haynes et al., 2007; Johnston et al., 2009; Zaretsky et al., 2009). B cell-dependent Bcl6 induction for Tfh cell differentiation was further evidenced by rescuing Tfh cell differentiation in μMT mice by constitutive expression of Bcl6 in antigen-specific CD4+ T cells (Johnston et al., 2009). B cell-dependent Tfh cell differentiation is, however, challenged by a recent study that found Tfh cells could develop in mice deficient for MHCII only on B cells if mice were given repeated Ag injection (Deenick et al., 2010). The interdependence of Tfh cell and GC B cell differentiation, both of which require Bcl6, adds an additional layer of complexity to assessing the cell autonomy of Tfh cell defects.

An alternative model has proposed that Tfh cell differentiation is not an independent developmental pathway, but instead Tfh cells are possibly a subsequent state of Th1, Th2, and Th17 cells (Awasthi and Kuchroo, 2009; Bauquet et al., 2009; Murphy and Stockinger, 2010; Zaretsky et al., 2009). Several studies have shown that Tfh cells can exhibit features of Th1, Th2, and Th17 cells (Bauquet et al., 2009; Fazilleau et al., 2009; Johnston et al., 2009; Reinhardt et al., 2009; Zaretsky et al., 2009). Indeed, such cytokine production is important for appropriate induction of B cell class switch recombination. Nevertheless, Bcl6 is capable of repressing Th1, Th2, and Th17 cell programs (Nurieva et al., 2009), and Tfh cells generally express low amounts of Th1, Th2, or Th17 cell-associated cytokines and transcription factors, particularly in humans (Breitfeld et al., 2000).

Given that Bcl6 is a critical regulator for Tfh cell differentiation, it is crucial to understand the regulation of Bcl6 induction in CD4+ T cells. Therefore, in this study, we probed the differentiation of CD4+ T cells in vivo to determine molecular and cellular cues for Bcl6 expression and the kinetics of Tfh cell differentiation. We demonstrate that Tfh cell differentiation occurs early after viral infection, as an independent differentiation program that is dependent on ICOS signals during DC priming, independent of B cells. We further show that an APC transition then occurs

from DCs to B cells and ICOS signaling is again required a second time for maintenance of Bcl6 and Tfh cells. These results allow for the development of an integrated model of Tfh cell differentiation.

RESULTS

Early Development of Bcl6+CXCR5+ Tfh Cells In Vivo

Tfh cells are readily identifiable at the peak of the CD4+ T cell response to an acute LCMV infection as CXCR5^{hi}SLAM^{lo} BTLA^{hi}PD1^{hi}Bcl6$^+$ virus-specific CD4+ T cells. Equivalent results are observed with either LCMV gp-specific TCR transgenic CD4+ T cells (SMARTA or SM) (Figure 1A) or polyclonal CD4+ T cells (Figure S1 available online). Tfh (CXCR5$^+$) antiviral CD4+ T cells at the peak of the immune response express Bcl6 (p = 0.0002) (Figure 1B).

To probe the molecular and cellular requirements of Tfh cell differentiation, we examined the kinetics of Tfh cell development in vivo, with a particular focus on when Bcl6 protein expression was induced, because Bcl6 is required for Tfh cell differentiation and expression of Bcl6 is sufficient to induce Tfh cell differentiation. SM CD4+ T cells were transferred into B6 mice and analyzed 3 and 4 days after LCMV infection. Strikingly, robust numbers of Bcl6$^+$CXCR5$^+$CD4$^+$ T cells were present at day 3 (Figure 1C). Surface CXCR5 expression was induced 15- to 30-fold higher on Bcl6$^+$ cells than naive CD4+ T cells (p = 0.0001, day 3; p = 0.0005, day 4; Figure 1D). Tfh cells are Bcl6$^+$CXCR5$^+$CD4$^+$ T cells. Because PD-1 is a third canonical Tfh cell marker (Chtanova et al., 2004; Haynes et al., 2007; Linterman et al., 2009), we examined PD-1 expression. Day 3 Bcl6$^+$CXCR5$^+$ SM expressed highly elevated PD-1 compared to the CXCR5$^-$ SM population (CXCR5$^+$ versus CXCR5$^-$ PD-1 MFI, p = 4.0×10^{-5}; Figures 1E and 1G). Tfh cell differentiation was further evidenced by analysis of ICOS expression, which is highly expressed on Tfh cells in humans and mice (Breitfeld et al., 2000; Vinuesa et al., 2005). Indeed, day 3 CXCR5$^+$ SM expressed significantly more ICOS (p = 0.0005 versus CXCR5$^-$ SM; Figure S1D). Interestingly, early Tfh cells also expressed more CD69 than effector Th antiviral CD4+ T cells (CXCR5$^+$ versus CXCR5$^-$ CD69 MFI, p = 0.0001; Figures 1F and 1H). CD69 inhibits S1P1-dependent egress of T cells from lymphoid tissues (Pham et al., 2008) and probably enhances retention of Tfh cells in and near B cell follicles (Fazilleau et al., 2007). Taken together, our Bcl6 data indicated that the Tfh cell developmental program commences early during CD4+ T cell priming in vivo.

DC Priming: SAP, CD40, and B Cell-Independent Tfh Cell Commitment

Tfh cells are crucial for the development of germinal centers, from which long-lived plasma cells and memory B cells are predominantly generated (Allen et al., 2007; Good-Jacobson et al., 2010). Germinal center formation requires SLAM-associated protein (SAP, encoded by *Sh2d1a*) expression in CD4+ T cells (Crotty et al., 2003; Qi et al., 2008). SAP is an intracellular signaling molecule that binds to members of the SLAM family of receptors, which are prominently expressed on T and B cells (Cannons et al., 2011). SAP deficiency in CD4+ T cells severely impairs the duration of cognate interactions with B cells, whereas the absence of SAP does not affect CD4+ T cell-DC
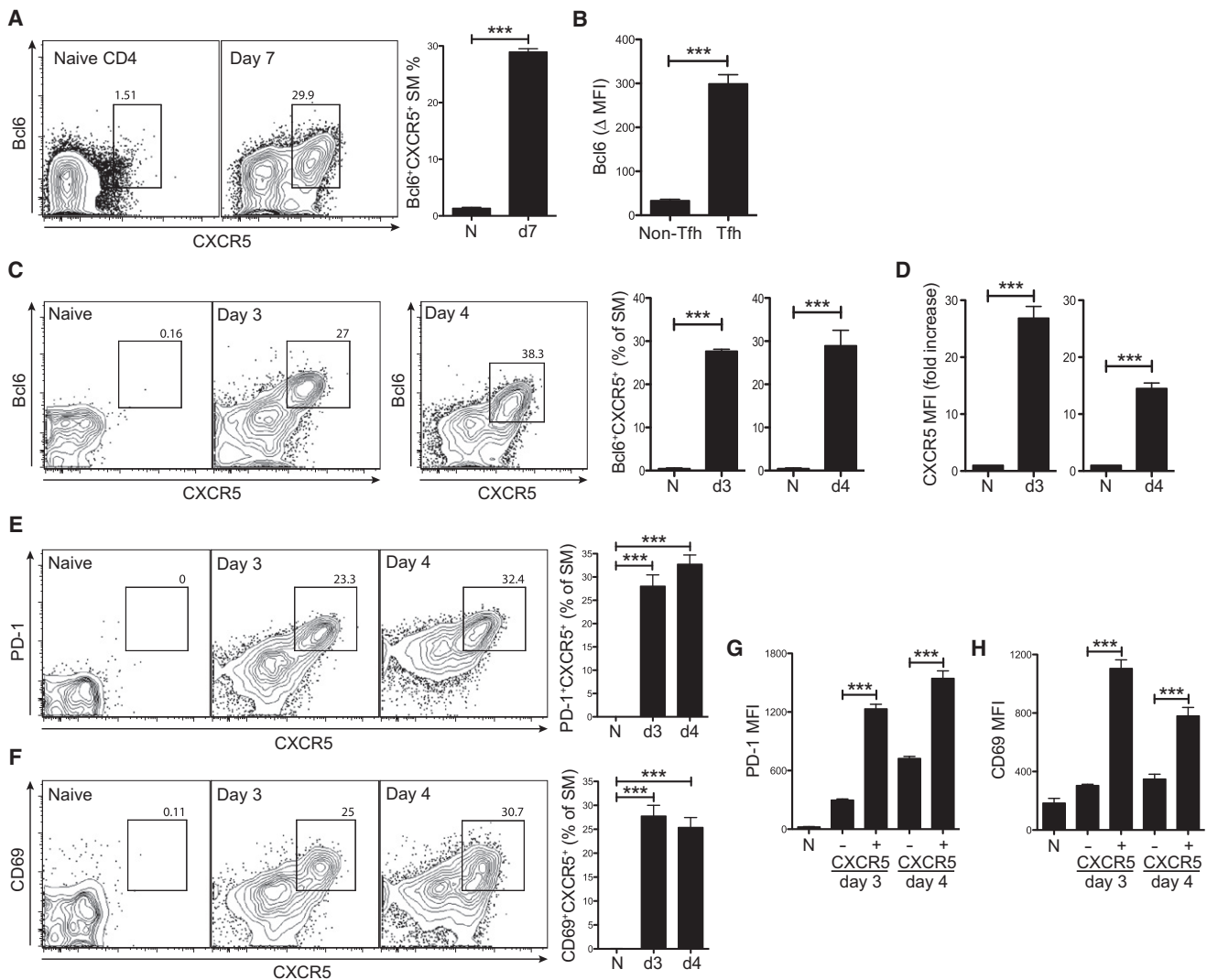
Figure 1. Bcl6$^+$CXCR5$^+$ Tfh Cell Differentiation Occurs within 72 hr In Vivo

(A–D) LCMV-specific SM CD4$^+$ T cells were transferred into B6 mice that were subsequently infected with LCMV.

(A) Bcl6$^+$CXCR5$^+$ Tfh cells analyzed 7 days postinfection (p.i.) ("d7") versus naive ("N," CD44^{lo}CD62^{hi}) CD4$^+$ T cells of uninfected control mice.

