

# STAT5 is a potent negative regulator of T<sub>FH</sub> cell differentiation

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**Follicular helper T cells (T<sub>FH</sub> cells) constitute the CD4<sup>+</sup> T cell subset that is specialized to provide help to germinal center (GC) B cells and, consequently, mediate the development of long-lived humoral immunity. T<sub>FH</sub> cell differentiation is driven by the transcription factor Bcl6, and recent studies have identified cytokine and cell–cell signals that drive Bcl6 expression. However, although T<sub>FH</sub> dysregulation is associated with several major auto-immune diseases, the mechanisms underlying the negative regulation of T<sub>FH</sub> cell differentiation are poorly understood. In this study, we show that STAT5 inhibits T<sub>FH</sub> cell differentiation and function. Constitutive STAT5 signaling in activated CD4<sup>+</sup> T cells selectively blocked T<sub>FH</sub> cell differentiation and GCs, and IL-2 signaling was a primary inducer of this pathway. Conversely, STAT5-deficient CD4<sup>+</sup> T cells (mature STAT5<sup>fl/fl</sup> CD4<sup>+</sup> T cells transduced with a Cre-expressing vector) rapidly up-regulated Bcl6 expression and preferentially differentiated into T<sub>FH</sub> cells during T cell priming in vivo. STAT5 signaling failed to inhibit T<sub>FH</sub> cell differentiation in the absence of the transcription factor Blimp-1, a direct repressor of Bcl6 expression and T<sub>FH</sub> cell differentiation. These results demonstrate that IL-2, STAT5, and Blimp-1 collaborate to negatively regulate T<sub>FH</sub> cell differentiation.**

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Abbreviations used: APC, allophycocyanin; GC, germinal center; LCMV, lymphocytic choriomeningitis virus; MFI, mean fluorescence intensity; RV, retroviral expression vector.

The germinal center (GC) reaction is an essential step in the development of humoral immunity, in which B cells undergo affinity maturation and differentiation into memory cells and long-lived plasma cells (Gatto and Brink, 2010). Follicular helper T cells (T<sub>FH</sub> cells) are CD4<sup>+</sup> T cells that migrate into B cell follicles and provide specialized help to GC B cells (Crotty, 2011). Impaired T<sub>FH</sub> cell differentiation results in a loss of GCs and T-dependent antibody responses (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009). Conversely, excessive T<sub>FH</sub> cell differentiation can drive the production of autoantibodies and is associated with several autoimmune diseases (Hu et al., 2009; Linterman et al., 2009).

Recent studies have investigated the signals that regulate T<sub>FH</sub> cell differentiation. T<sub>FH</sub> cells possess a distinctive gene program (Crotty, 2011), and the transcription factor Bcl6 is necessary for T<sub>FH</sub> cell differentiation in vivo (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009). Multiple rounds of interaction with antigen-presenting cells are necessary for

T<sub>FH</sub> cell differentiation (Johnston et al., 2009; Deenick et al., 2010; Choi et al., 2011; Goenka et al., 2011), and multiple signals are involved. In particular, ICOS–ICOSL interaction (Akiba et al., 2005; Gigoux et al., 2009; Hu et al., 2009; Choi et al., 2011) and contributions from IL-6 (Nurieva et al., 2008, 2009; Eto et al., 2011; Harker et al., 2011) are important for Bcl6 expression and T<sub>FH</sub> cell differentiation in most murine in vivo conditions. However, the signals that negatively regulate T<sub>FH</sub> cell differentiation are not well understood. One repressor of Bcl6 is the transcription factor Blimp-1, which is expressed by non-T<sub>FH</sub> effector CD4<sup>+</sup> T cells such as T<sub>H</sub>1, T<sub>H</sub>2, and induced regulatory T cells (T<sub>reg</sub> cells; Fazilleau et al., 2009; Johnston et al., 2009; Ma et al., 2009; Yusuf et al., 2010; Choi et al., 2011; Cretney et al., 2011). Bcl6 and Blimp-1 are mutually antagonistic; together, they constitute a regulatory axis that determines commitment

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to  $T_{FH}$  or non- $T_{FH}$  effector  $CD4^+$  T cell differentiation (Johnston et al., 2009; Crotty et al., 2010). Consequently, negative regulators of  $T_{FH}$  cell differentiation may act by directly targeting Bcl6 or by inducing Blimp-1 or other factors.

