

# IL-2 Receptor $\beta$ -Dependent STAT5 Activation Is Required for the Development of Foxp3<sup>+</sup> Regulatory T Cells<sup>1</sup>

Matthew A. Burchill,<sup>2\*</sup> Jianying Yang,<sup>2\*</sup> Christine Vogtenhuber,<sup>†</sup> Bruce R. Blazar,<sup>†</sup> and Michael A. Farrar<sup>3\*</sup>

*IL-2*<sup>-/-</sup> mice develop autoimmunity despite having relatively normal numbers of regulatory T cells (Tregs). In contrast, we demonstrate that *IL-2*<sup>-/-</sup> × *IL-15*<sup>-/-</sup> and *IL-2Rβ*<sup>-/-</sup> mice have a significant decrease in Treg numbers. Ectopic expression of *foxp3* in a subset of CD4<sup>+</sup> T cells rescued Treg development and prevented autoimmunity in *IL-2Rβ*<sup>-/-</sup> mice, suggesting that IL-2Rβ-dependent signals regulate *foxp3* expression in Tregs. Subsequent analysis of IL-2Rβ-dependent signal transduction pathways established that the transcription factor STAT5 is necessary and sufficient for Treg development. Specifically, T cell-specific deletion of STAT5 prevented Treg development; conversely, reconstitution of *IL-2Rβ*<sup>-/-</sup> mice with bone marrow cells expressing an IL-2Rβ mutant that exclusively activates STAT5 restored Treg development. Finally, STAT5 binds to the promoter of the *foxp3* gene suggesting that IL-2Rβ-dependent STAT5 activation promotes Treg differentiation by regulating expression of *foxp3*. *The Journal of Immunology*, 2007, 178: 280–290.

Regulatory T cells (Tregs)<sup>4</sup> have recently been the subject of intense research due to their critical role in preventing autoimmune disease. However, the molecular factors that regulate the development, homeostasis, and function of Tregs remain to be fully elucidated. Previous studies have demonstrated that the loss of IL-2, IL-2R $\alpha$ , or IL-2R $\beta$  leads to the unexpected development of lethal autoimmune disease (1–3). Subsequent studies by Malek et al. (4) established that the primary defect in IL-2R $\beta$ -deficient mice was the loss of functional Tregs, as they found that transfer of small numbers of wild-type (WT) Tregs into neonatal IL-2R $\beta$ -deficient mice completely prevented the onset of autoimmune disease. These findings suggested that IL-2/IL-2R signaling plays an important role in Treg biology.

Although IL-2 and IL-2R signaling are critical for the formation or maintenance of a functional Treg population, the exact role that IL-2R signaling plays in Treg biology has not yet been defined. Over the last few years, several studies have reported conflicting evidence about the role of IL-2 or IL-2R subunits in Treg development, homeostasis, or function. For example, Malek et al. (5) used a transgenic mouse line in which IL-2R $\beta$  expression was

restricted to the thymus to address the role of IL-2 in Treg development. When these thymus-restricted *IL-2Rβ* transgenic mice were crossed onto an IL-2R $\beta$ -deficient background, CD4<sup>+</sup>CD25<sup>+</sup> Tregs were restored in both the thymus and spleen, and autoimmunity was prevented. These results suggest that IL-2/IL-2R signaling is required only for the development of Tregs in the thymus and that IL-2 is not required to promote their homeostasis and function in the periphery. In contrast, other work suggested a role for IL-2 in maintaining CD4<sup>+</sup>CD25<sup>+</sup> Treg homeostasis. Specifically, administration of an IL-2-neutralizing Ab to WT mice significantly reduced the population of CD4<sup>+</sup>CD25<sup>+</sup> T cells in the periphery (6). These studies suggested that IL-2 signaling is critical for the survival of Tregs. Conversely, two other groups demonstrated that *IL-2*<sup>-/-</sup> mice have either identical (7) or only slightly reduced numbers (8) of Tregs when compared with WT mice, suggesting that IL-2 is not required for Treg development or homeostasis. Finally, several groups have claimed that IL-2 is primarily required for the activation and function of Tregs. For example, studies by Lafaille and colleagues (9) demonstrated that the transfer of CD4<sup>+</sup> T cells from *IL-2*<sup>-/-</sup> mice could prevent autoimmune disease in a mouse model that normally develops spontaneous experimental autoimmune encephalomyelitis. In contrast, transfer of *IL-2Rα*<sup>-/-</sup> CD4<sup>+</sup> T cells did not prevent experimental autoimmune encephalomyelitis in this model, suggesting a requirement for IL-2 to support suppressive capacity. Likewise, using in vitro suppression assays, two separate groups demonstrated that neutralization of IL-2 inhibits Treg suppressor function in vitro (10, 11). Taken together, the published literature reveals ongoing uncertainty regarding the role of IL-2 and the IL-2R in the development, homeostasis, and/or function of Tregs.

Although the past few years have witnessed advances in the functional and phenotypic characterization of Tregs, the reliance on CD25 as a marker to identify these cells is a potential confounding factor because it is also expressed on activated effector T cells. More recently, the transcription factor Foxp3 has been identified as a unique marker for Tregs in both humans and mice (12–17). Importantly, these studies further documented that Foxp3 expression was sufficient to convert CD4<sup>+</sup>CD25<sup>-</sup> T cells into CD4<sup>+</sup>CD25<sup>+</sup> T cells that exhibited potent suppressor function and thereby established Foxp3 as a master regulator of Treg lineage

\*Department of Laboratory Medicine and Pathology and Department of Pediatrics, Center for Immunology, Cancer Center and <sup>†</sup>Division of Hematology, Oncology, and Blood and Marrow Transplantation, University of Minnesota, Minneapolis, MN 55455

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<sup>2</sup> M.A.B. and J.Y. contributed equally to the results presented here.

<sup>3</sup> Address correspondence and reprint requests to Dr. Michael A. Farrar, Center for Immunology, University of Minnesota, 312 Church Street SE, 6-116 Nils Hasselmo Hall, Minneapolis, MN 55455. E-mail address: farra005@tc.umn.edu

<sup>4</sup> Abbreviations used in this paper: Treg, regulatory T cell; WT, wild type; LN, lymph node; ChIP, chromatin immunoprecipitation; LMC, littermate control mice;  $\gamma_c$ , common  $\gamma$ -chain; DN, double negative.

commitment and function. Therefore, to identify how IL-2 signaling affects Treg biology, we used intracellular staining for Foxp3 to more accurately identify Treg populations in mice in which IL-2 signaling has been disrupted. As recently reported (7, 8), we also observed that CD4<sup>+</sup>Foxp3<sup>+</sup> T cells are detectable in *IL-2*<sup>-/-</sup> mice. In contrast, we found that *IL-2Rβ*<sup>-/-</sup> and  $\gamma_c$ <sup>-/-</sup> mice have drastically reduced populations of CD4<sup>+</sup>Foxp3<sup>+</sup> cells in the thymus and peripheral lymphoid organs. Consistent with these findings, *IL-2*<sup>-/-</sup> × *IL-15*<sup>-/-</sup> mice show a comparable Treg defect as that observed in *IL-2Rβ*<sup>-/-</sup> mice. Thus, we demonstrate that IL-2 and IL-15 are redundant cytokines that can both signal through an IL-2Rβ/common  $\gamma_c$  ( $\gamma_c$ ) complex to promote the development of CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs.

Having established that IL-2Rβ-dependent pathways are required for Treg development, we proceeded to identify key downstream effectors of this process. We found that the introduction of Foxp3 via transgenesis into a subset of *IL-2Rβ*<sup>-/-</sup> CD4<sup>+</sup> T cells restored Treg development and functional Treg suppressor activity, thereby inhibiting activation of the remaining CD4<sup>+</sup>Foxp3<sup>-</sup> T cells and preventing autoimmunity in vivo. These results demonstrate that Foxp3 expression alone, and not additional IL-2Rβ-dependent signals, is sufficient for production of Tregs that have inhibitory activity. Furthermore, we provide evidence that IL-2Rβ-dependent STAT5 activation is the key signaling event involved in this process. First, we demonstrate that T cell-specific expression of STAT5 is required for Treg development. Second, we demonstrate that STAT5 activation, achieved via expression of a constitutively active STAT5 transgene, is sufficient to restore CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs in the thymus of *IL-2Rβ*<sup>-/-</sup> mice. Third, reconstitution of *IL-2Rβ*<sup>-/-</sup> mice with bone marrow cells expressing IL-2Rβ mutants that can only activate STAT5, and not other IL-2Rβ-dependent signaling pathways, restored a population of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs in both the thymus and spleen and was sufficient to prevent T cell activation. Finally, we demonstrate that STAT5 binds specifically to the *foxp3* promoter in CD4<sup>+</sup>CD25<sup>+</sup> Tregs. Taken together, our results provide strong evidence that IL-2Rβ-dependent STAT5 activation is required for Treg development and that it does so via direct effects on *foxp3* expression.

## Materials and Methods

### Mice

*IL-2*<sup>-/-</sup>, *IL-2Rα*<sup>-/-</sup>, *IL-2Rβ*<sup>-/-</sup>,  $\gamma_c$ <sup>-/-</sup>, and *IL-7Rα*<sup>-/-</sup> mice were obtained from The Jackson Laboratory and maintained in our breeding colony. In some experiments, *IL-2*<sup>-/-</sup> × *rag-2*<sup>+/-</sup> and *IL-2Rβ*<sup>-/-</sup> × *rag-2*<sup>+/-</sup> mice were used; *rag-2* heterozygosity had no effect on results since identical findings were obtained with *IL-2*<sup>-/-</sup> and *IL-2Rβ*<sup>-/-</sup> mice. *IL-15*<sup>-/-</sup> and *rag-2*<sup>-/-</sup> mice were obtained from Taconic Farms. *IL-2*<sup>-/-</sup> × *IL-15*<sup>-/-</sup> mice were generated by crossing *IL-2*<sup>-/-</sup> × *rag-2*<sup>-/-</sup> with *IL-15*<sup>-/-</sup> mice; *STAT5b-CA* × *IL-2Rβ*<sup>-/-</sup> mice were generated by crossing *STAT5b-CA* × *IL-2Rβ*<sup>+/-</sup> mice with *IL-2Rβ*<sup>-/-</sup> × *rag-2*<sup>-/-</sup> mice. *STAT5a/b*<sup>FL/FL</sup> and *CD4-Cre* mice were provided by Dr. L. Hennighausen (National Institutes of Health, Bethesda, MD) and Dr. K. Hogquist (University of Minnesota, Minneapolis, MN), respectively. *B6.C.H2bm12/KhEg* (termed *bm12*) mice were purchased from The Jackson Laboratory. C57BL/6 mice were purchased from the National Institutes of Health or Taconic Farms. The *foxp3* transgenic mice were generated as described (18) and provided by Dr. F. Ramsdell (Zymogenetics, Seattle, WA). All mouse strains used were backcrossed at least 10 generations onto the C57BL/6 genetic background except for *STAT5a/b*<sup>FL/FL</sup> mice. Mice used for analysis were 4–6 wk old, unless otherwise noted. The University of Minnesota Institutional Animal Care and Use Committee approved all animal experiments.