(B) Bcl6 protein expression in day 7 SM as Δ MFI (e.g., Bcl6 MFI$_{Tfh}$ − Bcl6 MFI$_{naive\ CD4}$).

(C) Bcl6$^+$CXCR5$^+$ Tfh cells, 3 and 4 days p.i. ("d3," "d4"). "N," uninfected mice. FACS plot shows SM CD4$^+$ T cells.

(D) Increase in SM CXCR5 MFI compared to that of naive (CD44^{lo}CD62^{hi}) CD4$^+$ T cells of uninfected mice.

(E–H) Day 3 and 4 after LCMV infection, PD-1 (E) and CD69 (F) coexpression with CXCR5 on SM CD4$^+$ T cells.

(G and H) PD-1 (G) and CD69 (H) MFIs of CXCR5$^+$ SM versus CXCR5$^-$ SM.

Data are representative of three or more independent experiments; n = 4 per time point for all panels. ***p < 0.001. Error bars are SEM. See also Figure S1.

interactions (Cannons et al., 2010; Qi et al., 2008). We therefore examined whether SAP has a role in early Tfh cell differentiation in vivo. $Sh2d1a^{-/-}$ SM CD4$^+$ T cells were transferred into C57BL/6 mice (B6) and analyzed for Tfh cell differentiation after acute LCMV infection. Both CXCR5 induction and Bcl6$^+$CXCR5$^+$ Tfh cell development (Figure 2A) by $Sh2d1a^{-/-}$ SM CD4$^+$ T cells were normal at day 3 and day 4 in vivo. Thus, Bcl6 expression and Tfh cell differentiation is induced in CD4$^+$ T cells prior to sustained B cell-T cell interactions.

CD40 is also required for germinal center development (MacLennan, 1994) and is prominently expressed on B cells. Therefore we analyzed whether CD40L signals direct early Tfh

cell development. We tested the early Tfh cell commitment of SM CD4$^+$ T cells in $Cd40^{-/-}$ mice. Robust Bcl6 induction was observed in the absence of CD40, and the frequency of Bcl6$^+$CXCR5$^+$ CD4$^+$ T cells was normal (Figure 2B). However, lack of CD40 results in a severe truncation of the B cell response by day 8 (Figure S2A) and resulted in a loss of Tfh cells (Figure S2B).

The finding of early Bcl6 induction revealed a conundrum, because Tfh cells were previously demonstrated to require the presence of cognate B cells in the work of our lab (Johnston et al., 2009) and others (Haynes et al., 2007; Zaretsky et al., 2009). We have confirmed those earlier results (WT versus μMT Tfh cells = 50% versus 18%; p = 1.8 × 10^{-5}; Figure 2C)
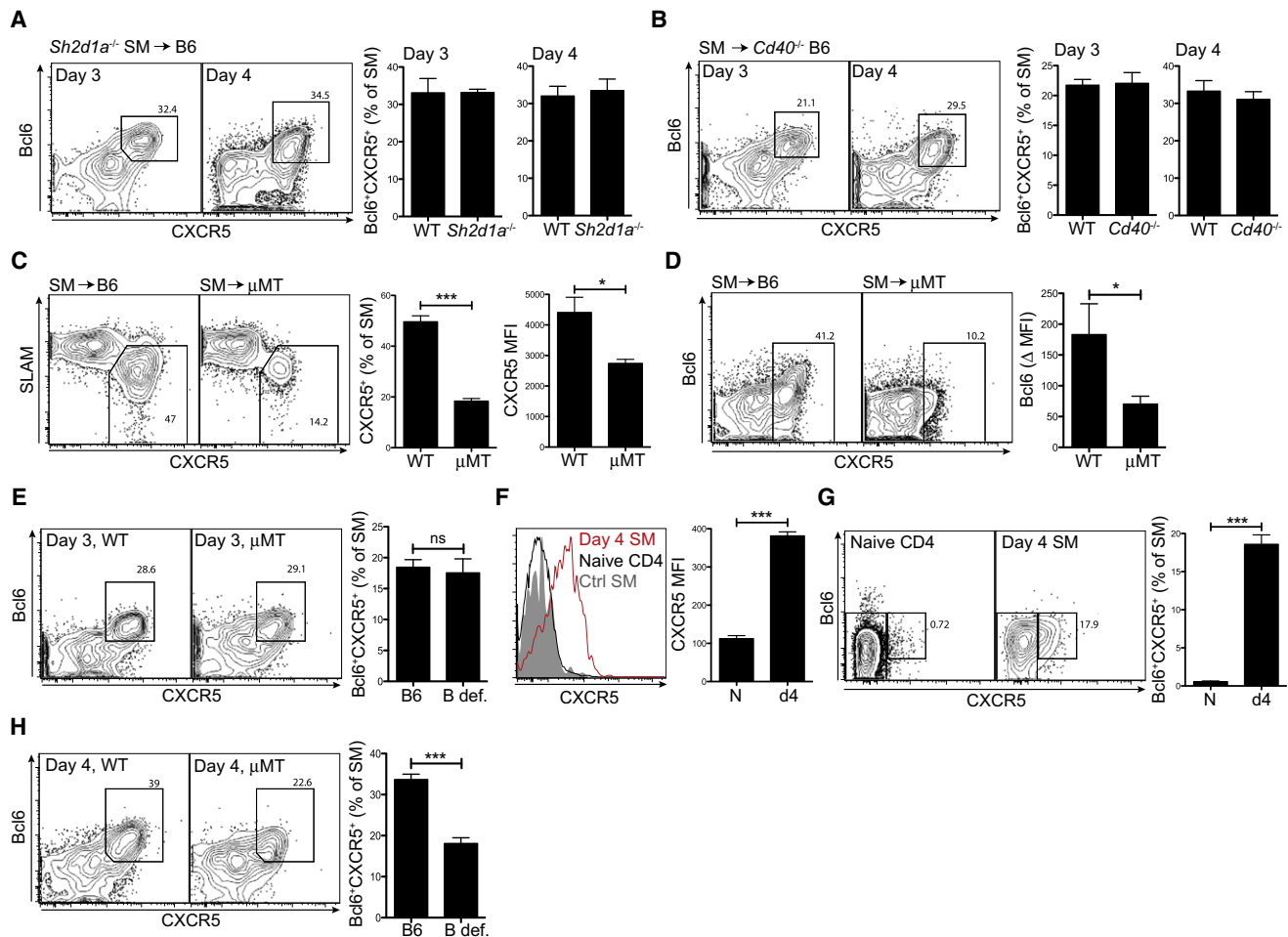
Figure 2. Early Bcl6⁺CXCR5⁺ Tfh Cell Differentiation Is B Cell Independent and Does Not Require SAP- or CD40L-Mediated Signals

(A) Naive $Sh2d1a^{-/-}$ or WT SM CD4⁺ T cells were transferred into B6 mice subsequently infected with LCMV. Bcl6⁺CXCR5⁺ Tfh cell differentiation analyzed at 3 and 4 days p.i. FACS plots show $Sh2d1a^{-/-}$ SM and gates identify Bcl6⁺CXCR5⁺ Tfh cells.

(B) WT SM CD4⁺ T cells were transferred into either B6 or $Cd40^{-/-}$ mice subsequently infected with LCMV. Tfh cell differentiation was analyzed as shown in (A).

(C and D) SM CD4⁺ T cells were transferred into B6 or B cell-deficient (μMT) mice and infected with LCMV, and the CD4⁺ T cell response was analyzed 7 days later.

(C) SM CD4⁺ T cells are shown. Gate indicates SLAM^{lo}CXCR5^{hi} Tfh cells.

(D) SM cells are shown and gates identify Bcl6⁺CXCR5⁺ Tfh cells. Bcl6 protein calculated Δ MFI (e.g., Bcl6 MFI$_{Tfh}$ − Bcl6 MFI$_{naive\ CD4}$).

(E and H) SM CD4⁺ T cells were transferred into B6 and μMT (or MD4-μMT. B def.) mice. Mice were then infected with LCMV and analyzed 3 (E) and 4 (H) days p.i. FACS plots show total SM cells, and gates identify Bcl6⁺CXCR5⁺ Tfh cells. Data are a composite of three or more independent experiments (n = 15–16 per group for day 3, n = 17–20 per group for day 4).

(F and G) LCMV Gp61 peptide-pulsed DCs were activated in vitro with LPS and transferred into B6 mice that had received naive SM CD4⁺ T cells. SM were analyzed 4 days after DC immunization.

(F) Left, SM CD4⁺ T cells in DC-immunized (red line) versus unimmunized mice (gray filled) compared to that of endogenous naive (CD44^{lo}) CD4⁺ T cells in DC-immunized mice (black line). Right, CXCR5 MFIs calculated. Day 4 SM ("day 4") versus SM in unimmunized mice ("N").

(G) Bcl6⁺CXCR5⁺ Tfh cells.

(A–D, F, and G) Data are representative of two independent experiments. n = 4 per group.

n.s., no statistically significant difference (p > 0.05). *p < 0.05, ***p < 0.001. Error bars are SEM. See also Figure S2.

and determined that Bcl6 protein expression by CD4⁺ T cells was severely reduced at the peak of the antiviral CD4⁺ T cell response in the absence of B cells (p = 0.014; Figure 2D). This led us to directly examine whether B cells were required for early Tfh cell differentiation. Clear Bcl6 induction and Bcl6⁺CXCR5⁺ Tfh cell differentiation was present in the absence of total (μMT) or cognate B cells (MD4-μMT) at day 3 in vivo (B def.; Figure 2E). Thus, early Tfh cell differentiation is B cell independent.

Given that Tfh cell differentiation initiates early and is B cell independent, DCs were implicated. To determine whether DCs could directly induce Tfh cell differentiation, we conducted in vivo experiments with peptide-pulsed DCs. Indeed, DC immunization was sufficient to induce CXCR5 expression (p = 3.2 × 10^{-5}; Figure 2F) and Bcl6 expression (p = 0.0001; Figure 2G) by CD4⁺ T cells. This was not due to transfer of antigen from DCs to B cells; comparable CXCR5 induction was observed in

DC-immunized $H2^{-/-}$ recipients (Figures S2C and S2D). These results strongly indicated that DC priming is sufficient to induce Bcl6 expression and initial Tfh cell differentiation.

