STAT5 is a potent negative regulator of $T_{FH}$ cell differentiation

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Follicular helper T cells ($T_{FH}$ cells) constitute the CD4+ 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. $T_{FH}$ 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 $T_{FH}$ dysregulation is associated with several major autoimmune diseases, the mechanisms underlying the negative regulation of $T_{FH}$ cell differentiation are poorly understood. In this study, we show that STAT5 inhibits $T_{FH}$ cell differentiation and function. Constitutive STAT5 signaling in activated CD4+ T cells selectively blocked $T_{FH}$ cell differentiation and GCs, and IL-2 signaling was a primary inducer of this pathway. Conversely, STAT5-deficient CD4+ T cells (mature STAT5fl/fl CD4+ T cells transduced with a Cre-expressing vector) rapidly up-regulated Bcl6 expression and preferentially differentiated into $T_{FH}$ cells during T cell priming in vivo. STAT5 signaling failed to inhibit $T_{FH}$ cell differentiation in the absence of the transcription factor Blimp-1, a direct repressor of Bcl6 expression and $T_{FH}$ cell differentiation. These results demonstrate that IL-2, STAT5, and Blimp-1 collaborate to negatively regulate $T_{FH}$ cell differentiation.

The germinatal 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 ($T_{FH}$ cells) are CD4+ T cells that migrate into B cell follicles and provide specialized help to GC B cells (Crotty, 2011). Impaired $T_{FH}$ 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 $T_{FH}$ 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 $T_{FH}$ cell differentiation. $T_{FH}$ cells possess a distinctive gene program (Crotty, 2011), and the transcription factor Bcl6 is necessary for $T_{FH}$ 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 $T_{FH}$ cell differentiation (Johnston et al., 2009; Deenick et al., 2010; Choi et al., 2011; Goenk 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 $T_{FH}$ cell differentiation. However, the signals that negatively regulate $T_{FH}$ cell differentiation are not well understood. One repressor of Bcl6 is the transcription factor Blimp-1, which is expressed by non-$T_{FH}$ effector CD4+ T cells such as $T_{H}1$, $T_{H}2$, and induced regulatory T cells ($T_{reg}$ 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
to T<sub>FH</sub> or non-T<sub>FH</sub> effector CD4<sup>+</sup> T cell differentiation (Johnston et al., 2009; Crotty et al., 2010). Consequently, negative regulators of T<sub>FH</sub> cell differentiation may act by directly targeting Bcl6 or by inducing Blimp-1 or other factors.

STAT5 signaling selectively inhibits T<sub>FH</sub> cell differentiation and function

It remains unclear how diverse signals combine to specify commitment to T<sub>FH</sub> or effector (T<sub>H1</sub>, T<sub>H2</sub>, T<sub>H17</sub>, etc.) CD4<sup>+</sup> T cell differentiation. Given the importance of STATs in regulating effector CD4<sup>+</sup> 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<sub>FH</sub> 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<sup>+</sup> 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.

Results and Discussion

STAT5 signaling selectively inhibits T<sub>FH</sub> cell differentiation and function

Figure 1.

STAT5 signaling selectively inhibits T<sub>FH</sub> cell differentiation and function. CD45.1<sup>+</sup> SMARTA.TCR transgenic (SM) CD4<sup>+</sup> 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<sup>+</sup> T cells. (C) Representative FACs plots gated on SM cells (CD4<sup>+</sup> CD45.1<sup>+</sup>), with T<sub>FH</sub> cells (SLAM<sup>low</sup> CXCR5<sup>high</sup>) boxed. Quantitation of SM T<sub>FH</sub> 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<sup>+</sup>), 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<sup>+</sup> T cells from a naive C57BL/6J mouse included as a control. (F) Quantitation of the percentage of SM cells that produced IFN-γ after PMA/ionomycin stimulation in vitro. Error bars depict the standard error of the mean.
Furthermore, STAT5-WT+ and STAT5-CA+ SM cells produced high levels of the TH1-associated cytokine IFN-γ (Fig. 1F), suggesting that non-TFH effector cell differentiation was not impaired by enhanced STAT5 signaling. Collectively, these data indicated that STAT5 signaling selectively inhibited TFH cell differentiation during an acute viral infection.

Lack of STAT5 signaling enhances Tfh cell differentiation

We hypothesized that STAT5 was a physiological inhibitor of Tfh cell differentiation and consequently that a lack of STAT5 signaling during CD4+ T cell priming would enhance Tfh 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+ T cells by transducing splenic STAT5 fl/fl SM CD4+ T cells with a Cre-expressing RV (Cre). Phospho-STAT5 protein was absent in STAT5-deficient (STAT5fl/fl Cre+) cells shortly thereafter, host mice were infected with Armstrong strain LCMV.

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

One possible mechanism by which STAT5 signaling could impair effector CD4+ T cell differentiation was induction of Treg cell differentiation. In some settings, STAT5 signaling drives Treg 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. 1E and not depicted).