### Intracellular flow cytometry

Spleen, thymus, and lymph nodes were harvested and single-cell suspensions were generated by disruption with ground glass slides. Before enumeration, RBC were removed from spleen preparations by ammonium

chloride lysis. Abs against the following cell surface markers were obtained from eBioscience: CD8 (53-6.7), CD25 (PC61.5), and glucocorticoid-induced TNFR family-related gene (DTA-1). In some experiments, potential contaminating cells were removed by gating out cells expressing B220, CD19, GR-1, CD11b, TER-119, NK1.1, and TCRγδ. Additional Abs directed at the cell surface molecule CD4 (RM4-5) were obtained from BD Pharmingen. Following surface staining, samples were fixed and permeabilized overnight using the PE anti-mouse/rat Foxp3 staining set obtained from eBioscience. Following fixation, cells were stained with either PE-labeled rat/anti-mouse Foxp3 (FJK-16s) or PE-labeled rat IgG2a,κ (P3) isotype control. Labeled cells were then collected and analyzed using a LSRII flow cytometer (BD Biosciences). Analysis of flow cytometry data was conducted using FlowJo software (Tree Star). Staining for intracellular phospho-STAT5 and phospho-Erk was done as previously described (19).

### Proliferation assays

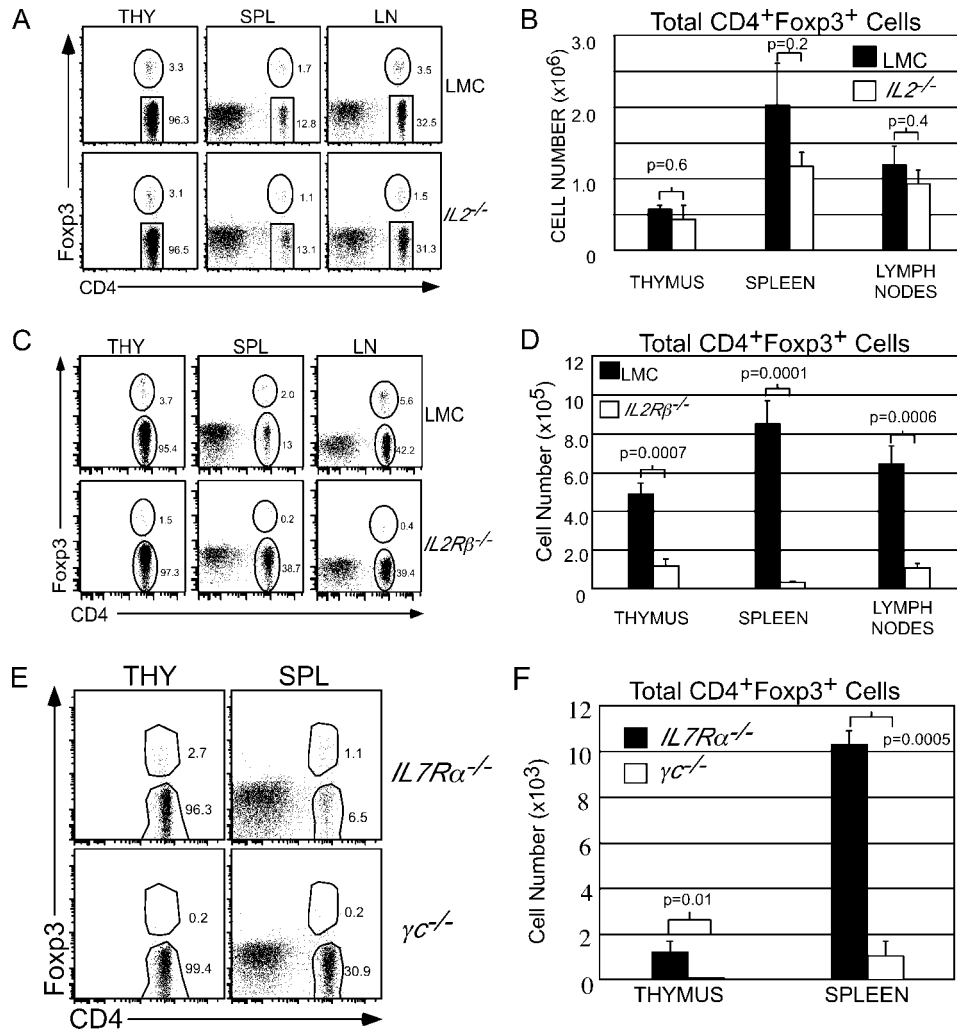
For proliferation assays involving *IL-2Rβ*<sup>-/-</sup> × *foxp3tg* mice, CD4<sup>+</sup>CD25<sup>-</sup> T cells were isolated from lymph nodes (LN) using a wire mesh to obtain a single-cell suspension. Cells were resuspended in medium consisting of DMEM (BioWhittaker), 10% FBS (HyClone), 50 μM 2-ME (Sigma-Aldrich), 10 mM HEPES buffer, 1 mM sodium pyruvate (Invitrogen Life Technologies), amino acid supplements (1.5 mM L-glutamine, L-arginine, and L-asparagine; Sigma-Aldrich), and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin; Sigma-Aldrich). Importantly, the percentage of Foxp3<sup>+</sup> cells in the sorted CD4<sup>+</sup> *IL-2Rβ*<sup>-/-</sup> × *foxp3tg* cells, CD4<sup>+</sup>*foxp3tg* cells, and WT CD4<sup>+</sup>CD25<sup>+</sup> cells was roughly equivalent (63–75% Foxp3<sup>+</sup>). LN cells were depleted of NK cells (hybridoma PK136) and CD8<sup>+</sup> T cells (hybridoma 2.43) by incubation with mAb, followed by passage through a goat anti-mouse and goat anti-rat Ig-coated column (Cedarlane Laboratories). CD25<sup>+</sup> T cells were depleted using anti-CD25 PE (hybridoma PC61; BD Pharmingen) and MACS anti-PE MicroBeads followed by LS MACS separation columns (Miltenyi Biotec). T cell- and NK cell-depleted stimulator cells were prepared from *bm12* spleens, incubated with anti-Thy1.2 mAb (hybridoma 30H-12) and NK mAb (hybridoma PK136) followed by incubation with rabbit complement (Neffenegger). Cells were irradiated at 30 Gy. CD4 T cells from WT, *foxp3tg*, *IL-2Rβ*<sup>-/-</sup>, and *IL-2Rβ*<sup>-/-</sup> × *foxp3tg* mice were enriched from the spleen using anti-CD4 PE (hybridoma RM4-5) followed by MACS separation. Cells were further purified using a FACSAria sorter to 99% purity. One hundred thousand CD4<sup>+</sup>CD25<sup>-</sup> T cells were mixed with equal numbers of *bm12* stimulators and indicated numbers of sorted CD4<sup>+</sup> T cells from the various strains in 200 μl in 96-well plates. Cultures were incubated for 7 days in DMEM at 37°C and 10% CO<sub>2</sub> and pulsed with [<sup>3</sup>H]thymidine at 1 μCi/well (Amersham Biosciences) for the last 16–18 h of culture. Cells were harvested and counted in the absence of scintillation fluid using a beta plate reader (Packard Instrument) and analyzed in quadruplicates.

### Retroviral bone marrow chimeras

The murine WT *IL-2Rβ* cDNA was used as a template to generate both *IL-2Rβ-STAT5* and *IL-2Rβ-ΔAH* constructs. In brief, the *IL-2Rβ-ΔAH* construct encodes for a receptor that is truncated after serine-326. The *IL-2Rβ-STAT5* construct was made by overlap extension PCR and encodes a chimeric receptor consisting of the first 326 aa of the murine WT *IL-2Rβ* construct fused to a sequence encoding the distal aa 494–505. *WT-IL-2Rβ*, *IL-2Rβ-STAT5*, and *IL-2Rβ-ΔAH* cDNAs were cloned into a modified pMIGR retroviral vector and retroviral particles were produced using 293T cells and a replication-deficient helper virus. Four-week-old *IL-2Rβ*<sup>-/-</sup> donor mice were treated with 5-fluorouracil to enrich for long-term hemopoietic stem cells, and 4 days posttreatment bone marrow was harvested from donor mice. High titer viral supernatants were used to infect donor bone marrow with either *WT-IL-2Rβ*, *IL-2Rβ-STAT5*, or *IL-2Rβ-ΔAH* constructs as previously described (20). Following retroviral infection, donor bone marrow was introduced into sublethally irradiated *rag2*<sup>-/-</sup> recipient mice (650 rad). Eight weeks after reconstitution, thymii and spleens were harvested from recipient mice and analyzed by flow cytometry.

### Chromatin immunoprecipitation (ChiP) assays

Spleen and LN were isolated from C57BL/6 mice and single-cell suspensions were obtained by disrupting organs with ground glass slides. Cells were resuspended in 1 × PBS containing 2% FBS (Atlas Biologicals). Isolated cells were then enumerated and stained with biotin-labeled CD8 (53-6.7), CD19 (MB19-1), CD11b (M1/70), CD45R (RA3-6B2), DX5 (DX5), Ly6C (RB6-8C5), I-A<sup>b</sup> (M5/114.15.2), IgM (eB121-15F9), and Ter-119 obtained from eBioscience and γδ TCR (GL3) obtained from BD Pharmingen. Following Ab staining, the cells were incubated with streptavidin-labeled MicroBeads and fractionated with an LS column (Miltenyi Biotec).