B cell-independent Tfh cell differentiation led us to analyze whether Tfh cells require sequential antigen presentation by DCs and B cells, with early Tfh cell commitment being instructed by DCs and Tfh cell maintenance determined by B cells. Whereas normal Bcl6$^+$CXCR5$^+$ Tfh cell differentiation was present in the absence of cognate B cells (μMT or MD4-μMT mice) at day 3 p.i. (Figure 2E), the frequency of Bcl6$^+$CXCR5$^+$CD4$^+$ T cells was significantly reduced at day 4 (p = 1.79 × 10^{-9}; Figure 2H), which implied that a major APC transition from DCs to B cells occurs for CD4$^+$ T cells approximately 3 to 4 days after an acute infection. This is consistent with a recent report that Tfh cells were present in the absence of B cells when mice were given continuous antigen via peptide injection (Deenick et al., 2010). Our data thus far indicate that early induction of Bcl6 and Tfh cell development occurs during interaction with DCs and is independent of SAP- or CD40-mediated signals, whereas maintenance of Bcl6 expression and stable commitment to Tfh cell differentiation normally requires additional interactions with cognate B cells.

ICOS Instructs Tfh Cell Differentiation

These observations led us to explore DC signals that could initiate Bcl6 induction and instruct Tfh cell differentiation. Interest in early signals controlling Tfh cell differentiation led us to examine ICOS signaling. Reduced numbers of Tfh cells are observed in the absence of ICOS signals (Akiba et al., 2005; Gigoux et al., 2009). However, it has not been addressed whether ICOS-mediated signals are required for Bcl6 induction and initiation of Tfh cell differentiation. To determine this, we analyzed whether $Icos^{-/-}$ SM CD4$^+$ T cells undergo early Tfh cell differentiation in vivo. Strikingly, Bcl6 induction was severely reduced in the absence of ICOS (p = 7.8 × 10^{-5} at day 3, p = 0.00093 at day 4; Figures 3A–3C). CD4$^+$ T cell activation and proliferation were not affected by the absence of ICOS (Figures S3A–S3D).

$Icos^{-/-}$ SM failed to differentiate to Tfh cells, with near-background Tfh cell frequencies at day 3 (p = 0.0001; Figure 3D). Bcl6^{hi} $Icos^{-/-}$ SM CD4$^+$ T cells were absent (Figure S3F). At day 4, a similarly severe loss of $Icos^{-/-}$ Tfh cells was seen (p = 4.65 × 10^{-6}; Figure 3D). The rare $Icos^{-/-}$ Tfh cells remaining had aberrantly low levels of Bcl6 (p = 3.95 × 10^{-7}; Figure 3C) and CXCR5 (p = 7.4 × 10^{-7}; Figure 3E and Figure S3E). Thus, ICOS-mediated signals are crucial for Bcl6 induction during CD4$^+$ T cell priming for initiation of Tfh cell differentiation.

### Sequential ICOS Requirements for Tfh Cells and Germinal Centers

Given the early Tfh cell defect of $Icos^{-/-}$ CD4$^+$ T cells, we then analyzed the impact of the Tfh cell defect on the magnitude of the germinal center B cell response in $Icos^{-/-}$ mice after an acute VACV infection. Germinal centers were impaired at day 8 in $Icos^{-/-}$ mice compared to WT controls (p = 0.0004; Figure 4A). This was consistent with severe germinal center defects reported in ICOS-deficient mice in response to nonreplicating antigens (Akiba et al., 2005; McAdam et al., 2001; Tafuri et al., 2001). CD4$^+$ T cell activation was normal (CD44^{hi}CD62L^{lo}CD4$^+$ T cell frequency; 24.0% ± 2.3% WT versus 26.3% ± 7.3% $ICOS^{-/-}$).

The defective germinal centers after VACV infection were probably a direct consequence of the reduced availability of T cell help, as indicated by the fact that Tfh cell numbers were significantly reduced in $Icos^{-/-}$ mice at day 8 after VACV infection (p = 0.002; Figure 4B). Remaining $Icos^{-/-}$ Tfh cells had significantly reduced CXCR5 expression (p = 0.0015; Figure 4B). Two populations of Tfh cells can be distinguished phenotypically in vivo (Haynes et al., 2007; Yusuf et al., 2010). Tfh cells physically within germinal centers (GC Tfh cells) have the highest PD-1 expression (Haynes et al., 2007) and can be distinguished on the basis of GL7 expression in mice with an acute viral infection (Yusuf et al., 2010). Generation of GC Tfh cells was impaired in the absence of ICOS-mediated signals in mice 8 days postinfection (p = 0.042; Figure 4C). Defective Tfh cell differentiation and CXCR5 induction were well correlated with a loss of Bcl6 expression by the $Icos^{-/-}$ CD4$^+$ T cells (p = 0.0004; Figure 4D). $Icos^{-/-}$ Tfh cell and GC Tfh cell defects were even more severe by day 12 after VACV infection (Figures 4E–4G). $Icos^{-/-}$ Tfh cell frequencies were merely 27% of normal (p = 4.2 × 10^{-6}; Figure 4E), and $Icos^{-/-}$ GC Tfh cells were barely detectable (p = 0.0001; Figure 4F). As a result, $Icos^{-/-}$ germinal centers decayed rapidly, with a 95% loss of GC B cells at day 12 after VACV infection (p = 0.0005; Figure 4G and Figure S4A). Similar defects were found after LCMV infection of $Icos^{-/-}$ mice (Figures S4B–S4D).

Experiments above show a requirement for ICOS early during DC priming. DCs and B cells both express ICOSL, the ligand for ICOS. $Icosl^{-/-}$ mice were unable to support Tfh cell differentiation, as shown by the fact that wild-type SM cells transferred into $Icosl^{-/-}$ recipients failed to develop a Bcl6^{hi} population (data not shown). A major APC transition from DCs to B cells occurs at 3–4 days p.i. (Figure 2). Therefore, we explored whether ICOSL signals were required both early and late during Tfh cell differentiation. ICOS signals after CD4$^+$ cells priming were blocked by in vivo treatment with anti-ICOSL (αICOSL) starting at day 3 p.i. (Figure S4E). ICOSL blockade after priming resulted in a significant loss of Tfh cells (~25%, p = 0.0004; Figure 4H) and GC Tfh cells (~50%, p = 0.003; Figure 4I) by day 8 p.i. Importantly, Bcl6-expressing CD4$^+$ T cells were substantially reduced (p = 0.0001; Figure 4J). Germinal centers were severely reduced (p = 0.005; Figure 4K), whereas overall B cells and activated CD4$^+$ T cell numbers were largely unaffected (data not shown). Taken together, these data demonstrate that there are two independent Tfh cell requirements for ICOS during sequential interactions with different APCs. ICOSL is first required on DCs for Tfh cell induction and ICOSL is then required again during T cell-B cell interactions for maintenance of Bcl6 expression.

ICOS Induction of Bcl6 Leads to Bcl6 Induction of CXCR5

Overall, the experiments elaborated above showed that Tfh cell development is present by day 3 in vivo and ICOS-mediated signals are essential. However, it was not known why ICOS is required. Is the signaling hierarchy such that ICOS induces Bcl6 first and then Bcl6 induces CXCR5, or is Bcl6 induction dependent on CXCR5 expression? Or does ICOS directly induce both CXCR5 and Bcl6 independently? To address these questions, we determined whether ICOS could induce CXCR5 expression on CD4$^+$ T cells in the absence of Bcl6. Bcl6 expression in SM
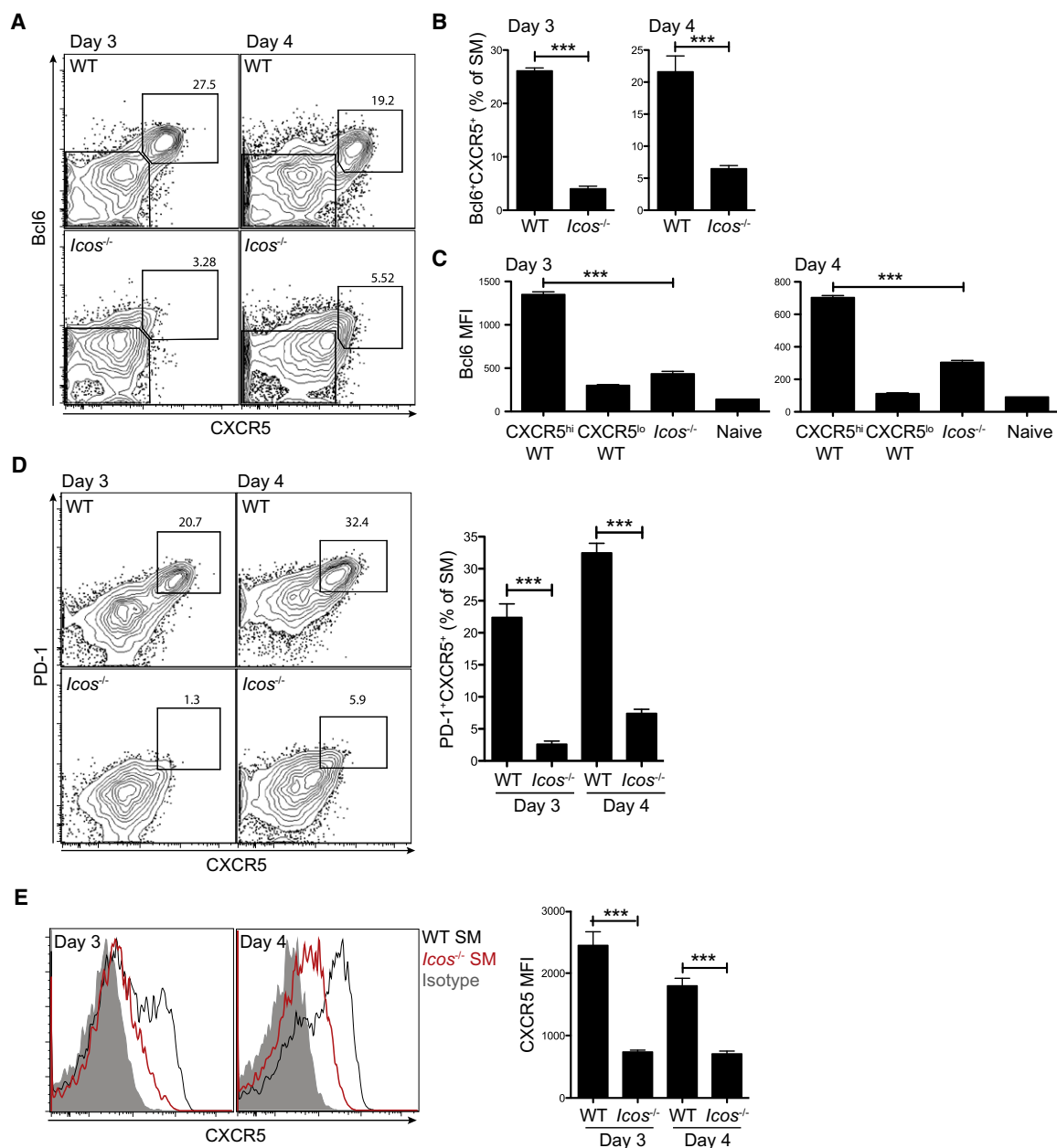
Figure 3. Early Tfh Cell Commitment Requires ICOS Signals for Bcl6 Expression