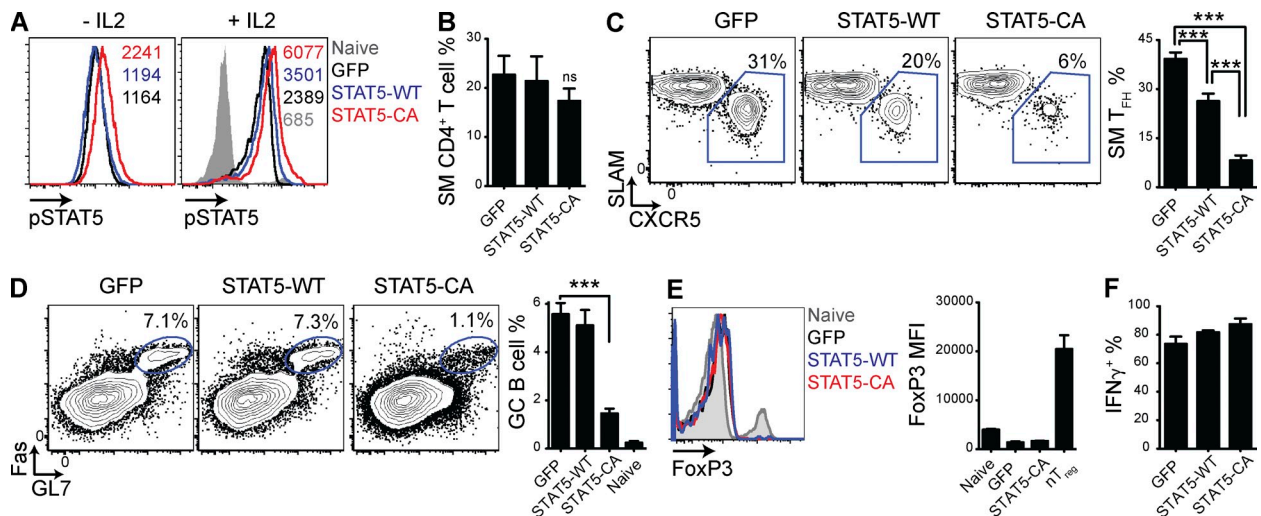
STAT-mediated cytokine signaling pathways are important regulators of effector lymphocyte differentiation (Zhu et al., 2010). In B cells, STAT5 and STAT3 regulate Bcl6 and Blimp-1, but in both cases, the type of regulation is controversial. STAT5 has been reported to induce Bcl6 expression (Scheeren et al., 2005) and, in other studies, to repress Bcl6 expression (Walker et al., 2007; Duy et al., 2011). Similarly, STAT3 signaling in B cells has been reported to drive Blimp-1 expression (Reljic et al., 2000; Diehl et al., 2008; Kwon et al., 2009) and to drive Bcl6 expression (Arguni et al., 2006). STAT5 has also recently been shown to play an important role in effector  $CD8^+$  T cell persistence (Tripathi et al., 2010). In  $CD4^+$  T cells, STAT3 signaling is required for  $T_{H17}$  cell differentiation (Hirahara et al., 2010) and may contribute to  $T_{FH}$  cell differentiation (Nurieva et al., 2008, 2009; Poholek et al., 2010; Eto et al., 2011). STAT5 signaling represses  $T_{H17}$  cell differentiation (Yang et al., 2011) but enhances the differentiation of multiple effector  $CD4^+$  T cell subsets, including  $T_{reg}$  cells (Wei et al., 2008),  $T_{H2}$  cells (Zhu et al., 2010), and  $T_{H1}$  cells (Liao et al., 2011).

In this study, we found that STAT5 signaling blocked  $T_{FH}$  cell differentiation and that this inhibition was induced by IL-2 and dependent on Blimp-1. These results identify a key negative regulatory pathway of  $T_{FH}$  cell differentiation.

## RESULTS AND DISCUSSION

### STAT5 signaling selectively inhibits $T_{FH}$ cell differentiation and function

It remains unclear how diverse signals combine to specify commitment to  $T_{FH}$  or effector ( $T_{H1}$ ,  $T_{H2}$ ,  $T_{H17}$ , etc.)  $CD4^+$  T cell differentiation. Given the importance of STATs in regulating effector  $CD4^+$  T cell gene programs (Zhu et al., 2010) and conflicting reports of STAT5 regulating Bcl6 or Blimp-1 in B cells (Scheeren et al., 2005; Walker et al., 2007; Duy et al., 2011), we examined the role of STAT5 in  $T_{FH}$  cell differentiation. Because the primary limiting factor for STAT activation and signaling is the availability of activating cytokines, we used retroviral expression vectors (RVs) expressing only GFP ("GFP"), GFP and WT STAT5b (STAT5-WT), or GFP and a constitutively active mutant of STAT5b (STAT5-CA; Onishi et al., 1998). SMARTA TCR transgenic  $CD4^+$  T cells (SM cells), specific for the lymphocytic choriomeningitis virus (LCMV) epitope GP<sub>66-77</sub> bound by MHC class II I-A<sup>b</sup>, were transduced with these RVs. STAT5-CA<sup>+</sup> cells exhibited increased phospho-STAT5 protein in the absence of IL-2 (93% increase relative to GFP<sup>+</sup> cells; Fig. 1 A; Onishi et al., 1998). Both STAT5-CA<sup>+</sup> and STAT5-WT<sup>+</sup> cells had augmented levels of phospho-STAT5 protein after stimulation with IL-2 (150% and 47% increase relative to GFP<sup>+</sup> cells, respectively; Fig. 1 A). Sorted transduced cells were adoptively transferred into WT C57BL/6J host mice.



**Figure 1. STAT5 signaling selectively inhibits  $T_{FH}$  cell differentiation and function.**  $CD45.1^+$  SMARTA TCR transgenic (SM)  $CD4^+$  T cells were transduced with RVs expressing GFP and WT STAT5b (STAT5-WT), GFP and a constitutively active form of STAT5b (STAT5-CA), or only GFP (GFP). (A) Representative histograms of phospho-STAT5 levels in transduced SM cells (GFP<sup>+</sup>), without stimulation (left) or after stimulation with IL-2 (right). Phospho-STAT5 MFIs are indicated. Data are representative of two independent experiments. (B–F) Transduced SM cells (those expressing GFP) were adoptively transferred into C57BL/6J mice that were subsequently infected with LCMV (see Materials and methods). Splenocytes were analyzed 8 d after infection. Data are a composite of four (B–D) or two (E and F) independent experiments and total  $n = 11–16$ /group (B–D) or 4/group (E and F). (B) Quantitation of SM cells as a percentage of all  $CD4^+$  T cells. (C) Representative FACS plots gated on SM cells ( $CD4^+ CD45.1^+$ ), with  $T_{FH}$  cells (SLAMF7<sup>low</sup> CXCR5<sup>high</sup>) boxed. Quantitation of SM  $T_{FH}$  cells as a percentage of all SM cells. GFP versus STAT5-WT: \*\*\*,  $P = 0.0002$ ; GFP versus STAT5-CA: \*\*\*,  $P < 0.0001$ ; STAT5-WT versus STAT5-CA: \*\*\*,  $P < 0.0001$ . (D) Representative FACS plots gated on B cells ( $B220^+$ ), with GC B cells (Fas<sup>+</sup> GL7<sup>+</sup>) circled. Quantitation of GC B cells as a percentage of all B cells. Uninfected C57BL/6J mice (naive) are also shown. \*\*\*,  $P < 0.0001$ . (E) Representative histograms of FoxP3 expression in SM cells and in total  $CD4^+$  T cells from an uninfected C57BL/6J mouse (naive). Quantitation of FoxP3 MFI, with natural  $T_{reg}$  cells (n<sub>Treg</sub>;  $CD4^+ CD25^+ FoxP3^+$ ) from a naive C57BL/6J mouse included as a control. (F) Quantitation of the percentage of SM cells that produced IFN- $\gamma$  after PMA/ionomycin stimulation in vitro. Error bars depict the standard error of the mean.