**FIGURE 1.** IL-2R $\beta$  and  $\gamma_c$  chains are required for the development of CD4<sup>+</sup>Foxp3<sup>+</sup> cells. **A**, Shown are CD4<sup>+</sup>CD8<sup>-</sup>-gated thymocytes (THY), total splenocytes (SPL), or LN cells stained with Abs to CD4 and Foxp3. Numbers to the right of the outlined areas represent the percentage of cells in the designated gate. **B**, Shown are average total Foxp3<sup>+</sup> cell numbers from *C57BL/6* (■) and *IL-2<sup>-/-</sup>* (□) mice. **C**, Thymii, spleens, and LN were harvested from 4-wk-old *C57BL/6* or *IL-2R $\beta$ <sup>-/-</sup>* mice and stained with Abs to CD4 and Foxp3. Flow cytometry plots shown depict CD4<sup>+</sup>CD8<sup>-</sup>-gated thymocytes, lymphocyte-gated splenocytes, and LN cells. Numbers indicate the percentage of cells in the gate. **D**, Total CD4<sup>+</sup>Foxp3<sup>+</sup>-gated cells from both *C57BL/6* (■) and *IL-2R $\beta$ <sup>-/-</sup>* (□) mice. **E**, Thymii and spleens were harvested from 6- to 8-wk-old *IL-7R $\alpha$ <sup>-/-</sup>* and  $\gamma_c$ <sup>-/-</sup> mice and stained with Abs to CD4, CD25, and Foxp3. Numbers indicate the percentage of CD4<sup>+</sup>CD8<sup>-</sup> thymocytes or total splenocytes inside the depicted gate. **F**, Total numbers of CD4<sup>+</sup>Foxp3<sup>+</sup> cells from *IL-7R $\alpha$ <sup>-/-</sup>* (■) and  $\gamma_c$ <sup>-/-</sup> (□). Error bars, SEM. Data in each case are representative of three or more independent experiments using between two and five mice per group. Mice used were between 4 and 5 wk of age, except as otherwise noted. The *p* values for all figures were determined by a two-tailed Student's *t* test.

The enriched CD4 lymphocytes were then stained with allophycocyanin-labeled CD4 (L3T4) and PE-labeled CD25 (PC61), and sorted into CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup> populations to a purity of >95% using a FACSAria (BD Biosciences). Alternatively, the enriched CD4 lymphocytes were stained with PE-labeled CD25 and fractionated into CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup> populations using LS columns to a purity of >80%. Following fractionation, CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup> populations were fixed with a 1× PBS solution containing 27% formaldehyde, and the mixture was then sonicated to generate DNA fragments of <1 kb. The size of the DNA fragments after sonication was confirmed by running aliquots of sheared DNA on ethidium bromide-stained agarose gels; DNA fragments typically averaged between 250 and 500 bp with no detectable fragments >1 kb. ChIP assays were performed with anti-STAT5 (sc-835; Santa Cruz Biotechnology) or anti-Mek1 (sc-219; Santa Cruz Biotechnology) Abs as previously described (21) using a kit from Upstate Biotechnology. Real-time PCR was performed using the Brilliant QPCR core reagent kit (Stratagene). All PCR were conducted for one cycle at 95°C for 10 min, followed by 45 cycles at 95°C (15 s) and 60°C (90 s). Serial dilutions of genomic DNA were used to obtain a standard curve used to quantify levels of DNA in ChIP assays. Oligonucleotides and TaqMan probes were obtained from Integrated DNA Technologies and are listed as follows: 5' promoter

primer, 5'-CTCACTCAGAGACTCGCAGCA-3'; 3' promoter primer, 5'-GCAAGCATGCATATGATCACC-3'; TaqMan promoter probe, FAM-CCAGCCATTCTGAGACTCTCTGATCTGTG-BHQ1; 5' intron 1 primer, 5'-CAGAAAAATCTGGCCAAGTT-3'; 3'-intron 1 primer, 5'-AGGACCTGAATTGGATATGGT-3'; TaqMan intron 1 probe, FAM-ACCTCCATA CAGCTTCTAAGAAACAGTCAAACA-BHQ1; 5' intron 11, 5'-CTGTGAG TTGGAAGCCAG-3'; 3' intron 11 primer, 5'-CTGAGCTATCTGTCCAGTT CC-3'; and TaqMan intron 11 probe, FAM-AACTGTAAATCTGTAATC CCAGCATGTGTACABHQ1.

## Results

### IL-2R $\beta$ signaling is critical for Treg development

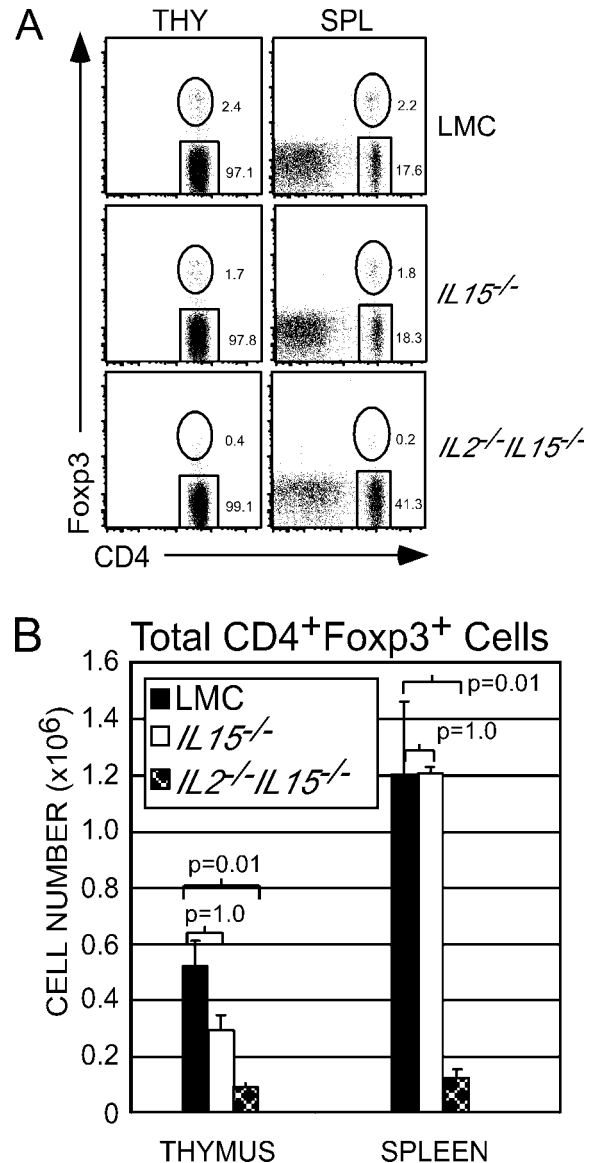
To investigate the role of IL-2 and IL-2R in Treg development, we compared littermate control mice (LMC) with *IL-2<sup>-/-</sup>*, *IL-2R $\beta$ <sup>-/-</sup>*, and  $\gamma_c$ <sup>-/-</sup> mice for the presence of CD4<sup>+</sup>Foxp3<sup>+</sup> cells. As recently reported (7, 8), we observed comparable populations of CD4<sup>+</sup>Foxp3<sup>+</sup> cells in LMC and *IL-2<sup>-/-</sup>* mice (Fig. 1A). Importantly, the absolute number of CD4<sup>+</sup>Foxp3<sup>+</sup> cells in *IL-2<sup>-/-</sup>*

mice was not significantly different from the number of CD4<sup>+</sup>Foxp3<sup>+</sup> cells observed in LMC mice (Fig. 1B). Similar results were found in *IL-2R $\alpha$* <sup>-/-</sup> mice (data not shown). In contrast, we found that *IL-2R $\beta$* <sup>-/-</sup> mice have a significant decrease in both the percentage and absolute cell numbers of CD4<sup>+</sup>Foxp3<sup>+</sup> cells in the thymus and in the periphery (Fig. 1, C and D). Specifically, *IL-2R $\beta$* <sup>-/-</sup> mice show a 4-fold decrease in numbers of thymic Tregs relative to LMC; the decrease is even more dramatic in the spleen and LN (27- and 6-fold decrease, respectively, relative to LMC mice). Analysis of  $\gamma_c$ -deficient mice revealed an even more striking defect with a virtual complete absence of Tregs in both the thymus and spleen (Fig. 1, E and F). A potential concern with this latter experiment is that  $\gamma_c$ <sup>-/-</sup> mice have dramatically reduced numbers of all lymphocyte subsets due to a requirement for  $\gamma_c$  in IL-7R-dependent early B and T cell development (22, 23). Hence, the abrogation of Treg development observed in  $\gamma_c$ <sup>-/-</sup> mice may be an indirect effect of the paucity of effector T cells in these mice. This is particularly relevant because Sakaguchi and colleagues (6) have provided evidence that IL-2 produced by activated effector T cells may be involved in the maintenance of Tregs. To address this issue, we analyzed Treg development in *IL-7R $\alpha$* <sup>-/-</sup> mice. Like  $\gamma_c$ <sup>-/-</sup> animals, *IL-7R $\alpha$* <sup>-/-</sup> mice have an identical block in thymocyte development (at the double-negative (DN)2 to DN3 transition) and exhibit a similar paucity of mature T cells (24). However, as shown in Fig. 1E, *IL-7R $\alpha$* <sup>-/-</sup> mice contain a substantial population of CD4<sup>+</sup>Foxp3<sup>+</sup> cells in both the thymus and the periphery that are significantly greater in total cell numbers than the few cells observed in  $\gamma_c$ <sup>-/-</sup> mice (albeit still reduced in numbers relative to WT mice, Fig. 1F). Importantly, the ratio of CD4<sup>+</sup>Foxp3<sup>+</sup>:CD4<sup>+</sup>Foxp3<sup>-</sup> T cells in *IL-7R $\alpha$* <sup>-/-</sup> mice was greater than or equal to that seen in WT mice, demonstrating that Treg development was not impaired. These results suggest that one or more cytokines that bind both IL-2R $\beta$  and  $\gamma_c$  are required for the development of Tregs.

Other than IL-2, the only cytokine known to signal through an IL-2R $\beta$ / $\gamma_c$  receptor complex is IL-15. *IL-15*<sup>-/-</sup> mice do not develop autoimmunity, and we have observed that they have virtually identical numbers of Tregs as WT mice. In contrast, *IL-2*<sup>-/-</sup> × *IL-15*<sup>-/-</sup> mice show a comparable decrease in Treg numbers as that observed in *IL-2R $\beta$* <sup>-/-</sup> mice (6-fold ↓ in the thymus and 12-fold ↓ in the spleen; Fig. 2). Thus, IL-2 and IL-15 are redundant cytokines, either of which can signal through an IL-2R $\beta$ / $\gamma_c$  receptor complex to promote Treg development.