WT or $Icos^{-/-}$ SM CD4$^+$ T cells were transferred into B6 mice subsequently infected with LCMV.

(A and B) Bcl6$^+$CXCR5$^+$ Tfh cell development was analyzed 3 and 4 days p.i. by FACS (A) and quantified (B).

(C) Bcl6 protein expression was quantified in WT CXCR5$^+$ SM and WT CXCR5$^-$ SM versus $Icos^{-/-}$ SM CD4$^+$ T cells.

(D) PD-1 expression on WT and $Icos^{-/-}$ SM CD4$^+$ T cells.

(E) Left, CXCR5 expression on WT (black) and $Icos^{-/-}$ (red) SM CD4$^+$ T cells, compared to isotype control (gray filled). Right, CXCR5 MFIs.

Data are representative of three independent experiments; n = 4 per group. ***$p < 0.001$. Error bars are SEM. See also Figure S3.

CD4$^+$ T cells was prevented by expression of Blimp1 (Prdm1-RV; Figure S5A). Mice received a 1:1 mixture of control and Prdm1-RV$^+$ SM cells (Figure 5A). Although SM CD4$^+$ T cell activation (CD44^{hi}; Figure S5D) and proliferation (Figure 5B) was normal at day 4 after LCMV infection, Bcl6 expression was efficiently blocked (p = 4.79×10^{-5} at day 3; Figure S5C and Figure 5E, right; p = 0.0008 at day 4; Figures 5C and 5D, right). Importantly, CD4$^+$

T cells were unable to upregulate CXCR5 expression in the absence of Bcl6 (p = 6.0×10^{-5} at day 3; Figure S5B and Figure 5E, left; p = 0.0008 at day 4; Figures 5C and 5D, left).

In another set of experiments, Bcl6 expression was directly quenched in SM CD4$^+$ T cells by expressing Bcl6 shRNA in the context of a natural micro RNA backbone for optimal processing (shRNAmir). Whereas both expansion (Figure 5F) and activation
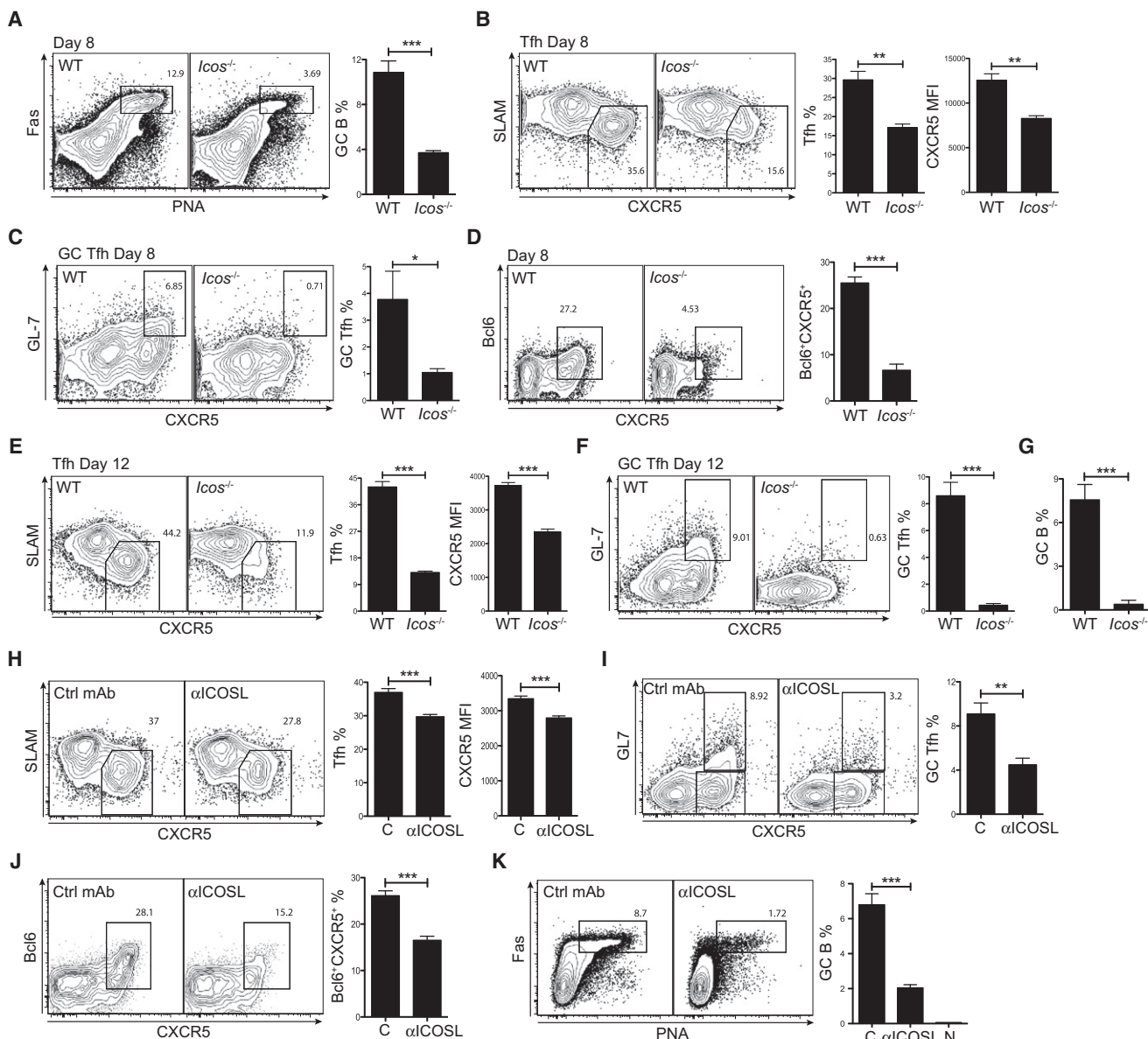
Figure 4. ICOS-Dependent Tfh Cell Maintenance and Germinal Center Formation

B6 and $Icos^{-/-}$ mice were infected with vaccinia virus (VACV). Mice were analyzed 8 days p.i. (A–D) and 12 days p.i. (E–G).

(A) Representative FACS plots of germinal center B cells ($Fas^{+}PNA^{+}$). Total $B220^{+}$ B cells are shown. Right, GC B cells were quantified as percent of total B cells.

(B) Representative FACS plots of WT and $Icos^{-/-}$ polyclonal Tfh cells. Activated $CD4^{+}$ T cells ($CD44^{hi}CD62L^{lo}$) are shown and gates identify $SLAM^{lo}CXCR5^{+}$ Tfh cells. Tfh cell frequencies (percent of activated $CD4^{+}$ T cells [$CD44^{hi}CD62L^{lo}$]) and CXCR5 MFIs were quantified.

(C) Representative FACS plots of WT and $Icos^{-/-}$ polyclonal GC Tfh cells. Activated $CD4^{+}$ T cells ($CD44^{hi}CD62L^{lo}$) are shown and gates identify $GL7^{+}CXCR5^{hi}$ GC Tfh cells. GC Tfh cell frequencies were quantified (percent of activated $CD4^{+}$ T cells [$CD44^{hi}CD62L^{lo}$]).

(D) Representative FACS plots of Bcl6 expression by $CD4^{+}$ T cells in B6 and $Icos^{-/-}$ mice. $Bcl6^{+}CXCR5^{+}$ Tfh cells were quantified (percent of activated $CD4^{+}$ T cells).

(E–G) Tfh cells (E), GC Tfh cells (F), and germinal center B cells (G) were analyzed 12 days p.i., as for day 8 and described in (A)–(C). Data are representative of two independent experiments for day 8 and day 12; n = 4 per group.

(H–K) B6 mice were infected with VACV and treated with anti-ICOSL (αICOSL) or isotype control mAb at day 3, 5, and 7 as schematically shown in Figure S4E. Tfh cells (H), GC Tfh cells (I), Bcl6 expression (J), and GC B cells (K) were analyzed 8 days p.i. Abbreviations: C, isotype control mAb-treated mice; αICOSL, anti-ICOSL-treated mice; N, naive uninfected mice. n = 5–6 per group.

Data are representative of two independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001. Error bars are SEM. See also Figure S4.