Shortly thereafter, host mice were infected with Armstrong strain LCMV.

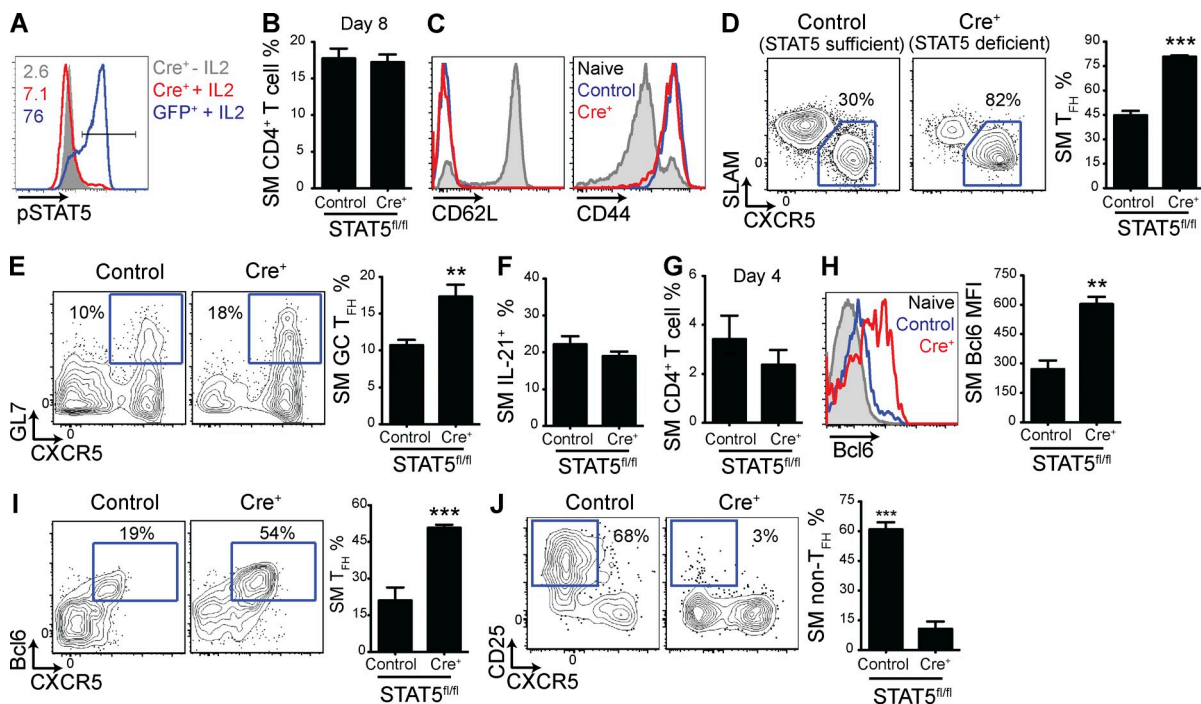
GFP<sup>+</sup>, STAT5-WT<sup>+</sup>, and STAT5-CA<sup>+</sup> SM cells all expanded normally in response to acute LCMV infection (Fig. 1 B). However, STAT5-CA<sup>+</sup> SM cells largely failed to differentiate into T<sub>FH</sub> cells (78% fewer T<sub>FH</sub> cells;  $P < 0.0001$ ; Fig. 1 C). Overexpression of WT STAT5 also reduced T<sub>FH</sub> cell differentiation (33% fewer T<sub>FH</sub> cells;  $P = 0.0002$ ; Fig. 1 C). Mice that received STAT5-CA<sup>+</sup> SM cells had fewer GC B cells (71% fewer GC B cells;  $P < 0.0001$ ; Fig. 1 D), consistent with a substantial loss of T<sub>FH</sub> cell help.

One possible mechanism by which STAT5 signaling could impair effector CD4<sup>+</sup> T cell differentiation was induction of T<sub>reg</sub> cell differentiation. In some settings, STAT5 signaling drives T<sub>reg</sub> cell differentiation via induction of FoxP3 (Wei et al., 2008). However, we found that SM cells transduced with the STAT5-WT RV or the STAT5-CA RV did not express FoxP3 and did not detectably suppress the endogenous immune response (Fig. 1 E and not depicted).

Furthermore, STAT5-WT<sup>+</sup> and STAT5-CA<sup>+</sup> SM cells produced high levels of the T<sub>FH</sub>1-associated cytokine IFN- $\gamma$  (Fig. 1 F), suggesting that non-T<sub>FH</sub> effector cell differentiation was not impaired by enhanced STAT5 signaling. Collectively, these data indicated that STAT5 signaling selectively inhibited T<sub>FH</sub> cell differentiation during an acute viral infection.