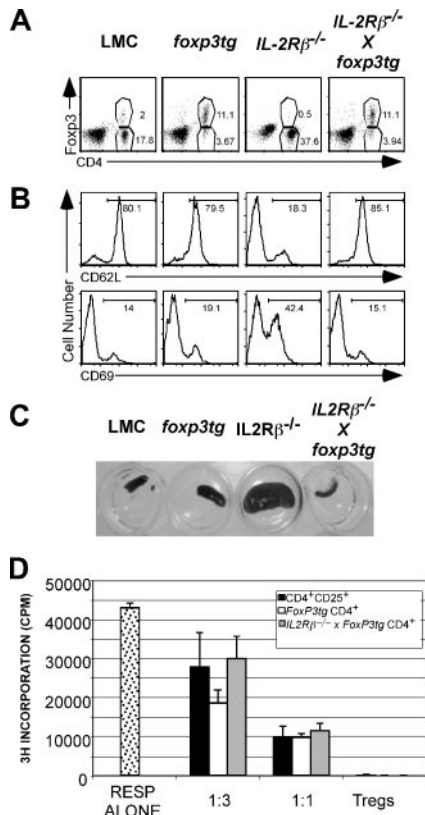
#### *Foxp3* expression restores a functional population of Tregs in *IL-2R $\beta$* <sup>-/-</sup> mice

One potential mechanism by which IL-2R $\beta$ -dependent signals could govern Treg development would be by influencing *foxp3* expression. To test this hypothesis, we made use of *foxp3* transgenic mice. The *foxp3* transgene (made by Ramsdell and colleagues, 18) was derived from a 30.8-kb cosmid encompassing the entire coding region of the *foxp3* gene as well as a significant portion of the upstream regulatory elements (18 kb). However, expression of the transgene does not mimic that of the endogenous *foxp3* gene, as it is expressed in most CD4<sup>+</sup> (75%) and CD8<sup>+</sup> (44%) T cells. We intercrossed *IL-2R $\beta$* <sup>-/-</sup> mice with *foxp3* transgenic mice (*foxp3*tg) and observed that the *foxp3* transgene effectively restores a large population of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells in *IL-2R $\beta$* <sup>-/-</sup> × *foxp3*tg mice (Fig. 3A). Notably, expression of the *foxp3* transgene in *IL-2R $\beta$* <sup>-/-</sup> mice prevented activation of the remaining CD4<sup>+</sup>Foxp3<sup>-</sup> T cells typically seen in *IL-2R $\beta$* <sup>-/-</sup> mice. For example, CD62L expression was restored, and CD69 levels were reduced, to levels seen on WT T cells (Fig. 3B). Furthermore, the lymphoproliferative disorder seen in *IL-2R $\beta$* <sup>-/-</sup>



**FIGURE 2.** IL-2 and IL-15 act as redundant factors to promote the development of Foxp3<sup>+</sup> cells. *A*, Shown are CD4<sup>+</sup>CD8<sup>-</sup>-gated thymocytes (THY) or total splenocytes (SPL) stained with Abs to CD4 and Foxp3. Numbers to the right of the outlined areas represent the percentage of cells in the designated gate. *B*, Shown are average total CD4<sup>+</sup>Foxp3<sup>+</sup> cell numbers from *C57BL/6* (■), *IL-15*<sup>-/-</sup> (□), and *IL-2*<sup>-/-</sup> × *IL-15*<sup>-/-</sup> (▨) mice. Error bars, SEM. Mice used in these experiments were between 4 and 5 wk of age. Data are representative of nine WT *C57BL/6* and *IL-15*<sup>-/-</sup> mice and four *IL-2*<sup>-/-</sup> × *IL-15*<sup>-/-</sup> mice.

mice was completely inhibited in *IL-2R $\beta$* <sup>-/-</sup> × *foxp3*tg mice as demonstrated by the reversal of splenomegaly (Fig. 3C) and the decreased total lymphocyte counts observed in *IL-2R $\beta$* <sup>-/-</sup> × *foxp3*tg mice relative to *IL-2R $\beta$* <sup>-/-</sup> controls (data not shown). Because peripheral T cells in *IL-2R $\beta$* <sup>-/-</sup> × *foxp3*tg mice express high levels of Foxp3, but are completely incapable of responding to IL-2R $\beta$ -dependent signals, we used them to examine whether IL-2R signaling is required to induce Treg suppressor function in vitro. For these experiments, we isolated WT CD4<sup>+</sup>CD25<sup>+</sup> T cells, CD4<sup>+</sup> T cells from *foxp3*tg mice, and CD4<sup>+</sup> T cells from *IL-2R $\beta$* <sup>-/-</sup> × *foxp3*tg mice and assayed their ability to inhibit proliferation of WT CD4<sup>+</sup>CD25<sup>-</sup> responder cells that had been stimulated with allogeneic APCs from *bm12* mice. As shown in



**FIGURE 3.** Foxp3 expression rescues Treg development in *IL-2Rβ<sup>-/-</sup>* mice. **A**, Spleens were harvested from 5- to 8-wk-old LMC, *IL-2Rβ<sup>-/-</sup>*, *foxp3tg*, and *IL-2Rβ<sup>-/-</sup> × foxp3tg* mice and stained with Abs to CD4 and Foxp3. **B**, CD4<sup>+</sup>-gated T cells were stained with Abs to CD69 and CD62L. **C**, Shown are spleens from 5-wk-old LMC, *foxp3tg*, *IL-2Rβ<sup>-/-</sup>*, and *IL-2Rβ<sup>-/-</sup> × foxp3tg* mice. Data are representative of five mice. **D**, One hundred thousand CD4<sup>+</sup>CD25<sup>-</sup> responder T cells from *C57BL/6* mice were mixed with equal numbers of allogeneic *bm12* splenocytes to induce proliferation. FACS-sorted CD4<sup>+</sup> cells from *foxp3* transgenic mice (□), *IL-2Rβ<sup>-/-</sup> × foxp3tg* mice (▣), or CD4<sup>+</sup>CD25<sup>+</sup> T cells from *C57BL/6* mice (■) were cultured at the indicated Treg to responder cell ratio. Data are presented as mean [<sup>3</sup>H]thymidine incorporation of quadruplicate cultures and error bars represent SEM. Data presented are representative of three independent experiments.

Fig. 3D, the CD4<sup>+</sup> T cells from *IL-2Rβ<sup>-/-</sup> × foxp3tg* mice suppressed responder cell proliferation to an equivalent degree as that achieved when using WT CD4<sup>+</sup>CD25<sup>+</sup> T cells. CD4<sup>+</sup> T cells from *foxp3tg* mice also inhibited proliferation of WT effector cells, whereas CD4<sup>+</sup> T cells from *IL-2Rβ<sup>-/-</sup>* mice did not (data not shown). These results demonstrate that Foxp3 expression is sufficient, in the absence of other IL-2Rβ-dependent signals, to effectively inhibit effector T cell proliferation in vitro.

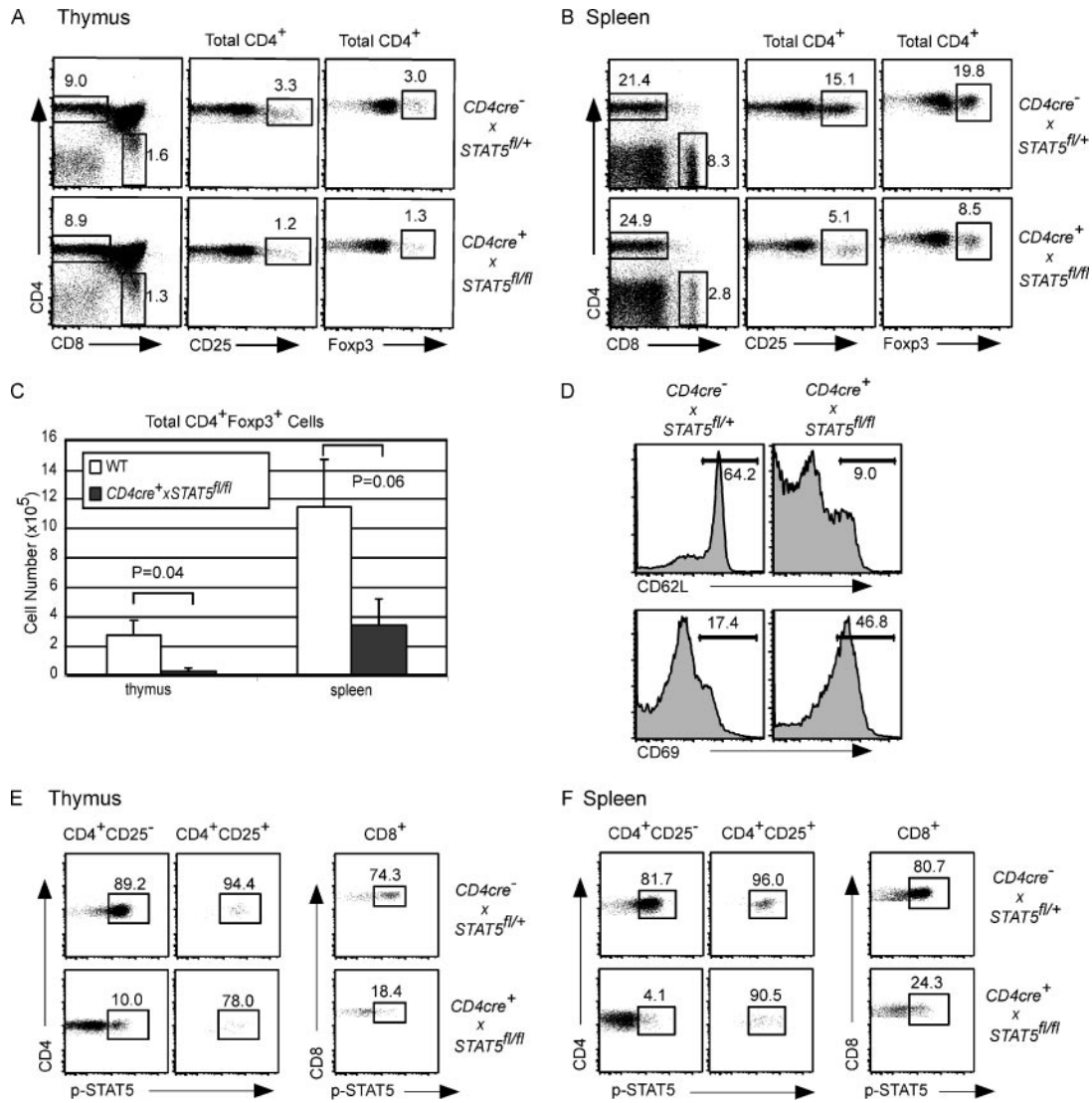
#### STAT5 signaling is required for Treg development