(CD44^{hi}; Figure S5E) were normal for Bcl6-shRNA-transduced SM CD4^{+} T cells, CXCR5 was not expressed when Bcl6 was suppressed (p = 0.001; Figure 5G). Collectively, our data define

a molecular hierarchy in Tfh cell commitment initiated at T cell priming, wherein ICOS signals to induce Bcl6, which subsequently induces high CXCR5 expression.
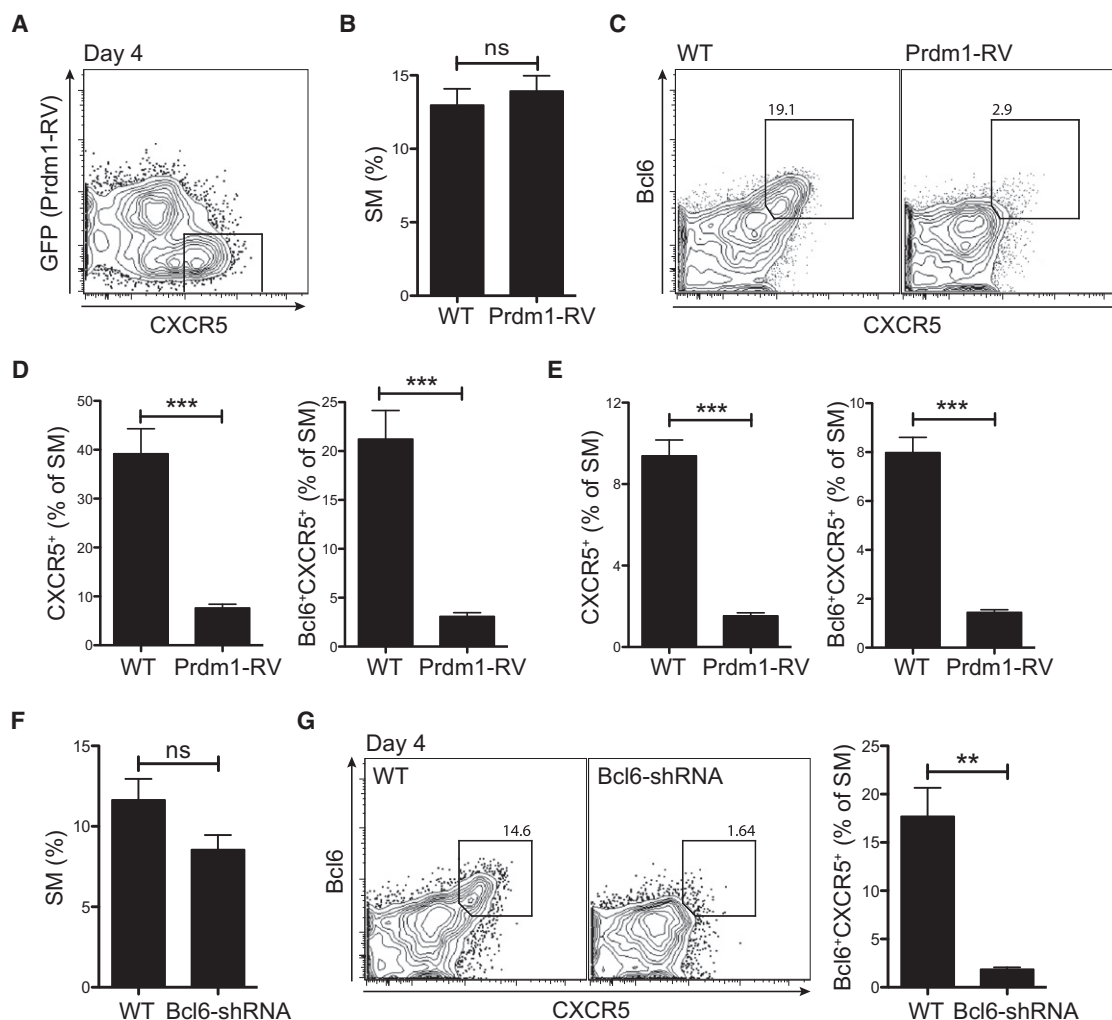
Figure 5. ICOS → Bcl6 → CXCR5

(A–E) Blimp1-expressing (Prdm1-RV$^+$, GFP$^+$) and nontransduced (GFP$^-$) SM CD4$^+$ T cells were transferred into the same (A) or separate (B–E) B6 recipient mice.

(A) SM CD4$^+$ T cells at day 4 after LCMV infection.

(B) WT SM versus Prdm1-RV$^+$ SM CD4$^+$ T cell expansion (percent of total CD4$^+$ T cells).

(C and D) CXCR5 expression by Prdm1-RV$^+$ SM and WT SM, day 4 p.i.

(C) Representative FACS plots showing WT (GFP$^-$) and Prdm1-RV$^+$ (GFP$^+$). Gates identify CXCR5$^+$Bcl6$^+$ SM.

(D) Quantitation of CXCR5$^+$ and CXCR5$^+$Bcl6$^+$ SM.

(E) CXCR5$^+$ SM and Bcl6$^+$CXCR5$^+$ Tfh cell frequencies day 3 p.i.

(F and G) Bcl6 shRNA-RV$^+$ (GFP$^+$Ametrine$^+$) and nontransduced (GFP$^-$Ametrine$^-$) SM CD4$^+$ T cells were transferred into separate B6 recipient mice subsequently infected with LCMV.

(F) SM CD4$^+$ T cell expansion, day 4 p.i. (percent of total CD4$^+$ T cells).

(G) Representative FACS plots of CXCR5 and Bcl6 expression by WT SM and Bcl6 shRNA$^+$ SM CD4$^+$ T cells. Total SM cells are shown and gates identify Bcl6$^+$CXCR5$^+$ SM.

Data are representative of three (A–E) and two (F–G) independent experiments; n = 4 per group per time point. **p < 0.01, ***p < 0.001. Error bars are SEM. See also Figure S5.

Tfh versus Effector Th Cell Commitment: High-Affinity IL-2 Receptor versus ICOS Signals

Given the rapid in vivo differentiation of Bcl6$^+$CXCR5$^+$CD4$^+$ T cells, we next examined when the earliest developmental features of Tfh cells versus effector Th cells can be distinguished. We previously reported that Bcl6 and Blimp1 are antagonistic transcription factors differentially expressed in Tfh and effector Th cells, respectively (Johnston et al., 2009). By using a Blimp1-YFP reporter, we now show that this bifurcation is dramatically polarized. Day 8 effector SM cells fall into two clear populations: Blimp1$^+$ effector Th cells and Blimp1$^-$CXCR5$^+$ Tfh cells (Figure 6A). The Blimp1$^-$ Tfh cells express more Bcl6 than Blimp1$^+$ effector Th cell counterparts (p = 9.0 × 10^{-7}; Figure 6B). This reinforces the concept that Blimp1 and Bcl6 serve as a bimodal transcription factor switch in CD4$^+$ T cell differentiation (Fazilleau et al., 2009; Johnston et al., 2009).
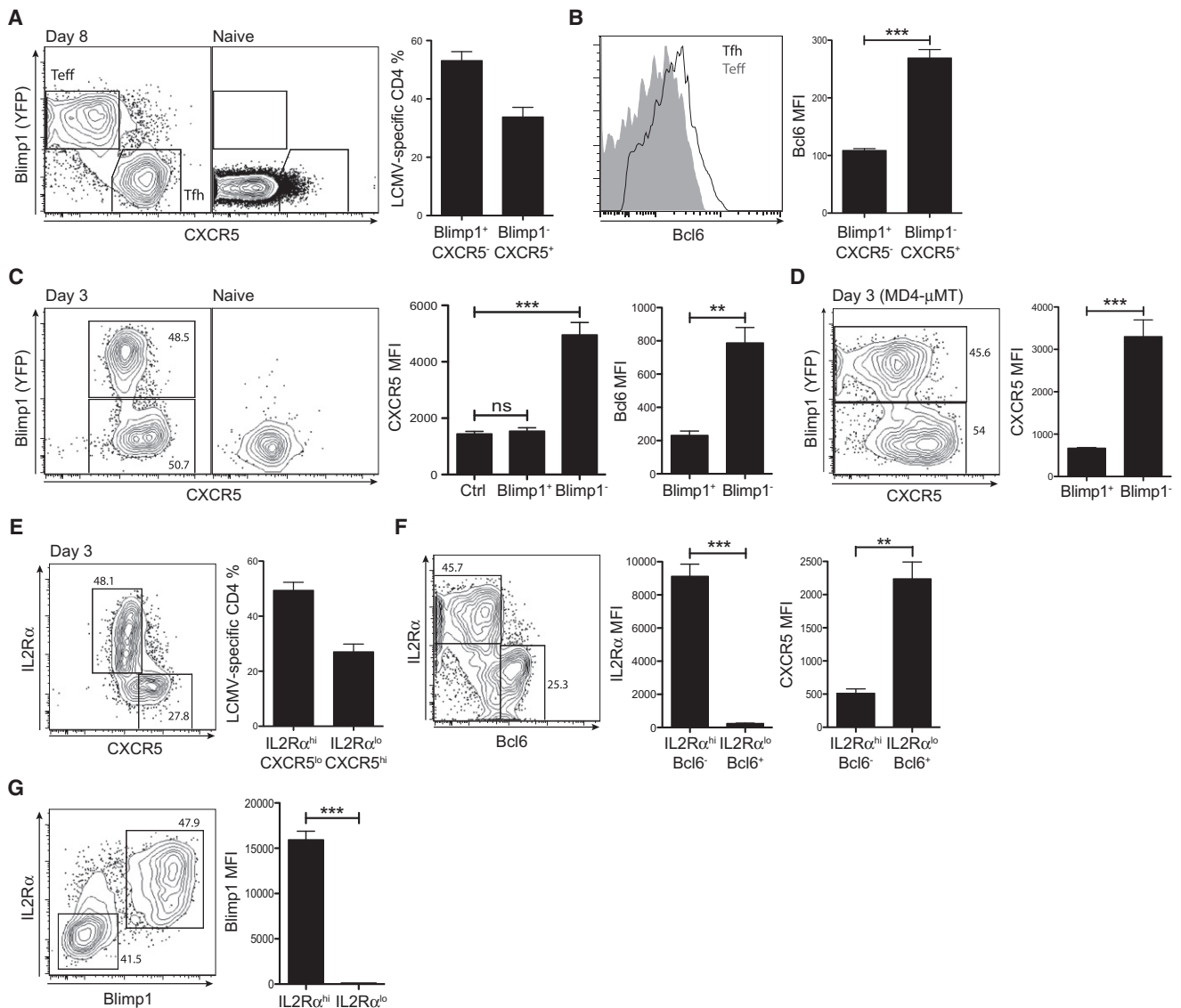
Figure 6. Rapid Bcl6 versus Blimp-1 Bifurcation of CD4$^+$ T Cell Differentiation In Vivo

(A) Blimp1 and CXCR5 expression by Blimp1-YFP BAC tg SM CD4$^+$ T cells was analyzed at 8 days p.i. in B6 recipient mice. Gates identify Blimp1$^+$CXCR5$^-$ effector Th cells and Blimp1$^-$CXCR5$^+$ Tfh cells.