### Lack of STAT5 signaling enhances T<sub>FH</sub> cell differentiation

We hypothesized that STAT5 was a physiological inhibitor of T<sub>FH</sub> cell differentiation and consequently that a lack of STAT5 signaling during CD4<sup>+</sup> T cell priming would enhance T<sub>FH</sub> cell differentiation. However, insufficient STAT5 signaling in the thymus results in a loss immunological self tolerance (Malek et al., 2002; Burchill et al., 2007). To avoid this complication, we conditionally deleted STAT5 in mature CD4<sup>+</sup> T cells by transducing splenic STAT5<sup>fl/fl</sup> SM CD4<sup>+</sup> T cells with a Cre-expressing RV (Cre). Phospho-STAT5 protein was absent in STAT5-deficient (STAT5<sup>fl/fl</sup> Cre<sup>+</sup>) cells



**Figure 2. Lack of STAT5 signaling enhances T<sub>FH</sub> cell differentiation.** STAT5<sup>fl/fl</sup> SM cells were transduced with an RV expressing Cre recombinase (Cre) or were not transduced but treated similarly (control). (A) Representative histograms of phospho-STAT5 levels in SM cells, with and without IL-2 stimulation. The percentage of cells that was phospho-STAT5<sup>+</sup> is indicated. (B–J) Cre<sup>+</sup> and control SM cells were adoptively transferred into C57BL/6J mice that were subsequently infected with LCMV. Splenocytes were analyzed 8 (B–F) or 4 (G–J) d after infection. Data are a composite of two independent experiments, and  $n = 8$ /group. (B) Quantitation of SM cells as a percentage of all CD4<sup>+</sup> T cells. (C) Representative histograms gated on SM cells or on CD4<sup>+</sup> T cells from an uninfected C57BL/6J mouse (naive). (D) Representative FACS plots gated on SM cells, with T<sub>FH</sub> cells (SLAMF6<sup>low</sup> CXCR5<sup>high</sup>) boxed. Quantitation of SM T<sub>FH</sub> cells as a percentage of total SM cells. \*\*\*,  $P < 0.0001$ . (E) Representative FACS plots gated on SM cells, with GC T<sub>FH</sub> cells (CXCR5<sup>high</sup> GL7<sup>high</sup>) boxed. Quantitation of SM GC T<sub>FH</sub> cells as a percentage of total SM cells. \*\*,  $P = 0.01$ . (F) IL-21 production by SM cells after PMA/ionomycin stimulation in vitro. Quantitation of IL-21<sup>+</sup> SM cells as a percentage of total SM cells. (G) Quantitation of SM cells as a percentage of total CD4<sup>+</sup> T cells. (H) Representative histograms of Bcl6 expression, gated on SM cells or on CD4<sup>+</sup> T cells from an uninfected C57BL/6J mouse (naive). Quantitation of SM cell Bcl6 MFI. \*\*,  $P = 0.0012$ . (I) Representative FACS plots gated on SM cells, with T<sub>FH</sub> (Bcl6<sup>high</sup> CXCR5<sup>high</sup>) boxed. Quantitation of SM T<sub>FH</sub> cells as a percentage of all SM cells. \*\*\*,  $P < 0.0001$ . (J) Representative FACS plots gated on SM cells, with non-T<sub>FH</sub> cells (CD25<sup>high</sup> CXCR5<sup>low</sup>) boxed. Quantitation of non-T<sub>FH</sub> SM cells as a percentage of total SM cells. \*\*\*,  $P < 0.0001$ . Error bars depict the standard error of the mean.

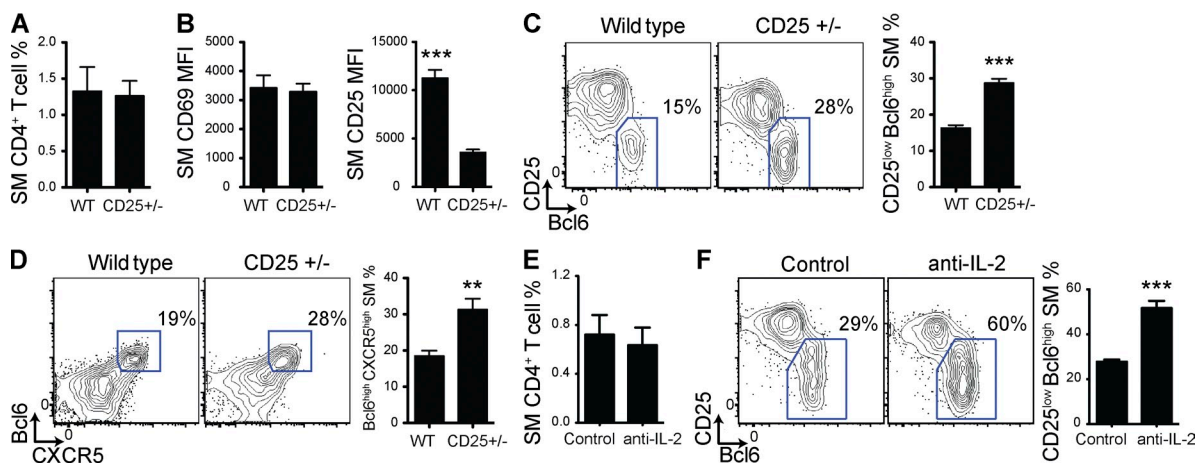
(Fig. 2 A), in agreement with PCR data (not depicted). STAT5-deficient SM cells or untransduced but similarly treated (control) SM cells were adoptively transferred into C57BL/6J host mice that were subsequently infected with LCMV. STAT5-deficient SM cells expanded as well as control SM cells and expressed surface markers consistent with normal activation (Fig. 2, B and C). Although the STAT5 signaling cytokines IL-2 and IL-7 are drivers of T cell survival and proliferation (Rochman et al., 2009), these results show that deletion of STAT5 from mature CD4<sup>+</sup> T cells is a viable approach to examine the role of STAT5 in effector CD4<sup>+</sup> T cell differentiation, consistent with recent CD4 T cell studies using STAT5<sup>fl/fl</sup> (Tripathi et al., 2010) or IL-2<sup>-/-</sup> CD4 T cells (Liao et al., 2011).