The IL-2R activates a number of downstream signaling pathways including those involving Erk, PI3K, and STAT5. Having previously demonstrated that STAT5 is preferentially activated in Tregs (25), we hypothesized that STAT5 signaling may play a critical role in Treg development. In fact, we and others have previously demonstrated that mice in which the first exon of *STAT5a* and *STAT5b* is disrupted (referred to as *STAT5a/b<sup>Ex1/Ex1</sup>* mice) have a decreased number of cells with regulatory activity (26, 27). However, because these studies predated the availability of methods to stain for intracellular *foxp3*, we reexamined this issue. We found that although *STAT5a/b<sup>Ex1/Ex1</sup>* mice do have a reduced percentage

of Tregs, substantial numbers of these cells could still be detected. However, we also observed that lymphocytes from these mice express significant amounts of truncated STAT5a/b protein (data not shown). Importantly, this truncated STAT5 protein has recently been shown to exhibit functional activity (28). To circumvent this issue, we made use of mice generated by Hennighausen and colleagues (28), in which the entire *STAT5a* and *STAT5b* genes are flanked by *loxP* sites (referred to as *STAT5a/b<sup>FL/FL</sup>* mice), allowing for Cre-mediated deletion of both *STAT5a* and *STAT5b* genes. We crossed these animals to *CD4-Cre* transgenic mice in which Cre protein is expressed in CD4<sup>+</sup>CD8<sup>+</sup> and CD4<sup>+</sup> thymocytes, as well as in peripheral CD4<sup>+</sup> T cells (29). Consistent with recent reports (30), we observed a significant decrease in CD8<sup>+</sup> T cells in the thymus and the periphery of *CD4-cre × STAT5a/b<sup>FL/FL</sup>* mice. Notably, CD4<sup>+</sup>Foxp3<sup>+</sup> T cells were also clearly reduced in both the thymus and spleen of *CD4-cre × STAT5a/b<sup>FL/FL</sup>* mice when compared with *STAT5a/b<sup>FL/FL</sup>* or *CD4-cre<sup>-</sup> × STAT5a/b<sup>FL/+</sup>* controls (Fig. 4, A–C). Consistent with the reduction in Treg numbers, we observed a significant increase in the percentage of activated CD4<sup>+</sup> T cells in these mice based on decreased CD62L and increased CD69 expression (Fig. 4D). However, it was also clear that not all CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs were eliminated in *CD4-Cre × STAT5a/b<sup>FL/FL</sup>* mice. One explanation for the remaining Tregs is that STAT5 may not have been efficiently eliminated in that cell lineage. To examine this issue, we stimulated T cells from *CD4-Cre × STAT5a/b<sup>FL/FL</sup>* mice with IL-2 and IL-7, which results in STAT5 phosphorylation that can be detected by flow cytometry. Because the intracellular staining protocols for Foxp3 and phospho-STAT5 require different fixation conditions that are not compatible, we used CD25 as a surrogate marker to identify Tregs. Phospho-STAT5 was clearly detected in CD4<sup>+</sup>CD25<sup>-</sup>, CD4<sup>+</sup>CD25<sup>+</sup>, and CD8<sup>+</sup> T cells from LMC (Fig. 4, E and F). However, in *CD4-Cre × STAT5a/b<sup>FL/FL</sup>* mice, the majority of CD4<sup>+</sup>CD25<sup>-</sup> and CD8<sup>+</sup> T cells had deleted STAT5a/b protein (95 and 70%, respectively) as demonstrated by the failure to detect phospho-STAT5 protein following IL-2/IL-7 stimulation. Importantly, our results with loss of STAT5 protein are in perfect agreement with previous findings by Yao et al. (30), who reported that >90% of double-positive thymocytes in *CD4-Cre × STAT5a/b<sup>FL/FL</sup>* mice have deleted the *STAT5a* and *STAT5b* genes. In contrast, CD4<sup>+</sup>CD25<sup>+</sup> T cells from *CD4-Cre × STAT5a/b<sup>FL/FL</sup>* mice exhibited minimal to no deletion of STAT5a/b as demonstrated by their robust ability to induce phospho-STAT5 upon IL-2/IL-7 stimulation in both the thymus and the spleen (Fig. 4, E and F). Similar results were obtained when using glucocorticoid-induced TNFR family-related gene as a cell surface marker to identify Tregs (data not shown). These results indicate that the only remaining Tregs are those that had not deleted STAT5 and thus strongly support a role for STAT5 in the development of Tregs.

#### STAT5 activation restores CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs in *IL-2Rβ<sup>-/-</sup>* mice

Having demonstrated that STAT5 is required for Treg development, we decided to examine whether STAT5 activation was sufficient to rescue Treg development in *IL-2Rβ<sup>-/-</sup>* mice. For these studies, we made use of transgenic mice that express a constitutively active form of STAT5b (STAT5b-CA) (21, 26, 31). Expression of *STAT5b-CA* is regulated by a compound promoter element consisting of the *lck* proximal promoter with the *Eμ* enhancer inserted into it. We have previously demonstrated that this transgene is expressed early during thymocyte development (at the early T cell progenitor stage) and that it continues to be expressed in both mature CD4<sup>+</sup> and CD8<sup>+</sup> T cells (21, 26). As shown in Fig. 5,

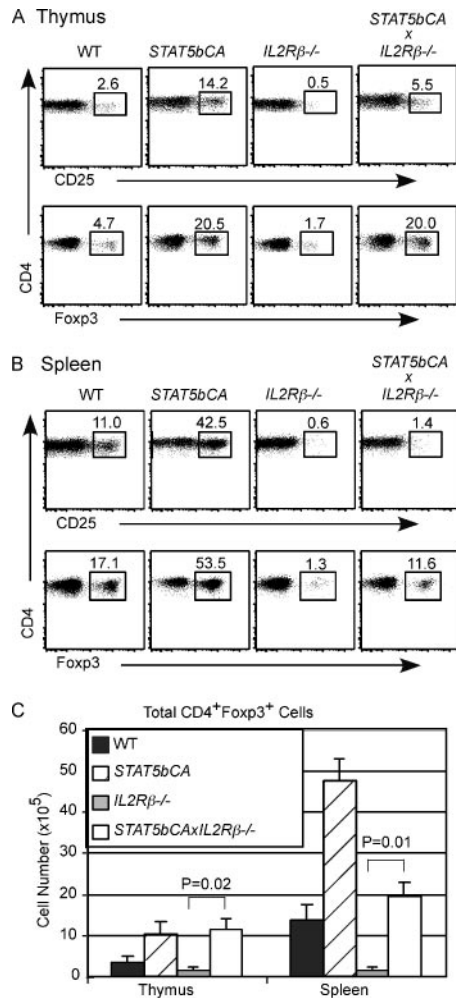


**FIGURE 4.** STAT5 signaling is necessary for the development of Tregs. *A* and *B*, *Left panels*, show thymocytes (*A*) or splenocytes (*B*) from LMC (*CD4-Cre*<sup>-</sup> × *STAT5a/b*<sup>FL/+</sup> or *STAT5a/b*<sup>FL/FL</sup>) and *CD4-Cre*<sup>+</sup> × *STAT5a/b*<sup>FL/FL</sup> mice stained with Abs to CD4 and CD8. *Center and middle panels*, CD4-gated thymocytes (*A*) or splenocytes (*B*) stained with Abs to CD25 or Foxp3. *C*, Shown are total numbers of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells from either LMC (□) or *CD4-Cre* × *STAT5a/b*<sup>FL/FL</sup> mice. *D*, Shown are histograms of CD4-gated T cells from LMC or *CD4-Cre* × *STAT5a/b*<sup>FL/FL</sup> mice stained with Abs to CD62L or CD69. *E* and *F*, Thymocytes (*E*) or splenocytes (*F*) were stimulated with 1000 U/ml IL-2 and 30 ng/ml IL-7 for 30 min and then stained with Abs to CD4, CD8, and CD25. Cells were then fixed, permeabilized, and stained for phospho-STAT5. Shown is a representative example of three or more experiments.

expression of the *STAT5b-CA* transgene restored a substantial population of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs in the thymus of *IL-2Rβ*<sup>-/-</sup> mice. Likewise, CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs could still be detected in the spleen of *STAT5b-CA* × *IL-2Rβ*<sup>-/-</sup> mice. However, these Tregs no longer expressed CD25 (Fig. 5*B*) and could not prevent T cell lymphoproliferation in vivo or animal death (data not shown). Thus, in many respects the CD4<sup>+</sup>CD25<sup>-</sup>Foxp3<sup>+</sup> Tregs observed in the spleen and lymph nodes of *STAT5b-CA* × *IL-2Rβ*<sup>-/-</sup> mice are very similar to the Tregs observed in *IL-2*<sup>-/-</sup> mice, which also fail to express CD25 and do not prevent autoimmunity (8).

One potential reason for the failure to prevent autoimmunity in *STAT5b-CA* × *IL-2Rβ*<sup>-/-</sup> mice is that the *lck* proximal promoter, which drives expression of the *STAT5b-CA* transgene, is known to become inactive in mature T cells and typically results in decreased expression of transgenes in mature T cells (32). Indeed, the expression of the *STAT5b-CA* transgene is quite low in peripheral T cells (26). Moreover, expression of the *STAT5b-CA* transgene in CD8<sup>+</sup> and CD4<sup>+</sup>Foxp3<sup>-</sup> T cells renders them more resistant to

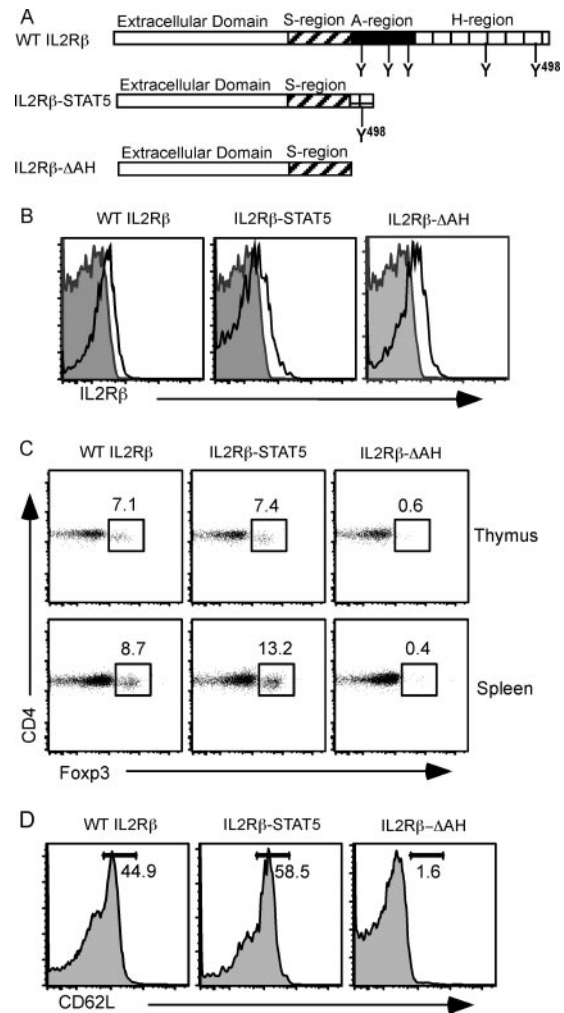
Treg-mediated suppression (data not shown). Finally, Rudensky and colleagues (8) have shown that Tregs develop in *IL-2*<sup>-/-</sup> mice (Fig. 1*A*) but that they fail to prevent autoimmunity in these mice because they exhibit reduced fitness compared with *IL-2*<sup>-/-</sup> effector T cells. We propose that a parallel situation exists in *STAT5b-CA* × *IL-2Rβ*<sup>-/-</sup> mice in which *STAT5b-CA* × *IL-2Rβ*<sup>-/-</sup> Tregs exhibit reduced fitness relative to *STAT5b-CA* × *IL-2Rβ*<sup>-/-</sup> effector T cells. Supporting this proposal, we have recently observed that expression of the *STAT5b-CA* transgene in  $\gamma_c$ <sup>-/-</sup> mice restores peripheral T cell numbers including CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs; importantly, these mice do not exhibit T cell activation, as determined by CD69 and CD62L expression, nor do they develop autoimmune disease (data not shown). Thus, in a situation where all T cells are equally affected ( $\gamma_c$ <sup>-/-</sup>) *STAT5b-CA* expression is sufficient to induce a population of functional Tregs that are capable of preventing autoimmune disease.