(B) Left, Bcl6 expression in Blimp1$^+$CXCR5$^-$ (gray filled) versus Blimp1$^-$CXCR5$^+$ (black line) SM CD4$^+$ T cells. Right, Bcl6 MFIs.

(C) Day 3 p.i., analysis of Blimp1, Bcl6, and CXCR5 expression by Blimp1-YFP BAC Tg SM CD4$^+$ T cells in B6 recipients. Representative FACS plots of SM cells from LCMV-infected (Day3) and uninfected (Naive) recipients are shown. CXCR5 and Bcl6 expression by Blimp1$^+$ versus Blimp1$^-$ SM CD4$^+$ T cells were quantified. Ctrl, isotype control stain.

(D) MD4-μMT recipient mice of Blimp1-YFP BAC tg SM CD4$^+$ T cells, day 3 p.i. Representative FACS plot of SM cells and CXCR5 quantitation.

(E–G) Blimp1-YFP SM CD4$^+$ T cells were transferred into B6 recipients and analyzed for IL2Rα expression 3 days after LCMV infection. IL2Rα^{hi} versus IL2Rα^{lo} SM CD4$^+$ T cells are identified in costains with CXCR5 (E), Bcl6 (F), and Blimp1 (G).

Data in all panels are representative of three or more independent experiments; n = 3–4 per group. **p < 0.01, ***p < 0.001. Error bars are SEM.

Blimp1 expression is commonly considered a late event in effector T cell differentiation (Martins and Calame, 2008; Rutishauser et al., 2009). However, given that Bcl6$^+$CD4$^+$ T cells could be identified at day 3 in vivo, we examined whether Blimp1$^+$ effector CD4$^+$ T cells might be also identified early in the immune response. Indeed, already by day 3, antiviral CD4$^+$ T cells segregated into two distinct cell types: Blimp1$^+$ or Blimp1$^-$, with significant CXCR5 induction only on Blimp1$^-$ populations

(p = 1.09 × 10^{-5}; Figure 6C). Importantly, day 3 Blimp1$^+$ and Blimp1$^-$ populations (Blimp1 MFI difference, p = 8.1 × 10^{-5}) showed reciprocal Bcl6 expression (Bcl6 MFI difference, p = 0.004; Figure 6C). The early bifurcation in Blimp1$^+$CXCR5$^{lo/-}$ effector Th cell versus Bcl6$^+$CXCR5^{hi} Tfh cell differentiation occurred via DC priming, as shown by the fact that it was able to occur in the absence of cognate B cells (46% Blimp1$^+$ and 53% Blimp1$^-$ SM in B6; Figure 6C; 46% Blimp1$^+$ and 54% Blimp1$^-$ SM in MD4-μMT;

Figure 6D). Thus, DC priming instructs a bifurcation between Blimp1- and Bcl6-expressing CD4$^+$ T cells by day 3 in vivo.

Blimp1 can be induced by IL-2 (Malek and Castro, 2010; Martins and Calame, 2008). Therefore we examined expression of the high-affinity IL-2 receptor alpha subunit (IL2Rα, CD25) at day 3 p.i. Two clear SM cell populations were distinguishable: IL2Rα^{hi}CXCR5^{lo} and IL2Rα^{lo}CXCR5^{hi} (Figure 6E). Strikingly, 98% less IL2Rα was expressed on Bcl6$^+$CD4$^+$ T cells compared to Bcl6^{lo}CD4$^+$ T cells (p = 0.0002; Figure 6F). Conversely, Blimp1 was strictly expressed in the cells with high IL2Rα expression (p = 0.00008; Figure 6G). In contrast to ICOS-mediated Bcl6 induction (Figure 3), these data demonstrate a competing pathway, via which IL-2-mediated Blimp1 upregulation occurs through IL2Rα signaling.

The dramatic segregation in CD4$^+$ T cell differentiation observed by day 3 led us to examine an even earlier in vivo time point. Surprisingly, at 48 hr p.i., Tfh versus effector Th cell differentiation was already distinguishable. First, we determined that IL2Rα expression is not homogeneous at this time point (Figure 7A). Although a large percentage of antiviral CD4$^+$ T cells were IL2Rα^{hi}, a significant percentage was IL2Rα^{int} (p = 1.5 × 10^{-7}; Figure 7A). Importantly, IL2Rα^{int} cells exhibited the highest CXCR5 MFI (p = 4.9 × 10^{-6}; Figure 7B) and, indeed, expressed the highest amounts of Bcl6 (p = 0.00001; Figure 7C). The early IL2Rα^{hi}CXCR5^{lo} versus IL2Rα^{int}CXCR5^{hi} SM cell polarization was also observed in the absence of cognate B cells (MD4-μMT, p = 0.00004; Figure 7D). Again, the IL2Rα^{int} SM cells expressed the highest amounts of Bcl6 (p = 0.0002; Figure 7E). Therefore, early differential IL2Rα expression occurs during CD4$^+$ T cell priming by DCs and is associated with distinct differentiation fates of the CD4$^+$ T cells. Given that Bcl6 induction was already identifiable by 48 hr in vivo and was segregated in the IL2Rα^{int} SM population, we examined whether Blimp1 was expressed at this very early time point. Indeed, at 48 hr p.i., strong Blimp1 expression was already occurring in 35% of antiviral CD4$^+$ T cells (approximately half of the IL2Rα^{hi} cells; Figure 7F). Notably, Blimp1 expression specifically occurred in the CD4$^+$ T cells that expressed the highest amount of IL2Rα (p = 1.5 × 10^{-6} IL2Rα^{int} versus IL2Rα^{hi}Blimp1$^-$; p = 2.7 × 10^{-10} IL2Rα^{int} versus IL2Rα^{int}Blimp1$^-$; Figure 7G), which is the opposite of the Bcl6 expression pattern (p = 6.2 × 10^{-7} IL2Rα^{int} versus IL2Rα^{hi}; Figure 7H). Virus-specific CD4$^+$ T cells at this 48 hr time point had predominantly undergone only two (40%) or three (37%) cell divisions (Figure 7I). IL2Rα^{int}Bcl6$^+$ cells were equally present in both the two and three cell division populations (Figure 7J). Collectively, our data indicate that differential ICOS versus IL2Rα signaling within the first two cell divisions is a major decision point directing Bcl6 or Blimp1 expression and Tfh versus effector Th cell differentiation during T cell priming.

DISCUSSION

Accumulating evidence has determined that Bcl6 is a critical determinant for the development and function of Tfh cells; however, the mechanisms by which Bcl6 is induced and Tfh cells develop have remained mostly unclear. Here we have used a variety of genetic and in vivo cellular approaches to show that Bcl6 induction and Tfh cell differentiation is initiated upon DC priming in vivo and requires ICOS. Notably,

Bcl6$^+$CXCR5$^+$CD4$^+$ T cells can be identified within the first two cell divisions in vivo, with strong polarization between Bcl6-expressing and Blimp1-expressing Th cells by 3 days p.i. These findings revise our understanding of both the mechanism and kinetics of Tfh cell differentiation.

Previous studies have shown that Tfh cells are a distinguishable cell type in vivo required for germinal center development and that the emergence of Tfh cells is fully dependent on Bcl6 expression (Crotty, 2011). Nevertheless, there has been little consensus regarding the nature of Tfh cell differentiation, in part resulting from lack of information about the earliest events in Tfh cell differentiation in vivo. One commonly held model of Tfh cell differentiation was that the Tfh cell program is a secondary program acquired later by some Th1, Th2, or Th17 cells, and Tfh cells do not exist as an independent cell type. Our data do not support that model. A detailed examination of the kinetics of development of Tfh cells in vivo revealed that Bcl6 expression occurs in a subset of CD4$^+$ T cells within two cell divisions after infection. These findings support models of Tfh cell differentiation whereby Tfh cell development is not contingent on a CD4$^+$ T cell first acquiring Th1, Th2, or Th17 cell characteristics.

Our findings also clarify the relationship between Tfh cells and APCs, particularly B cells and dendritic cells. Previous studies found that Tfh cells were largely dependent on B cells. We confirmed that the presence of Tfh cells at time points later than 1 week postinfection was dependent on B cells, specifically cognate B cells. In addition, B cell interaction with Tfh cells is required for maintenance of Bcl6 expression in Tfh cells. Nevertheless, Tfh cell differentiation initiates independently of B cells, as evidenced by normal Bcl6 induction in B cell-deficient mice at day 3 p.i. In parallel experiments, we reasoned that presence of Bcl6$^+$CD4$^+$ T cells within two divisions in vivo implicated DCs as the responsible APCs for the CD4$^+$ T cells, given the rarity of cognate B cells early. Indeed, DC immunization experiments revealed that DC priming can induce Bcl6 and CXCR5 in CD4$^+$ T cells. B cells become the primary APCs after approximately day 3 to 4 p.i., probably because of the increased availability of antigen-specific B cells as APCs when they undergo extensive clonal expansion, as well as the colocalization of the antigen-specific B cells and Tfh cells. This APC shift is probably also due to death of the majority of the antigen-presenting mature DCs by 4 days after an acute viral infection or protein immunization, limiting MHCII-presented antigen in the lymphoid tissue predominantly to B cells after this time. B cells can influence T cell responses in a variety of situations (Lund and Randall, 2010). This phenomenon of distinct stimulation requirements for initiation of differentiation and then maintenance of differentiation is a common feature of many developmental processes in biology, including CD4$^+$ T cell differentiation, as observed by the instability of iTreg or Th17 cell differentiation in the absence of cofactors or sustained signals. A distinct attribute of Tfh cell differentiation is the requirement for two different APC types at two stages of differentiation.