Deletion of STAT5 markedly enhanced SM T<sub>FH</sub> cell differentiation (80% more T<sub>FH</sub> cells;  $P < 0.0001$ ; Fig. 2 D). T<sub>FH</sub> cell differentiation is a multistep, multistage process (Crotty, 2011), and T<sub>FH</sub> cells that have progressed into GCs, GC T<sub>FH</sub> cells, can be identified by PD-1 or GL7 staining (Haynes et al., 2007; Yusuf et al., 2010; Kitano et al., 2011; Lee et al., 2011; Goenka et al., 2011). GC T<sub>FH</sub> cell abundance was also increased in the absence of STAT5 (55% more GC T<sub>FH</sub> cells;  $P = 0.01$ ; Fig. 2 E). STAT5-deficient T<sub>FH</sub> cells expressed normal levels of IL-21, a key T<sub>FH</sub>-produced cytokine that sustains the GC reaction (Fig. 2 F; Crotty, 2011). Production of IL-2 in both T<sub>FH</sub> and non-T<sub>FH</sub> effector cells was also maintained in the absence of STAT5 signaling (not depicted). These data showed that T<sub>FH</sub> proliferation, survival, and function were not dependent on STAT5 signaling and,

more importantly, that STAT5 was a physiological inhibitor of T<sub>FH</sub> cell differentiation.

Recently, we and others found that activated CD4<sup>+</sup> T cells rapidly bifurcate into T<sub>FH</sub> or non-T<sub>FH</sub> effector cell differentiation programs during priming (Choi et al., 2011; Kitano et al., 2011). Consequently, we examined the effect of STAT5 deficiency on commitment to T<sub>FH</sub> cell differentiation. 4 d after LCMV infection, STAT5-deficient (Cre<sup>+</sup>) SM cells had again expanded as well as control SM cells (Fig. 2 G), yet the absence of STAT5 signaling strongly enhanced Bcl6 expression and T<sub>FH</sub> cell differentiation (123% increase in Bcl6 mean fluorescence intensity [MFI;  $P = 0.0012$ ] and 128% more T<sub>FH</sub> cells [ $P < 0.0001$ ]; Fig. 2, H and I). These results indicate that STAT5 acts early in T cell priming during an acute viral infection to block Bcl6 expression and thereby prevent commitment to T<sub>FH</sub> cell differentiation.

A key function of STAT5 in T cells is to mediate signaling by IL-2. Intriguingly, activated CD4<sup>+</sup> T cells that have recently begun T<sub>FH</sub> cell differentiation express lower levels of the high affinity subunit of the IL-2 receptor, IL-2R $\alpha$  (CD25; Choi et al., 2011). We noted that STAT5 deficiency resulted in a reduction in IL-2R $\alpha$  expression (82% reduction in CD25<sup>high</sup> CXCR5<sup>low</sup> non-T<sub>FH</sub> SM cells;  $P < 0.001$ ; Fig. 2 J), consistent with previous studies (Nakajima et al., 1997; Malek et al., 2002). Overall, these results suggested that STAT5 inhibited commitment to T<sub>FH</sub> cell differentiation during T cell priming and that IL-2 may induce this pathway.



**Figure 3. IL-2 signaling inhibits T<sub>FH</sub> cell differentiation during T cell priming.** (A–D) WT and CD25<sup>+/-</sup> SM CD4<sup>+</sup> T cells were adoptively transferred into C57BL/6J mice that were infected with LCMV 1 d later. Splenocytes were analyzed 2 (A–C) or 3 (D) d after infection. Data are representative of two independent experiments, and  $n = 5$ /group. (A) Quantitation of SM cells as a percentage of total CD4<sup>+</sup> T cells. (B) Quantitation of SM cell CD69 and CD25 MFI. \*\*\*,  $P < 0.0001$ . (C) Representative FACS plots gated on SM cells, with T<sub>FH</sub>-committed cells (CD25<sup>low</sup> Bcl6<sup>high</sup>) boxed. Quantitation of T<sub>FH</sub>-committed SM cells as a percentage of total SM cells. \*\*\*,  $P < 0.0001$ . (D) Representative FACS plots gated on SM cells, with T<sub>FH</sub>-committed cells (Bcl6<sup>high</sup> CXCR5<sup>high</sup>) boxed. Quantitation of T<sub>FH</sub>-committed SM cells as a percentage of total SM cells. \*\*,  $P = 0.01$ . (E and F) WT SM CD4<sup>+</sup> T cells were adoptively transferred into C57BL/6J mice. Host mice were treated with anti-IL-2 neutralizing antibodies or isotype-matched control antibodies and infected with LCMV 1 d later. Splenocytes were analyzed 2 d after infection. Data are representative of two independent experiments, and  $n = 5$ /group. (E) Quantitation of SM cells as a percentage of total CD4<sup>+</sup> T cells. (F) Representative FACS plots gated on SM cells, with T<sub>FH</sub>-committed cells (CD25<sup>low</sup> Bcl6<sup>high</sup>) boxed. Quantitation of T<sub>FH</sub>-committed SM cells as a percentage of total SM cells. \*\*\*,  $P = 0.001$ . Error bars depict the standard error of the mean.

**IL-2 signaling inhibits Bcl6 expression during T cell priming**

To directly test the role of IL-2 on commitment to T<sub>FH</sub> cell differentiation, we first used SM cells that were heterozygous for deletion of IL-2R $\alpha$  (CD25<sup>+/-</sup>). When transferred into C57BL/6J mice that were subsequently infected with LCMV, activation and expansion of CD25<sup>+/-</sup> SM cell was equivalent to that of WT SM cells (Fig. 3, A and B). Strikingly, the twofold reduction in IL-2R $\alpha$  expression resulted in preferential T<sub>FH</sub> cell differentiation by CD25<sup>+/-</sup> cells as early as 48 h after LCMV infection (110% more CD25<sup>low</sup> Bcl6<sup>high</sup> cells; P < 0.001; Fig. 3 C). Similar results were obtained 72 h after LCMV infection (P < 0.01; Fig. 3 D), by which time SM cells had bifurcated into T<sub>FH</sub> and T<sub>H1</sub> effector cells.