**FIGURE 5.** STAT5b activation restores Treg development in the thymus of *IL-2Rβ<sup>-/-</sup>* mice. Shown are CD4<sup>+</sup>CD8<sup>-</sup>-gated thymocytes (A) or splenocytes (B) stained with Abs to CD4, CD25, and Foxp3 from WT controls; STAT5b-CA transgenic mice; *IL-2Rβ<sup>-/-</sup>* mice; and *STAT5b-CA* × *IL-2Rβ<sup>-/-</sup>* mice. C, Shown are total numbers of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells in WT controls, STAT5b-CA transgenic mice, *IL-2Rβ<sup>-/-</sup>* mice, and *STAT5b-CA* × *IL-2Rβ<sup>-/-</sup>* mice. Error bars, SEM. Data are representative of three separate experiments.

To circumvent these possible problems, we expressed a chimeric *IL-2Rβ* construct that selectively activates STAT5 in bone marrow cells from *IL-2Rβ<sup>-/-</sup>* mice (Fig. 6A). Reconstitution of *rag2<sup>-/-</sup>* mice with these transduced bone marrow cells results in the generation of T cells that should induce STAT5 phosphorylation at physiological levels when exposed to endogenously produced IL-2. Control constructs encoding either the full-length WT *IL-2Rβ* chain or a signaling defective receptor in which the cytoplasmic tail contained just the S region were also generated and tested using this approach (referred to as WT *IL-2Rβ* and *IL-2Rβ-ΔAH*, respectively). We first examined the signaling properties of control and chimeric receptors by introducing them into the cytokine-dependent BAF cell line. IL-2 stimulation of the cells expressing the WT *IL-2Rβ* chain led to activation of the ERK and STAT5 signaling pathways. In contrast, the chimeric *IL-2Rβ-STAT5* receptor only activated STAT5, whereas the signaling defective *IL-2Rβ-ΔAH* receptor did not activate any of these pathways (data not shown). Thus, the chimeric *IL-2Rβ-STAT5* receptor selectively leads to STAT5 activation.

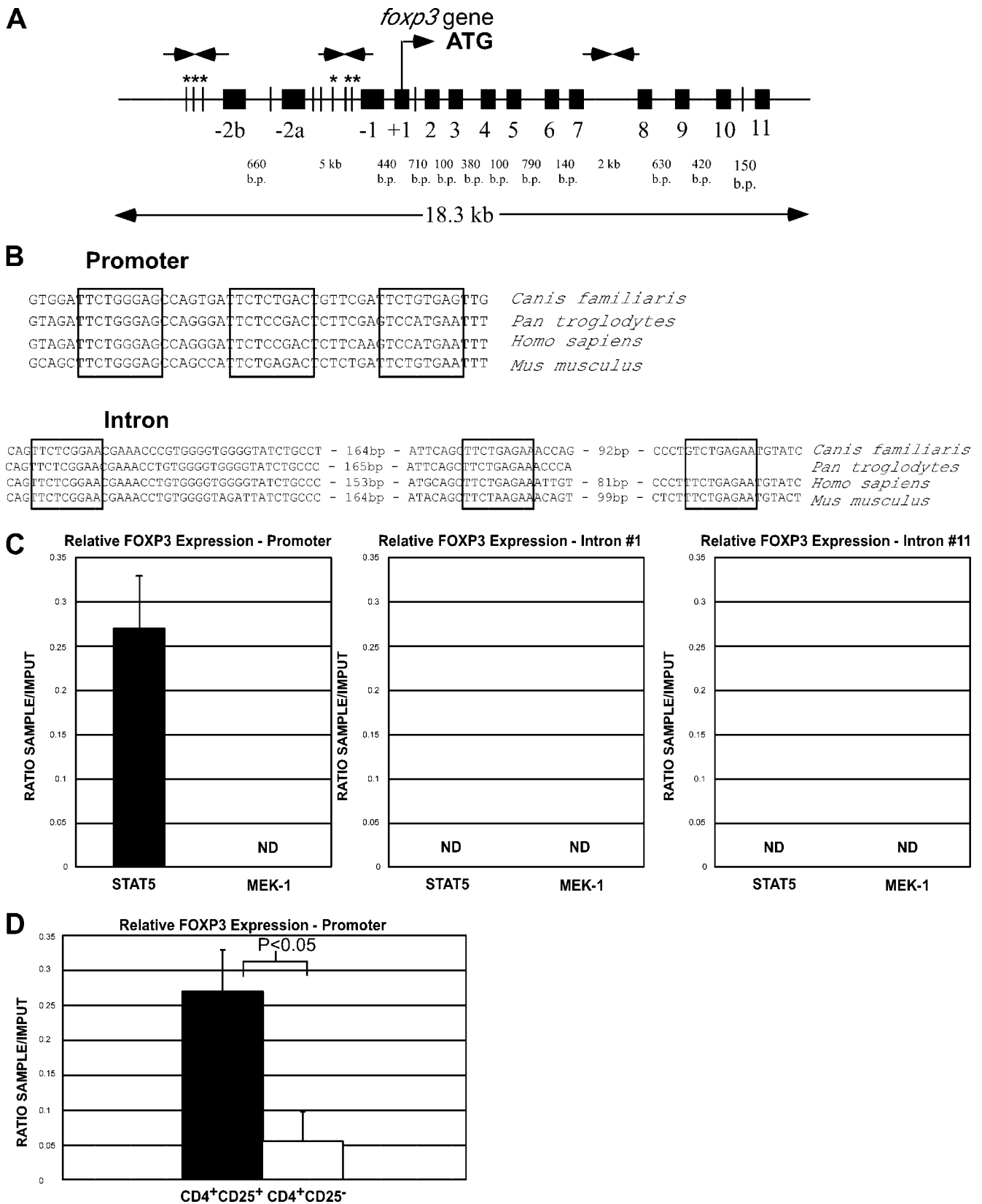
The WT *IL-2Rβ*, *IL-2Rβ-STAT5*, or *IL-2Rβ-ΔAH* constructs were then introduced into *IL-2Rβ<sup>-/-</sup>* bone marrow using a mod-



**FIGURE 6.** STAT5 signaling is sufficient to mediate *IL-2Rβ*-dependent Treg development. A, Schematic representation of *IL-2Rβ* constructs used for retroviral reconstitution experiments. B, Splenic and thymic cells from recipient mice were harvested at 9 wk postreconstitution and stained with Abs to *IL-2Rβ* (open histograms). Shaded histograms represent Ab staining on *IL-2Rβ*-deficient mice. C and D, Foxp3 and CD62L levels of expression were analyzed on CD4<sup>+</sup>-gated cells. Numbers above the outlined areas represent the percentage of cells in the designated gate. Data are representative of three independent recipient mice per group.

ified version of the pMIGR retroviral vector (20). Infected *IL-2Rβ<sup>-/-</sup>* bone marrow was then transferred into *rag2<sup>-/-</sup>* recipients; 9 wk following reconstitution, recipient mice were analyzed for the presence of CD4<sup>+</sup>Foxp3<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup> Treg populations. Cell surface staining for the *IL-2Rβ* chain demonstrated that equal percentages of splenic CD4<sup>+</sup> T cells in these bone marrow chimeric mice expressed the retrovirally driven *IL-2Rβ* chain (Fig. 6B). Mice reconstituted with the WT *IL-2Rβ* construct developed a significant population of CD4<sup>+</sup>Foxp3<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup> cells, indicating that reintroduction of the WT receptor could restore Tregs in *IL-2Rβ<sup>-/-</sup>* mice (Fig. 6C and data not shown). Similarly, the *IL-2Rβ-STAT5* construct also restored a comparable population of CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs. Importantly, most Tregs in the spleen of these mice expressed high levels of CD25, indicating that they resembled Tregs that are normally observed in WT mice. In contrast, the signaling defective *IL-2Rβ-ΔAH* construct did not restore a population of CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs.

We next examined T cell activation in these mice. Virtually all T cells (95%) in mice reconstituted with the signaling defective



**FIGURE 7.** STAT5 binds to the *foxp3* promoter in Tregs. *A*, Schematic representation of the *foxp3* gene. Arrows, Locations of PCR primers used for quantification of ChIP assays. Asterisks, The conserved STAT5 binding sites shown in *B* and analyzed by ChIP assays in *C*. *B*, Alignment of selected regions of *foxp3* gene among various species. *C*, Real-time PCR quantification of genomic DNA obtained from CD4<sup>+</sup>CD25<sup>+</sup> T cells immunoprecipitated with either anti-STAT5 (■) or anti-MEK-1. *Left panel*, The amount of PCR product normalized to input control of the *foxp3* promoter region containing the STAT5 binding sites noted in *A*. *Middle panel*, The amount of PCR product normalized to input control of the *foxp3* intron 1 region containing the STAT5 binding sites noted in *A*. *Right panel*, the amount of PCR product normalized to input control of the *foxp3* intron 11 region noted in *A*. Input samples amplified DNA from all regions tested. Error bars, SEM. Data are representative of two independent experiments. *D*, Comparison of STAT5 binding to the *foxp3* promoter sites in CD4<sup>+</sup>CD25<sup>+</sup> vs CD4<sup>+</sup>CD25<sup>-</sup> T cells. Error bars, SEM. Data are representative of two independent experiments.