Each simple model of Tfh cell differentiation (cytokine program, B cell-dependent program, and secondary program) has had strengths and weaknesses. Our studies here now allow for proposal of an integrated model of Tfh cell differentiation, focused on the central role of Bcl6 in this process. We have found that Bcl6 expression occurs early during DC priming,
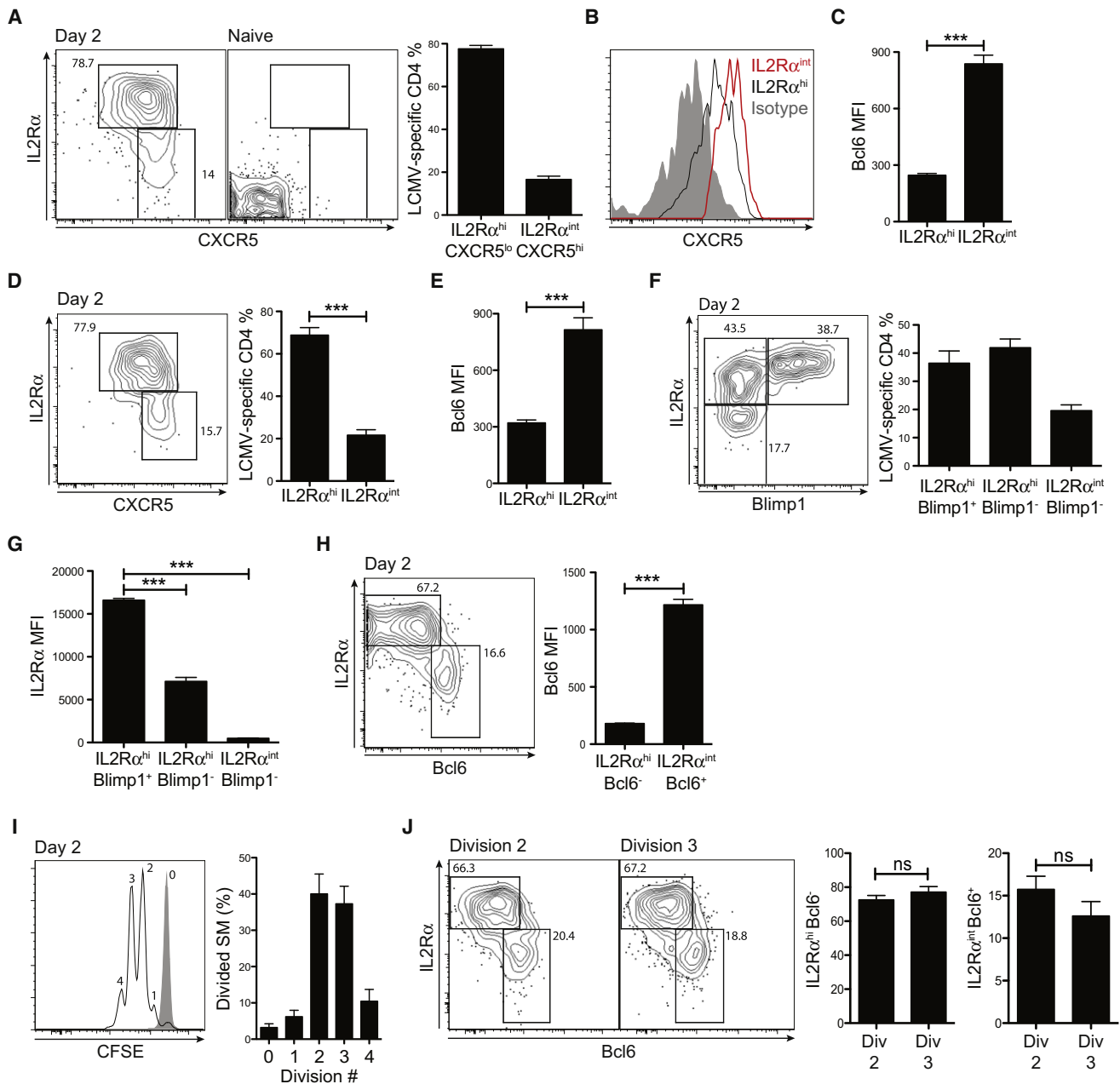
Figure 7. Tfh versus Effector Th Cells Are Distinguishable by the Second Cell Division, in Association with Differential Early IL-2Rα Expression Levels

Early virus-specific Tfh versus effector Th cell differentiation was analyzed at 48 hr after acute LCMV infection.

(A) Representative FACS plots of SM CD4$^+$ T cells from LCMV-infected (Day 2) and uninfected (Naive) mice. Gates identify IL2Rα^{hi}CXCR5^{lo} versus IL2Rα^{int}CXCR5^{hi} SM CD4$^+$ T cells.

(B) CXCR5 expression by IL2Rα^{hi} (black) versus IL2Rα^{int} (red) SM CD4$^+$ T cells. Isotype control shown by gray filled histogram.

(C) Bcl6 expression by IL2Rα^{hi} versus IL2Rα^{int} SM CD4$^+$ T cells.

(D) Expression of IL2Rα and CXCR5 by SM CD4$^+$ T cells in MD4-μMT recipients, day 2 p.i.

(E) Bcl6 expression by SM CD4$^+$ T cells in MD4-μMT recipients, day 2 p.i.

(F) IL2Rα and Blimp1 expression by Blimp1-YFP SM CD4$^+$ T cells in B6 recipients, day 2 p.i. Representative SM CD4$^+$ T cell FACS plot and quantitation are shown.

(G) IL2Rα expression (MFI) by SM CD4$^+$ T cell populations gated in (F).

(H) Bcl6 expression by SM CD4$^+$ T cells in B6 recipients at day 2 p.i. Gates identify IL2Rα^{hi} versus IL2Rα^{int} SM CD4$^+$ T cells. Bcl6 protein expression (MFI) by each population was calculated.

(I and J) CFSE-labeled SM CD4$^+$ T cells were transferred into B6 recipient mice subsequently infected with LCMV. Cell divisions and differentiation were analyzed at day 2 p.i.

(I) Cell divisions. Gray filled histogram show SM CD4$^+$ T cells in uninfected mice.

and Tfh cell maturation occurs quickly thereafter. The cytokine-only model does not suffice, on the basis of the requirement for ICOS signaling during DC priming for Bcl6 expression, as well as the limited impact of IL6, IL21 double deficiency (Eto et al., 2011). The B cell-dependence model does not suffice, on the basis of the early Tfh cell differentiation in the absence of B cells. The secondary program model fails on the basis of Bcl6 induction and Tfh cell differentiation occurring immediately upon DC priming. Nevertheless, aspects of each of these available models were accurate, supported by quality data from multiple published studies. Therefore, key attributes of each are incorporated into our integrated model based on their biological relevance (Figure S6). The secondary program model is inaccurate, but it is nevertheless true that Tfh cells may possess attributes of Th1, Th2, or Th17 cells in some conditions. IL-4, IFN-γ, and IL-17 can be important class switch factors produced by Tfh cells to control B cell antibody isotypes. We interpret these findings as evidence that Tfh cell differentiation that occurs at the time of DC priming is done in the context of a cytokine milieu that, depending on the conditions, can allow partial Th1 (or Th2 or Th17) cell features that coexist with the Tfh cell program. This may relate to the recently appreciated functional plasticity of CD4$^+$ T cells (Murphy and Stockinger, 2010).

ICOS is the only homolog of CD28 found in humans or mice. ICOS has functions distinct from CD28 because of two unique characteristics. Unlike CD28, ICOS is not expressed on resting T cells and is rapidly upregulated after TCR stimulation (Hutloff et al., 1999). Second, the signaling motifs in the cytoplasmic tail of ICOS are distinct from those of CD28 (Yong et al., 2009). The importance of ICOS for germinal centers and T cell-dependent antibody responses was rapidly recognized with *Icos*$^{-/-}$ mice (McAdam et al., 2001; Tafuri et al., 2001). Rare ICOS-deficient humans have a severe loss of antibody responses and memory B cells (Warnatz et al., 2006). In the absence of ICOS, reduced Tfh cell numbers are observed in both mice and humans (Akiba et al., 2005; Bossaller et al., 2006; Grimbacher et al., 2003). Conversely, overexpression of ICOS in mice resulting from a loss-of-function mutation in Roquin (*sanroque*) causes severe autoimmunity via unchecked germinal center development and autoantibody production (Vinuesa et al., 2005). Sanroque mice have dramatic increases in Tfh cell numbers (Linterman et al., 2009; Vinuesa et al., 2005). Although ICOS is clearly important for germinal centers and Tfh cell development, it has been unknown why ICOS is so central to this process. We reasoned that ICOS may be required for initiation of Tfh cell differentiation by induction of Bcl6 in CD4$^+$ T cells during DC priming. Consistent with that idea, a profound defect in early Tfh cell differentiation was demonstrated in the absence of ICOS. Notably, Bcl6 expression was substantially reduced, indicating that ICOS is required for Bcl6 upregulation and subsequent Bcl6 control of the Tfh cell differentiation program. Not only is ICOS required for Bcl6 induction for Tfh cells during DC priming, ICOS signals are again required a second time for Tfh cell maintenance of Bcl6, this time from B cells during T cell- B cell cognate interactions. Relevant to our studies, CXCR5$^+$CD4$^+$ T cells were

reduced in mice with B cell-specific deletion of ICOSL (ICOSL$^{fl/fl}$ CD19-Cre) (Nurieva et al., 2008). Our experiments indicate that the mechanism by which ICOS initiates Tfh cell differentiation during DC priming is through Bcl6 expression. We found that Bcl6 controls the downstream expression of CXCR5 in CD4$^+$ T cells, thereby establishing a molecular hierarchy of Tfh cell differentiation from ICOS to Bcl6 to CXCR5. This is consistent with evidence that miR17-92 can reduce CXCR5 expression in B cells (Yu et al., 2009). ICOS signals can promote IL-21 production (Bauquet et al., 2009; Hiramatsu et al., 2010), and IL-21 can enhance Bcl6 mRNA expression (Nurieva et al., 2009). However, several studies showed that Tfh cells develop in *Il21*$^{-/-}$ or *Il21r*$^{-/-}$ mice (Crotty, 2011). Nevertheless, IL-6 and IL-21 doubly deficient mice do exhibit reduced Tfh cell frequencies and reduced Bcl6 expression by Tfh cells, confirming that these cytokines do contribute to Tfh cell differentiation (Eto et al., 2011). Intracellular signals from ICOS to induce Bcl6 expression are probably augmented by cytokines to control Tfh versus effector Th cell differentiation.