Figure 2. Lack of STAT5 signaling enhances Tfh cell differentiation. STAT5fl/fl 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+ is indicated. (B–J) Cre+ 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+ T cells. (C) Representative FACS plots gated on SM cells, with TFH cells (SLAMlow CXCR5high) boxed. Quantitation of SM TFH cells as a percentage of total SM cells. ***, P < 0.0001. (D) Representative FACS plots gated on SM cells, with GC TFH cells (CXCR5high GL7high) boxed. Quantitation of SM GC TFH cells as a percentage of total SM cells. **, P = 0.01. (E) IL-21 production by SM cells after PMA/ionomycin stimulation in vitro. Quantitation of IL-21+ SM cells as a percentage of total SM cells. ***, P < 0.0001. (F) Representative FACS plots gated on SM cells, with TFH cells (SLAMhigh CXCR5high) boxed. Quantitation of SM TFH cells as a percentage of total SM cells. ***, P < 0.0001. (G) IL-21 production by SM cells after PMA/ionomycin stimulation in vitro. Quantitation of IL-21+ SM cells as a percentage of total SM cells. ***, P < 0.0001. (H) Representative FACS plots gated on SM cells, with GC TFH cells (CXCR5high GL7high) boxed. Quantitation of SM GC TFH cells as a percentage of total SM cells. ***, P < 0.0001. (I) Representative FACS plots gated on SM cells, with TFH cells (Bcl6high CXCR5high) boxed. Quantitation of non-TFH SM cells as a percentage of total SM cells. ***, P < 0.0001. Error bars depict the standard error of the mean.

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more importantly, that STAT5 was a physiological inhibitor of TFH cell differentiation.

Recently, we and others found that activated CD4+ T cells rapidly bifurcate into TFH or non-TFH 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 TFH cell differentiation. 4 d after LCMV infection, STAT5-deficient (Cre+) 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 TFH cell differentiation (123% increase in Bcl6 mean fluorescence intensity [MFI; P = 0.0012] and 128% more TFH 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 TFH cell differentiation.

A key function of STAT5 in T cells is to mediate signaling by IL-2. Intriguingly, activated CD4+ T cells that have recently begun TFH cell differentiation express lower levels of the high affinity subunit of the IL-2 receptor, IL-2Rα (CD25; Choi et al., 2011). We noted that STAT5 deficiency resulted in a reduction in IL-2Rα expression (82% reduction in CD25 high CXCR5 low non-TFH 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 TFH cell differentiation during T cell priming and that IL-2 may induce this pathway.

Deletion of STAT5 markedly enhanced SM TFH cell differentiation (80% more TFH cells; P < 0.0001; Fig. 2 D). TFH cell differentiation is a multistep, multistage process (Crotty, 2011), and TFH cells that have progressed into GCs, GC TFH 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 TFH cell abundance was also increased in the absence of STAT5 (55% more GC TFH cells; P = 0.01; Fig. 2 E). STAT5-deficient TFH cells expressed normal levels of IL-21, a key TFH-produced cytokine that sustains the GC reaction (Fig. 2 F; Crotty, 2011). Production of IL-2 in both TFH and non-TFH effector cells was also maintained in the absence of STAT5 signaling (not depicted). These data showed that TFH proliferation, survival, and function were not dependent on STAT5 signaling and, more importantly, that STAT5 was a physiological inhibitor of TFH cell differentiation.

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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-2Rα (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-2Rα 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>H</sub>1 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>H</sub>1 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 increased T<sub>FH</sub> cell differentiation.

**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 Ametrine 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>−</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 (SLAM<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 < 0.05; **P < 0.001; ***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<sub>FH</sub> cell differentiation. This STAT5 function appears to be primarily induced by IL-2, as reduced IL-2 signaling substantially increased T<sub>FH</sub> 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<sub>FH</sub> 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<sub>FH</sub> and T<sub>effector</sub> CD4<sup>+</sup> T cell differentiation. Importantly, our finding that a twofold reduction in IL-2Rα expression (CD25<sup>+/−</sup>) shifts CD4<sup>+</sup> T cells toward T<sub>FH</sub> 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<sub>FH</sub> cell differentiation and the development of GCs (Fig. 1 C), it is also intriguing to consider how these processes may impact CD4<sup>+</sup> T cell memory.

Given the association of dysregulated T<sub>FH</sub> activity with autoantibody production, manipulation of STAT5 activity to attenuate T<sub>FH</sub> 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<sub>FH</sub> 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-Shellev et al., 2003) and CD25-deficient mice (Il2ra<sup>−/−</sup>) were crossed to the C57BL/6J background by M. Farrar and colleagues at the National Institutes of Health, Bethesda, MD; Cui et al., 2004) and 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. 862 of 884 (97.5%) descriptive single nucleotide polymorphisms in backcrossed STAT5<sup>fl/fl</sup> mice were consistent with 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. 862 of 884 (97.5%) descriptive single nucleotide polymorphisms in backcrossed STAT5<sup>fl/fl</sup> mice were consistent with 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. 862 of 884 (97.5%) descriptive single nucleotide polymorphisms in backcrossed STAT5<sup>fl/fl</sup> mice were consistent with 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. 862 of 884 (97.5%) descriptive single nucleotide polymorphisms in backcrossed STAT5<sup>fl/fl</sup> mice were consistent with 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. 862 of 884 (97.5%) descriptive single nucleotide polymorphisms in backcrossed STAT5<sup>fl/fl</sup> mice were consistent with 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. 862 of 884 (97.5%) descriptive single nucleotide polymorphisms in backcrossed STAT5<sup>fl/fl</sup> mice were consistent with 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. 862 of 884 (97.5%) descriptive single nucleotide polymorphisms in backcrossed STAT5<sup>fl/fl</sup> mice were consistent with 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.
(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+ 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 (γY694; 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.