IL-2R $\beta$ - $\Delta$ AH construct expressed low levels of CD62L consistent with an activated phenotype (Fig. 6D, right panel). In contrast, T cell activation was greatly diminished in mice reconstituted with either the WT or IL-2R $\beta$ -STAT5 receptor as demonstrated by the increase in the percentage of T cells expressing high levels of CD62L (Fig. 6D, left and center panels). Identical conclusions were reached when using expression of CD69 as a marker of T cell activation (data not shown). Thus, Tregs generated following reconstitution with an IL-2R $\beta$  construct that can only activate STAT5 were capable of preventing T cell activation in vivo (although we cannot rigorously rule out the possibility that these Tregs may exhibit somewhat reduced suppressor function). These results demonstrate that STAT5 activation is sufficient to promote IL-2R $\beta$ -dependent development of CD4<sup>+</sup>CD25<sup>-</sup>Foxp3<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs in both the thymus and peripheral lymphoid organs.

#### STAT5 binds to the foxp3 gene promoter

The findings that *foxp3* expression or STAT5 activation are both sufficient to restore Treg development in IL-2R $\beta$ <sup>-/-</sup> mice suggested that STAT5 may act by directly regulating *foxp3* expression. To examine this issue, we first scanned the murine *foxp3* gene for potential STAT5 consensus binding sites. As shown in Fig. 7A, the murine *foxp3* gene contains 11 potential STAT5 binding sites. Because functional binding sites should be evolutionarily conserved, we compared the *foxp3* gene sequences from murine, human, monkey, and dog species and observed that six of these sites were conserved (Fig. 7B); these include three closely spaced sequences in the *foxp3* promoter and three sites located between murine exon-2a and exon-1 (Fig. 7A). To determine whether STAT5 actually binds to these conserved sites we conducted ChIP assays. As seen in Fig. 7C, immunoprecipitation with an anti-STAT5 Ab revealed STAT5 binding to the conserved sites in the promoter of the *foxp3* gene in murine CD4<sup>+</sup>CD25<sup>+</sup> T cells. In contrast, no binding could be seen to the three conserved sites in intron 2 or the distal site located in intron 11. No PCR product was observed when ChIP was done using an anti-Mek-1 control Ab. Finally, significant STAT5 binding to the *foxp3* gene was not observed in CD4<sup>+</sup>CD25<sup>-</sup> T cells, a cell population which is mostly comprised of CD4<sup>+</sup>Foxp3<sup>-</sup> T cells (Fig. 7D). Thus, these experiments provide evidence that STAT5 can play a role in regulating *foxp3* gene expression.

## Discussion

It has been well established that IL-2 and the IL-2R play important roles in Treg biology. However, whether IL-2 or the IL-2R affect the development, homeostasis, or suppressor function of Tregs has remained controversial. In a large part, this controversy stems from the use of CD25 as a marker for Tregs. For example, CD25 is also expressed on activated effector T cells; furthermore, CD25 is not expressed on all Tregs as was recently demonstrated using *foxp3*<sup>GFP</sup> or *foxp3*<sup>dsRed</sup> knock-in mice (33, 34). Herein, we use intracellular staining for Foxp3 to identify the role of IL-2 and the IL-2R on the development of Tregs. In agreement with D'Cruz and Klein (7) and Fontenot et al. (8), we find that IL-2 is not required for the development of Tregs in the thymus or for their maintenance in young IL-2<sup>-/-</sup> mice. In contrast, we observed that IL-2R $\beta$  and  $\gamma_c$  are required for Treg development. The surprising finding that the IL-2R $\beta$  and  $\gamma_c$  chains are required for Treg development, whereas IL-2 is not, raised the question of what cytokines signal through the IL-2R $\beta$ / $\gamma_c$  complex to promote Treg differentiation. The only other cytokine known to use the IL-2R $\beta$  chain for signaling is IL-15. Although IL-15<sup>-/-</sup> mice have no obvious defects in Treg numbers or function, IL-2<sup>-/-</sup>  $\times$  IL-15<sup>-/-</sup> mice have

a clear deficit in Tregs, which mimics that seen in IL-2R $\beta$ <sup>-/-</sup> mice. Thus, our findings point to a previously unappreciated role for IL-15 in Treg development and provide a compelling explanation for the continued presence of Tregs in IL-2<sup>-/-</sup> mice.

A remaining question is whether other cytokines can contribute to this process as well. It is difficult to directly compare results in  $\gamma_c$ <sup>-/-</sup> and IL-2R $\beta$ <sup>-/-</sup> mice due to the additional early blocks in thymocyte development that occur in  $\gamma_c$  null mice. However, the more stringent block in Treg development observed in  $\gamma_c$ <sup>-/-</sup> vs IL-2R $\beta$ <sup>-/-</sup> mice is at least consistent with the possibility that other  $\gamma_c$ -using cytokines (IL4, IL-7, IL9, or IL-21) might be able to contribute to STAT5-dependent Treg development. Supporting this conjecture, Fontenot et al. (8) did not report a significant difference in Treg numbers in very young IL-2R $\beta$ <sup>-/-</sup> mice (2 wk old). Their results differ from our findings in older (4–5 wk of age) IL-2R $\beta$ <sup>-/-</sup> and IL-2<sup>-/-</sup>  $\times$  IL-15<sup>-/-</sup> mice. The most likely explanation for this difference is that, in very young mice, Tregs will face less competition from naive T cells for binding to IL-7. A similar phenomenon has already been described for naive T cells in young mice, which appear to undergo a homeostatic expansion-like process due to less competition for endogenous IL-7 (35, 36). Thus, in the relatively lymphopenic conditions that exist in very young mice, developing Tregs might still be able to bind IL-7. In older mice, the increased number of naive T cells expressing high levels of IL-7R $\alpha$ , coupled with the reduced expression of IL-7R $\alpha$  on Tregs relative to other T cell subsets, would make it unlikely that Tregs could effectively compete for and respond to IL-7 signals.

The finding that both IL-2<sup>-/-</sup>  $\times$  IL-15<sup>-/-</sup> mice and IL-2R $\beta$ <sup>-/-</sup> mice lack Tregs provides strong evidence that signaling pathways downstream of the IL-2R $\beta$  receptor complex entrain Treg differentiation. In this study, we document that STAT5 activation is both necessary and sufficient for IL-2R $\beta$ -dependent Treg development. Previous studies using *STAT5a/b*<sup>Ex1/Ex1</sup> mice had provided evidence that STAT5 is required for Treg survival (26, 27). However, the discovery that these mice express a hypomorphic rather than a STAT5 null allele makes it impossible to draw conclusions about the role of STAT5 in Treg development based on studies using these mice (28). Using a conditional STAT5 knockout model, we demonstrate clear differences in the role that STAT5 plays in CD8<sup>+</sup> and CD4<sup>+</sup>Foxp3<sup>-</sup> T cells vs CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs. Specifically, we observe decreased numbers of CD4<sup>+</sup>Foxp3<sup>-</sup> and CD8<sup>+</sup> T cells in *CD4-Cre*  $\times$  *STAT5a/b*<sup>FL/FL</sup> mice (J. Yang, M. A. Burchill, and M. A. Farrar, unpublished results). Importantly, these cell populations also showed efficient deletion of STAT5 (70–95% deletion), indicating that STAT5 affects survival but not development of these naive T cell populations. We also observed a decrease in Treg numbers in *CD4-Cre*  $\times$  *STAT5a/b*<sup>FL/FL</sup> mice (Fig. 4, A and B). However, virtually none of the remaining Tregs had deleted STAT5, thereby strongly supporting a role for STAT5 in governing development of this cell population. Thus, our results establish that an IL-2R $\beta$ -activated STAT5-dependent signaling pathway plays a critical role in Treg development.

A key question in the field of Treg biology is what are the signals that govern *foxp3* transcription. Our findings that either forced *foxp3* expression or constitutive STAT5b activation in Tregs could rescue Treg development in the absence of IL-2R signaling suggested that these factors might be involved in a coherent signaling pathway. Specifically, our studies provide evidence that STAT5 binds to conserved sites within the *foxp3* promoter and thereby provide a direct link between IL-2R signals and *foxp3* expression. A potential concern is that the identified STAT5 binding sites do not exactly match the STAT5b

binding consensus motif (TTCXXXGAA). However, it is important to note that the three sites detected in the promoter region are separated by only 7 bp. Previous work by Leonard and colleagues (37) has demonstrated that STAT5 binding sites separated by 6–7 bp allows efficient binding of STAT5 to those sites as STAT5 tetramers rather than dimers. Moreover, they noted that these tetrameric sites frequently diverged from the optimal consensus STAT5-binding motif. Thus, the spacing of the STAT5 binding sites in the *foxp3* promoter suggest that formation of STAT5 tetramers may be important for STAT5 binding to the *foxp3* promoter.

A recent report by Zorn et al. (38) has also suggested that STAT5 regulates *foxp3* expression, although they suggested that the relevant binding sites are those found within the first intron of the *foxp3* gene. This conclusion was based on experiments in which they isolated the STAT5 consensus binding sites in the first intron, placed them in a luciferase reporter construct, and introduced this reporter construct along with a plasmid encoding an activated form of STAT5 into fibroblasts. In those studies, they found that the isolated STAT5 binding sites could lead to enhanced luciferase expression in a STAT5-dependent manner. In contrast, our ChIP studies using murine Tregs demonstrate that STAT5 does not bind to the intronic sites *in vivo*. Thus, we believe that the physiologically relevant STAT5 binding sites are those found within the promoter region of the *foxp3* gene and not the first intron. Finally, we note that STAT5 signaling by itself is not sufficient to induce *foxp3* expression because STAT5 activation did not convert all T cells into Tregs. Thus, an important question for the future will be the identification of other factors that interact with STAT5 to help initiate or maintain *foxp3* gene expression.

In conclusion, our data suggest a model for how IL-2 signals entrain Treg development. Namely, we propose that IL-2/IL-15 signal through an IL-2R $\beta$ / $\gamma$ <sub>c</sub> complex to transmit STAT5-dependent signals that directly impinge on *foxp3* transcription. A remaining question is where the IL-2/IL-15 comes from that initiates this process. A simple explanation would be that IL-15 produced constitutively by stromal cells may be sufficient to drive Treg development in the thymus. However, IL-2 must also contribute as *IL-15*<sup>-/-</sup> mice show only slight defects, if any, in thymic Treg generation. It is tempting to speculate that this relates to the higher self-reactivity of TCRs expressed by Tregs (39). In this model, the stronger T cell signals delivered by TCRs expressed on developing Tregs may be sufficient to induce IL-2R $\alpha$  expression allowing these cells to respond to either autocrine or paracrine IL-2, and thereby inducing STAT5 activation and subsequent *foxp3* transcription. Foxp3 expression would then act in conjunction with STAT5 to maintain high level IL-2R $\alpha$  expression (thereby allowing Tregs to respond to IL-2 produced by activated effector T cells) and inhibit subsequent TCR-dependent IL-2 secretion in mature Tregs. Although some aspects of this model await experimental verification, it provides a testable hypothesis that links the current elements known to be required for Treg development, namely, the TCR and CD28, the IL-2R complex, and the transcription factor Foxp3.