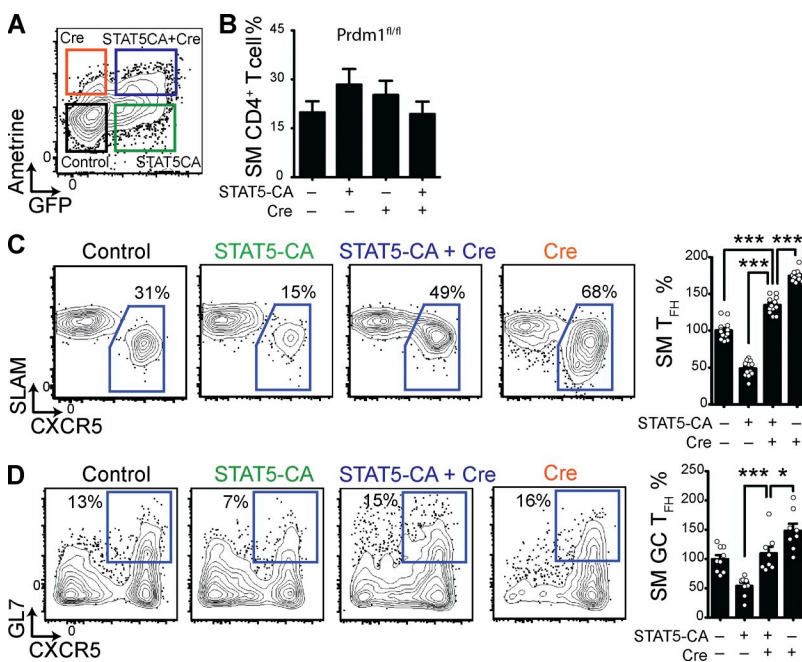
Next, we transferred WT SM cells into C57BL/6J mice treated with anti-IL-2 neutralizing antibodies and then infected with LCMV. IL-2 neutralization did not impair SM cell expansion (Fig. 3 E) but did significantly enhance commitment to T<sub>FH</sub> cell differentiation (86% increase; P = 0.0004; Fig. 3 F). Together, these data demonstrated that IL-2 is a key mediator of STAT5 signaling and inhibition of T<sub>FH</sub> cell differentiation during T cell priming and that reduced IL-2 signaling is sufficient to bias T cells away from T<sub>H1</sub> effector cell differentiation and toward T<sub>FH</sub> cell differentiation.

**STAT5-mediated inhibition of T<sub>FH</sub> cell differentiation is dependent on Blimp-1**

Because IL-2 and STAT5 regulate myriad genes in lymphocytes, it was important to identify the STAT5 targets responsible for inhibiting T<sub>FH</sub> cell differentiation. Because IL-2 can induce Blimp-1 expression in CD8<sup>+</sup> T cells (Martins and Calame, 2008; Kalia et al., 2010; Pipkin et al., 2010), we hypothesized that STAT5 signaling in CD4<sup>+</sup> T cells inhibited T<sub>FH</sub> cell differentiation by also inducing expression of Blimp-1.

We tested the ability of STAT5 signaling to inhibit T<sub>FH</sub> cell differentiation in the absence of Blimp-1 (encoded by the gene *Prdm1*) by cotransducing Prdm1<sup>fl/fl</sup> SM cells with both STAT5-CA RV (expressing GFP) and Cre RV (expressing the fluorescent protein Ametrine). STAT5-CA<sup>+</sup> Cre<sup>+</sup>, STAT5-CA<sup>+</sup> Cre<sup>-</sup>, STAT5-CA<sup>-</sup> Cre<sup>+</sup>, and STAT5-CA<sup>-</sup> Cre<sup>-</sup> (control) SM cells were purified and adoptively transferred into C57BL/6J mice that were subsequently infected with LCMV (Fig. 4 A). All populations of SM cells expanded equivalently (Fig. 4 B). Deletion of Blimp-1 alone (Cre<sup>+</sup>) enhanced T<sub>FH</sub> cell differentiation (Fig. 4 C), as previously demonstrated (Johnston et al., 2009). Consistent with the experiments shown in Fig. 2, expression of STAT5-CA in Cre<sup>-</sup> (Blimp-1 intact) Prdm1<sup>fl/fl</sup> SM cells inhibited T<sub>FH</sub> cell differentiation (Fig. 4 C). However, STAT5-CA and Cre cotransduced Prdm1<sup>fl/fl</sup> SM cells, which possessed constitutive STAT5 signaling but lacked Blimp-1, readily differentiated into T<sub>FH</sub> cells (176% more T<sub>FH</sub> cells than for Blimp-1-intact STAT5-CA<sup>+</sup> SM cells; P < 0.0001; Fig. 4 C). GC T<sub>FH</sub> cell differentiation was also restored in STAT5-CA<sup>+</sup> SM cells by the absence of Blimp-1 (104% increase; P < 0.0001; Fig. 4 D). These data indicated that STAT5 inhibition of T<sub>FH</sub> cell differentiation was mediated by Blimp-1, consistent with the recent finding that STAT5 can directly bind the *Prdm1* promoter in CD4<sup>+</sup> T cells after IL-2 stimulation (Yang et al., 2011). Additional STAT5 targets may also contribute (Liao et al., 2011), as the contribution of Blimp-1 was not complete (Fig. 4, C and D).