An important function of Bcl6 is repression of Blimp1. Tfh cells express Bcl6 and effector Th cells express Blimp1 (Fazilleau et al., 2009; Johnston et al., 2009). Blimp1 is capable of blocking Bcl6 expression and thereby extinguishing the Tfh cell differentiation program (Johnston et al., 2009). Our observation of the dramatic bimodal polarization of CD4$^+$ T cells to almost exclusive Blimp1$^+$ effector Th and Bcl6$^+$ Tfh cell types at the peak of the CD4$^+$ T cell response highlighted the stringency of the antagonism between these two transcription factors. Blimp1 is known to be expressed late in effector CD8$^+$ T cell differentiation and is considered a late marker of T cell differentiation. Surprisingly, polarized Bcl6 versus Blimp1 expression was found as early as 48 hr p.i., after only two cell divisions. We infer from these results that Bcl6 and Blimp1 are important early orchestrators of CD4$^+$ T cell differentiation, thereby driving antagonistic and self-reinforcing differentiation processes. Given our finding that ICOS signaling instructs Bcl6 expression and Tfh cell differentiation, the knowledge that an early bifurcation occurs between Tfh and effector Th cell developments during priming led us to ask what signaling molecule may instruct Blimp1 in association with canonical effector Th cell differentiation. One molecule known to affect Blimp1 expression is IL-2 (Malek and Castro, 2010). The high-affinity IL2Rα subunit is crucial for IL-2-mediated signaling and is expressed after TCR activation. Our study shows that Blimp1 is regulated by CD4$^+$ T cells according to differential IL2Rα expression during the priming phase of the immune response. At 48 hr p.i., the virus-specific CD4$^+$ T cell population possessed marked heterogeneity in IL2Rα expression, covering a 100- to 500-fold range of variation in expression. A large proportion of CD4$^+$ T cells highly expressed IL2Rα and had already upregulated Blimp1 expression. In contrast, cells possessing more moderate amounts of IL2Rα expression (IL2Rα^{int}) instead expressed Bcl6.

IL-2 can induce Blimp1 in both CD4$^+$ T cells and CD8$^+$ T cells (Malek and Castro, 2010). Interestingly, terminal effector CD8$^+$ T cells (short-lived effector cells, SLECs) express Blimp1 and

(J) Representative FACS plots are shown of SM CD4$^+$ T cells that have undergone two (left) and three (right) cell divisions. Gates identify IL2Rα^{hi}Bcl6$^-$ and IL2Rα^{int}Bcl6$^+$ SM CD4$^+$ T cells.

Data in all panels are representative of three or more independent experiments; n = 3–4 per group. **p < 0.01, ***p < 0.001. Error bars are SEM. See also Figure S6.

require Blimp1 for their full functionality (Kallies et al., 2009; Rutishauser et al., 2009). The question of how CD8+ T cell memory is generated has been a topic of intensive interest in cellular immunology. Recently it was determined that differential IL2Rα expression on virus-specific CD8+ T cells as early as 3.5 days after LCMV infection was associated with two separate cell fates. IL2Rα^{hi}CD8+ T cells express Blimp1 more than their IL2Rα^{int} counterparts and consequentially are biased to differentiate into terminal effector CD8+ T cells (SLEC) (Kalia et al., 2010; Pipkin et al., 2010). Notably, CD8+ T cells that are instead IL2Rα^{int} are destined to become memory precursor CD8+ T cells (MPEC), which express elevated Bcl6 (Rutishauser et al., 2009), in contrast to the Blimp1 expression by terminal effector CD8+ T cells. Therefore, CD8+ T cells undergo a fate choice decision process during priming, surprisingly analogous to the mechanism of CD4+ T cell Tfh cell differentiation we demonstrate here. These parallels indicate that mechanisms limiting early IL2Rα expression on T cells may enhance both Tfh cell differentiation and the development of T cell memory.

Our study illuminates the involvement of ICOS versus IL2Rα signals during priming for induction of Bcl6 expression and Tfh cell differentiation and highlights the importance of further examination of the intersections and interplay between these antagonizing pathways for determining T cell fates. How are these two upstream pathways regulated? ICOS-mediated signals for Tfh cells require the phosphoinositide 3-kinase (PI3K) pathway (Gigoux et al., 2009). Unlike CD28, ICOS fails to bind both Grb2 and Gads and therefore does not induce IL-2 production (Harada et al., 2003; Watanabe et al., 2006). It is plausible that higher TCR affinity results in prolonged T cell-DC interactions during the extended periods of CD4+ T cell-DC interaction in the first 24 hr of priming in vivo (Celli et al., 2007; Mempel et al., 2004). Higher TCR affinity is associated with a preference for Tfh cell differentiation over effector Th cell differentiation (Fazilleau et al., 2009). This may well be due to higher TCR affinity causing extended T cell-DC interactions, resulting in prolonged ICOS-ICOSL exposure at the time of DC priming, with CD4+ T cell integration of total ICOS signal strength over the duration of the APC interaction resulting in initiation of Tfh cell differentiation. We hypothesize that repeated but less prolonged early exposure of CD4+ T cells to antigen preferentially induces IL2Rα expression. Each of these processes can be an amplifying and self-reinforcing loop that would be capable of leading to rapidly polarized Bcl6-expressing Tfh cells and Blimp1-expressing effector Th cells. It is appealing to consider that a comparable ICOS signaling process could be occurring during T cell-B cell interactions. The higher amount of ICOS expression on Tfh cells combined with the duration of the T cell-B cell interaction determines the overall amount of ICOS signaling received by the Tfh cells. The duration of T cell-B cell interaction is dependent on the magnitude of SAP-dependent SLAM family receptor adhesion. Such an idea is consistent with the requirement for SAP to obtain GC Tfh cells and germinal centers. This is in keeping with the concept that Tfh cell differentiation is a multistage, multicomponent process (Crotty, 2011).

Collectively, our findings provide mechanistic insights for signaling pathways in regulation of two antagonizing transcription factors that balance Tfh versus effector Th cell differentiation. Given that most successful vaccines are dependent on CD4+ T cell provision of help to B cells to produce protective antibodies and B cell memory, further understanding of Tfh versus effector Th cells fate determination may be useful in rational vaccine design.

EXPERIMENTAL PROCEDURES

Mice and Viral Infections
Mice were either purchased from the Jackson Laboratory or obtained from in-house breeders (details in Supplemental Information). LCMV Armstrong and VACV$_{WR}$ (Western Reserve strain) stocks were prepared and quantified as previously described (McCausland et al., 2007; Sette et al., 2008). All animal experiments were conducted in accordance with approved animal protocols at LIAI.

Retrovirus Production and Cell Transfers
Prdm1 (Blimp1)-expressing retroviral vector (pMIG-GFP) was reported previously (Johnston et al., 2009). Bcl6 shRNA sequences were cloned into pLMP-GFP (Open Biosystem) and a custom pLMP-Ametrine, with additional modifications. Virions were produced from the Plat-E cell line as previously described (Johnston et al., 2009) with some modifications. VSV-g-expressing plasmid (pHDM-VSV-G) was cotransfected for each RV production for increased virion stability. Culture supernatants were obtained 2 days after transfections, filtered through 0.45 μm syringe filters, and ultracentrifuged at 24,000 rpm for 90 min. VSV-g-expressing chimeric retroviral stocks were saved at −80°C until use.

Naive or retrovirally transduced (details in Supplemental Information) SM CD4+ T cells were transferred into recipient mice by intravenous injections via the retro-orbital sinus. Cell transfer numbers for each time point were as follows: 1×10^6, $4–5 \times 10^5$, 2×10^5, and 5×10^3 SM CD4+ T cells for day 2, 3, 4, and 8 (or 7) experiments, respectively.

Anti-ICOSL Treatments
Anti-ICOSL (rat IgG2a, clone HK5.3) and isotype control (rat IgG2a) mAbs were purchased from BioXcell. C57BL/6 mice were given 100 μg of anti-ICOSL or isotype control mAb via both i.p. and retro-orbitally 3 days after VACV$_{WR}$ infection. Subsequent doses of 100 μg of mAbs were injected retro-orbitally at 5 and 7 days after infection.

Flow Cytometry
Single-cell suspensions were obtained by a gentle mechanical disruption of spleens. After ACK lysis, cell suspensions were surface stained in FACS buffer (PBS + 0.5% BSA) with monoclonal antibodies: SLAM (CD150, Biolegend); CD4, CD8, Fas, GL7 (BD Biosciences); CD44, CD45.1/2, IL2Rα (CD25), CD62L, CD69, PD-1 (J43), ICOS, B220 (eBioscience); and PNA (Vector Labs). CXCR5 stains and intracellular Bcl6 stains are described in Supplemental Information. All FACS samples were washed twice with FACS buffer, acquired with an LSRII (BD Biosciences), and then analyzed with FlowJo (Tree Star).

Statistics
Statistical analyses were done with Prism 5.0 (GraphPad). p values were calculated with two-tailed unpaired Student's t tests with a 95% confidence interval. Error bars on bar graphs depict the standard error of mean (SEM).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at doi:10.1016/j.immuni.2011.03.023.

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