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## Disclosures

The authors have no financial conflict of interest.

## References

- Sadlack, B., H. Merz, H. Schorle, A. Schimpl, A. C. Feller, and I. Horak. 1993. Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. *Cell* 75: 253–261.
- Willerford, D. M., J. Chen, J. A. Ferry, L. Davidson, A. Ma, and F. W. Alt. 1995. Interleukin-2 receptor  $\alpha$ -chain regulates the size and content of the peripheral lymphoid compartment. *Immunity* 3: 521–530.
- Suzuki, H., T. M. Kundig, C. Furlonger, A. Wakeham, E. Timms, T. Matsuyama, R. Schmits, J. J. Simard, P. S. Ohashi, H. Griesser, et al. 1995. Deregulated T cell activation and autoimmunity in mice lacking interleukin-2 receptor  $\beta$ . *Science* 268: 1472–1476.
- Malek, T. R., A. Yu, V. Vincek, P. Scibelli, and L. Kong. 2002. CD4 regulatory T cells prevent lethal autoimmunity in IL-2R $\beta$ -deficient mice. Implications for the nonredundant function of IL-2. *Immunity* 17: 167–178.
- Malek, T. R., B. O. Porter, E. K. Codias, P. Scibelli, and A. Yu. 2000. Normal lymphoid homeostasis and lack of lethal autoimmunity in mice containing mature T cells with severely impaired IL-2 receptors. *J. Immunol.* 164: 2905–2914.
- Setoguchi, R., S. Hori, T. Takahashi, and S. Sakaguchi. 2005. Homeostatic maintenance of natural Foxp3<sup>+</sup>CD25<sup>+</sup>CD4<sup>+</sup> regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. *J. Exp. Med.* 201: 723–735.
- D'Cruz, L. M., and L. Klein. 2005. Development and function of agonist-induced CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells in the absence of interleukin 2 signaling. *Nat. Immunol.* 6: 1152–1159.
- Fontenot, J. D., J. P. Rasmussen, M. A. Gavin, and A. Y. Rudensky. 2005. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat. Immunol.* 6: 1142–1151.
- Furtado, G. C., M. A. Curotto de Lafaille, N. Kutchukhidze, and J. J. Lafaille. 2002. Interleukin 2 signaling is required for CD4<sup>+</sup> regulatory T cell function. *J. Exp. Med.* 196: 851–857.
- de la Rosa, M., S. Rutz, H. Döringer, and A. Scheffold. 2004. Interleukin-2 is essential for CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cell function. *Eur. J. Immunol.* 34: 2480–2488.
- Thornton, A. M., E. E. Donovan, C. A. Piccirillo, and E. M. Shevach. 2004. Cutting edge: IL-2 is critically required for the *in vitro* activation of CD4<sup>+</sup>CD25<sup>+</sup> T cell suppressor function. *J. Immunol.* 172: 6519–6523.
- Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299: 1057–1061.
- Khattri, R., T. Cox, S. A. Yasayko, and F. Ramsdell. 2003. An essential role for Scurfin in CD4<sup>+</sup>CD25<sup>+</sup> T regulatory cells. *Nat. Immunol.* 4: 337–342.
- Fontenot, J. D., M. A. Gavin, and A. Y. Rudensky. 2003. Foxp3 programs the development and function of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells. *Nat. Immunol.* 4: 330–336.
- Chatila, T. A., F. Blaeser, N. Ho, H. M. Lederman, C. Voulgaropoulos, C. Helms, and A. M. Bowcock. 2000. JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic dysregulation syndrome. *J. Clin. Invest.* 106: R75–R81.
- Wildin, R. S., F. Ramsdell, J. Peake, F. Faravelli, J. L. Casanova, N. Buist, E. Levy-Lahad, M. Mazzella, O. Goulet, L. Perroni, et al. 2001. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat. Genet.* 27: 18–20.
- Bennett, C. L., J. Christie, F. Ramsdell, M. E. Brunkow, P. J. Ferguson, L. Whitesell, T. E. Kelly, F. T. Saulsbury, P. F. Chance, and H. D. Ochs. 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat. Genet.* 27: 20–21.
- Brunkow, M. E., E. W. Jeffery, K. A. Hjerrild, B. Paepers, L. B. Clark, S. A. Yasayko, J. E. Wilkinson, D. Galas, S. F. Ziegler, and F. Ramsdell. 2001. Disruption of a new forkhead/winged-helix protein, scurfy, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat. Genet.* 27: 68–73.
- Will, W. M., J. D. Aaker, M. A. Burchill, I. R. Harmon, J. J. O'Neil, C. A. Goetz, K. L. Hippen, and M. A. Farrar. 2006. Attenuation of IL-7 receptor signaling is not required for allelic exclusion. *J. Immunol.* 176: 3350–3355.
- Pear, W. S., G. P. Nolan, M. L. Scott, and D. Baltimore. 1993. Production of high-titer helper-free retroviruses by transient transfection. *Proc. Natl. Acad. Sci. USA* 90: 8392–8396.
- Goetz, C. A., I. R. Harmon, J. J. O'Neil, M. A. Burchill, T. M. Johanns, and M. A. Farrar. 2005. Restricted STAT5 activation dictates appropriate thymic B versus T cell lineage commitment. *J. Immunol.* 174: 7753–7763.
- DiSanto, J. P., W. Muller, D. Guy-Grand, A. Fischer, and K. Rajewsky. 1995. Lymphoid development in mice with a targeted deletion of the interleukin 2 receptor  $\gamma$  chain. *Proc. Natl. Acad. Sci. USA* 92: 377–381.
- He, Y. W., H. Nakajima, W. J. Leonard, B. Adkins, and T. R. Malek. 1997. The common  $\gamma$ -chain of cytokine receptors regulates intrathymic T cell development at multiple stages. *J. Immunol.* 158: 2592–2599.
- Peschon, J. J., P. J. Morrissey, K. I. Grabstein, F. J. Ramsdell, E. Maraskovsky, B. C. Gliniak, L. S. Park, S. F. Ziegler, D. E. Williams, C. B. Ware, et al. 1994. Early lymphocyte expansion is severely impaired in interleukin-7 receptor deficient mice. *J. Exp. Med.* 180: 1955–1960.
- Bensinger, S. J., P. T. Walsh, J. Zhang, M. Carroll, R. Parsons, J. C. Rathmell, C. B. Thompson, M. A. Burchill, M. A. Farrar, and L. A. Turka. 2004. Distinct IL-2 receptor signaling pattern in CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells. *J. Immunol.* 172: 5287–5296.

26. Burchill, M. A., C. A. Goetz, M. Prlic, J. J. O'Neil, I. R. Harmon, S. J. Bensinger, L. A. Turka, P. Brennan, S. C. Jameson, and M. A. Farrar. 2003. Distinct effects of STAT5 activation on CD4<sup>+</sup> and CD8<sup>+</sup> T cell homeostasis: development of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells versus CD8<sup>+</sup> memory T cells. *J. Immunol.* 171: 5853–5864.
27. Snow, J. W., N. Abraham, M. C. Ma, B. G. Herndier, A. W. Pastuszak, and M. A. Goldsmith. 2003. Loss of tolerance and autoimmunity affecting multiple organs in STAT5A/5B-deficient mice. *J. Immunol.* 171: 5042–5050.
28. Cui, Y., G. Riedlinger, K. Miyoshi, W. Tang, C. Li, C. X. Deng, G. W. Robinson, and L. Hennighausen. 2004. Inactivation of Stat5 in mouse mammary epithelium during pregnancy reveals distinct functions in cell proliferation, survival, and differentiation. *Mol. Cell. Biol.* 24: 8037–8047.
29. Lee, P. P., D. R. Fitzpatrick, C. Beard, H. K. Jessup, S. Lehar, K. W. Makar, M. Perez-Melgosa, M. T. Sweetser, M. S. Schlissel, S. Nguyen, et al. 2001. A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. *Immunity* 15: 763–774.
30. Yao, Z., Y. Cui, W. T. Watford, J. H. Bream, K. Yamaoka, B. D. Hissong, D. Li, S. K. Durum, Q. Jiang, A. Bhandoola, et al. 2006. Stat5a/b are essential for normal lymphoid development and differentiation. *Proc. Natl. Acad. Sci. USA* 103: 1000–1005.
31. Goetz, C. A., I. R. Harmon, J. J. O'Neil, M. A. Burchill, and M. A. Farrar. 2004. STAT5 activation underlies IL-7 receptor-dependent B cell development. *J. Immunol.* 172: 4770–4778.
32. Wildin, R. S., A. M. Garvin, S. Pawar, D. B. Lewis, K. M. Abraham, K. A. Forbush, S. F. Ziegler, J. M. Allen, and R. M. Perlmutter. 1991. Developmental regulation of *lck* gene expression in T lymphocytes. *J. Exp. Med.* 173: 383–393.
33. Fontenot, J. D., J. P. Rasmussen, L. M. Williams, J. L. Dooley, A. G. Farr, and A. Y. Rudensky. 2005. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* 22: 329–341.
34. Wan, Y. Y., and R. A. Flavell. 2005. Identifying Foxp3-expressing suppressor T cells with a bicistronic reporter. *Proc. Natl. Acad. Sci. USA* 102: 5126–5131.
35. Min, B., R. McHugh, G. D. Sempowski, C. Mackall, G. Foucras, and W. E. Paul. 2003. Neonates support lymphopenia-induced proliferation. *Immunity* 18: 131–140.
36. Schuler, T., G. J. Hammerling, and B. Arnold. 2004. Cutting edge: IL-7-dependent homeostatic proliferation of CD8<sup>+</sup> T cells in neonatal mice allows the generation of long-lived natural memory T cells. *J. Immunol.* 172: 15–19.
37. Soldaini, E., S. John, S. Moro, J. Bollenbacher, U. Schindler, and W. J. Leonard. 2000. DNA binding site selection of dimeric and tetrameric Stat5 proteins reveals a large repertoire of divergent tetrameric Stat5a binding sites. *Mol. Cell. Biol.* 20: 389–401.
38. Zorn, E., E. A. Nelson, M. Mohseni, F. Porcheray, H. Kim, D. Litsa, R. Bellucci, E. Raderschall, C. Canning, R. J. Soiffer, et al. 2006. IL-2 regulates FOXP3 expression in human CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells through a STAT dependent mechanism and induces the expansion of these cells in vivo. *Blood* 108: 1571–1579.
39. Hsieh, C. S., Y. Liang, A. J. Tyznik, S. G. Self, D. Liggitt, and A. Y. Rudensky. 2004. Recognition of the peripheral self by naturally arising CD25<sup>+</sup>CD4<sup>+</sup> T cell receptors. *Immunity* 21: 267–277.