The signals that negatively regulate T<sub>FH</sub> cell differentiation have not been well characterized. In this study, we found that STAT5 is a key physiological inhibitor of Bcl6 expression and thereby an inhibitor of T<sub>FH</sub> cell differentiation. The absence of STAT5 resulted in increased Bcl6 expression and



**Figure 4. STAT5 inhibition of T<sub>FH</sub> cell differentiation is mediated by Blimp-1.**

Prdm1<sup>fl/fl</sup> SM cells were transduced with STAT5-CA RV and/or with a variant of Cre RV expressing the fluorescent protein mAmetrine or were not transduced but treated similarly (control). Data are a composite of three (A–C) or two (D) independent experiments, and n = 11–12/group (A–C) or 8/group (D). (A) Representative FACS plot gated on viable (7AAD<sup>low</sup>) cells, with untransduced, singly transduced, and cotransduced cells boxed. (B–D) Transduced or control SM cells were adoptively transferred into C57BL/6J mice that were infected with LCMV 3–5 d later. Splenocytes were analyzed 8 d after infection. (B) Quantitation of SM cells as a percentage of total CD4<sup>+</sup> T cells. (C) Representative FACS plots gated on SM cells, with T<sub>FH</sub> cells (SLAMF6<sup>low</sup> CXCR5<sup>high</sup>) boxed. Quantitation of SM T<sub>FH</sub> cells as a percentage of total SM cells. Data have been normalized so that the mean control SM T<sub>FH</sub> percentage for each experiment is 100%. \*\*\*, P < 0.0001. (D) Representative FACS plots gated on SM cells, with GC T<sub>FH</sub> cells (GL7<sup>high</sup> CXCR5<sup>high</sup>) boxed. Quantitation of SM GC T<sub>FH</sub> cells as a percentage of total SM cells. Data have been normalized so that the mean control SM GC T<sub>FH</sub> percentage for each experiment is 100%. \*, P = 0.0330; \*\*\*, P < 0.0001. Error bars depict the standard error of the mean.

preferential  $T_{FH}$  cell differentiation. This STAT5 function appears to be primarily induced by IL-2, as reduced IL-2 signaling substantially increased  $T_{FH}$  cell differentiation. Because IL-2 and other STAT5 signaling cytokines are important mediators of T cell proliferation and survival, it was somewhat surprising that IL-2-deprived or STAT5-deficient  $T_{FH}$  cells expanded normally. In agreement with our observations, the size of the antiviral CD4<sup>+</sup> T cell response was unaffected by STAT5 deficiency in another study (Tripathi et al., 2010).

Our data suggest that STAT5 is not necessary for the CD4<sup>+</sup> T cell effector response per se, but is required to properly balance  $T_{FH}$  and  $T_{H1}$  effector CD4<sup>+</sup> T cell differentiation. Importantly, our finding that a twofold reduction in IL-2R $\alpha$  expression (CD25<sup>+/-</sup>) shifts CD4<sup>+</sup> T cells toward  $T_{FH}$  cell differentiation demonstrates that small changes in IL-2 availability can have a significant impact on T cell fate decisions in vivo. Bcl6 is also involved in the development of T cell memory (Ichii et al., 2004; Crotty et al., 2010; Pipkin et al., 2010; Pepper et al., 2011). Although in this study we focus on how STAT5 negatively regulates  $T_{FH}$  cell differentiation and the development of GCs (Fig. 1 C), it is also intriguing to consider how these processes may impact CD4 T cell memory.

Given the association of dysregulated  $T_{FH}$  activity with autoantibody production, manipulation of STAT5 activity to attenuate  $T_{FH}$  cell differentiation or function may be a useful tool in the treatment of autoimmune disease. Conversely, manipulation of this pathway may also be a valuable tool to augment  $T_{FH}$  activity and thus the potency of candidate vaccines for a variety of infectious diseases.

## MATERIALS AND METHODS

**Mice.** C57BL/6J mice, as well as Prdm1<sup>fl/fl</sup> mice (Shapiro-Shelef et al., 2003) and CD25-deficient mice (Il2ra<sup>-/-</sup>, B6.129S4-Il2ra<sup>tm1Dw</sup>/J; Willerford et al., 1995) fully backcrossed to C57BL/6J, were purchased from the Jackson Laboratory. CD4-cre mice were purchased from Taconic. SM TCR transgenic CD45.1<sup>+</sup> mice with a C57BL/6J background were bred at the La Jolla Institute for Allergy and Immunology (LIAI; McCausland et al., 2007). STAT5<sup>fl/fl</sup> mice were generated by L. Hennighausen and colleagues (National Institutes of Health, Bethesda, MD; Cui et al., 2004) and backcrossed to the C57BL/6J background by M. Farrar and colleagues at the University of Minnesota (Minneapolis, MN), resulting in >10 generations of backcrossing to B6. STAT5<sup>fl/fl</sup> mice were further backcrossed to SM mice on the C57BL/6J background. 862 of 884 (97.5%) descriptive single nucleotide polymorphisms in backcrossed STAT5<sup>fl/fl</sup> mice were consistent with the C57BL/6J background, as determined by whole genome microsatellite analysis performed through the University of California, Los Angeles Southern California Genotyping Consortium on mice from LIAI. STAT5<sup>fl/fl</sup> mice, Prdm1<sup>fl/fl</sup> mice, and CD25<sup>-/-</sup> mice were crossed with SM mice at LIAI.

STAT5 deletion efficiency was determined by quantitative PCR (forward primer, 5'-ATGGACTCACACCCACAAGGA-3'; and reverse primer, 5'-CACTGCTACAAGGCTACACAAAACC-3'). For CD25 SM experiments, CD25<sup>+/+</sup> littermate control SM mice were used with CD25<sup>+/-</sup> SM mice. All animal experiments were conducted in accordance with animal protocols approved by the LIAI Institutional Animal Care and Use Committee.

**RVs, transductions, and cell transfers.** Gene expression experiments were performed with GFP-expressing RV pMIG as well as a modification of pMIG expressing the fluorescent protein mAmetrine1.1 (Ai et al., 2008)

instead of GFP (provided by D. Vignali, St. Jude Children's Research Hospital, Memphis, TN). STAT5-expressing retroviruses were designed using previously described sequences for WT and constitutively active STAT5b (H299R + S711F; Onishi et al., 1998). Cre recombinase-expressing retrovirus has been previously described (Johnston et al., 2009). Transduction of STAT5<sup>fl/fl</sup> SM CD4<sup>+</sup> T cells with Cre RV resulted in a deletion efficiency of ~93%, as measured by qPCR analysis.

Virions were produced using the Plat-E cell line as previously described (McCausland et al., 2007). For retroviral transduction of CD4<sup>+</sup> T cells, CD4<sup>+</sup> T cells were purified from the splenocytes of naive mice by magnetic bead negative selection (Miltenyi Biotec) and suspended in D-10 (DMEM + 10% fetal calf serum, supplemented with 2 mM GlutaMAX [Invitrogen] and 100 U/ml penicillin/streptomycin [Invitrogen]) with 10 ng/ml recombinant human IL-2 and 50  $\mu$ M  $\beta$ -mercaptoethanol.  $2 \times 10^6$  cells per well were stimulated in 24-well tissue culture plates precoated with 8  $\mu$ g/ml anti-CD3 (clone 17A2; Bio X Cell) and anti-CD28 (clone 37.51; Bio X Cell). After 24 h, cells were transduced as described previously (McCausland et al., 2007). Where necessary, cells were cotransduced by simultaneous transduction with two separately prepared retrovirus stocks. After a total of 72 h of stimulation, CD4<sup>+</sup> T cells were split and transferred into new wells with fresh D-10, IL-2, and  $\beta$ -mercaptoethanol. After an additional 72 h, transduced CD4<sup>+</sup> T cells were highly purified by sorting for GFP and/or mAmetrine expression on a FACSDiva or FACSAria (BD). Transduction efficiencies ranged from 10–40% before sorting.  $2.0 \times 10^5$  or  $2.5 \times 10^4$  transduced SM cells were adoptively transferred into each C57BL/6J host mouse via the retroorbital sinus for day 4 and day 8 experiments, respectively. In some experiments, cells that were not transduced but treated similarly were used as control cells in parallel adoptive transfer experiments. For day 2 and day 3 experiments,  $10^6$  and  $5 \times 10^5$  freshly isolated SM CD4<sup>+</sup> T cells, respectively, were adoptively transferred into each C57BL/6J host mouse via the retroorbital sinus.

**Infections.** LCMV stocks were prepared and quantified as previously described (McCausland et al., 2007). Infection doses were  $10^6$ ,  $5 \times 10^5$ ,  $2 \times 10^5$ , and  $5 \times 10^4$  plaque-forming units of LCMV Armstrong per mouse for day 2, day 3, day 4, and day 8 experiments, respectively. Infections were performed by intraperitoneal injection.

**IL-2 neutralization in vivo.** Mice received either control rat IgG2a or rat anti-IL-2 (clone S4B6). Each mouse was treated with 1.0 mg antibody via intraperitoneal and retroorbital injection 24 h before LCMV infection and then again 24 h after LCMV infection.

**Flow cytometry.** Single-cell suspensions of spleen were prepared by standard gentle mechanical disruption. Surface staining for flow cytometry was performed with monoclonal antibodies against SLAM (CD150; BioLegend) and CD4, CD8, CD45.1, CD44, CD62L, CD25, PD-1, CD69, B220, Fas, and GL7 (eBioscience). Surface stains were performed for 30–60 min at 4°C in PBS supplemented with 0.5% bovine serum albumin and 0.1% sodium azide, unless specified otherwise.

CXCR5 staining was performed as described previously (Johnston et al., 2009) for day 8 experiments using purified anti-CXCR5 (BD) for 60 min, followed by biotinylated anti-rat IgG (Jackson ImmunoResearch Laboratories, Inc.), and then by allophycocyanin (APC)- or PE-labeled streptavidin (Invitrogen) at 4°C in PBS supplemented with 0.5% bovine serum albumin, 2% fetal calf serum, and 2% normal mouse serum. For day 2–4 experiments, CXCR5 staining was performed using biotinylated anti-CXCR5 (BD) for 30 min, followed by APC- or PE-labeled streptavidin at 4°C (Choi et al., 2011).

Intracellular cytokine staining was performed as described previously (McCausland et al., 2007) after stimulation with 20 ng/ml PMA (Sigma-Aldrich) and 1  $\mu$ M ionomycin (Sigma-Aldrich) in the presence of 2  $\mu$ g/ml brefeldin-A (BD) for 4 h. Directly conjugated antibodies against IFN- $\gamma$  and IL-2 (BD) were used. For IL-21, staining was performed using an IL-21R-FC chimeric protein (R&D Systems) followed by PE- or APC-labeled anti-human IgG (Jackson ImmunoResearch Laboratories, Inc.; Johnston et al., 2009). Intracellular staining for Bcl6 was performed as previously described

(Choi et al., 2011) with an Alexa Fluor 647–conjugated monoclonal antibody to Bcl6 (clone K112–91; provided by E. O'Donnell and D. Ernst, BD) and the FoxP3 ICS kit buffers and protocol (eBioscience).

For phospho-STAT5 FACS, sorted GFP<sup>+</sup> cells were fixed and permeabilized with Phosflow Lyse/Fix buffer and Phosflow Perm Buffer III (BD). Where indicated, cells were first stimulated with 20 ng/ml of recombinant human IL-2 for 30 min. Cells were stained with anti-phospho-STAT5 antibody (pY694; BD). Samples were acquired using a C6 Flow Cytometer (Accuri) or an LSRII (BD) and analyzed using FlowJo (Tree Star).

**Statistical analysis.** Statistical tests were performed using Prism 5.0c (GraphPad Software). P-values were calculated by two-tailed unpaired Student's *t* tests with a 95% confidence interval. Error bars depict the standard error of the mean. \*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.001.

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