Nonredundant roles for Stat5a/b in directly regulating Foxp3


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Abstract

Stats (signal transducers and activators of transcription) regulate multiple aspects of T cell fate. T regulatory (Treg) cells are a critical subset that limits immune responses, but the relative importance of Stat5a/b versus Stat3 for Treg cell development has been contentious. We observed that peripheral CD25+CD4+ T cells were reduced in Stat5ΔN mice; however, the levels of Foxp3, a transcription factor that is critical for Treg cells, were normal in splenic CD4+ T cells even though they were reduced in the thymus. In contrast, complete deletion of Stat5a/b (Stat5−/−) resulted in dramatic reduction in CD25- or Foxp3-expressing CD4+ T cells. An intrinsic requirement was demonstrated by reduction of Stat5a/b in CD4-expressing cells and by stem cell transplantation using Stat5−/− fetal liver cells. Stat5a/b were also required for optimal induction of Foxp3 in vitro and bound directly to the foxp3 gene. Reduction of Stat3 in T cells did not reduce the numbers of Treg cells in the thymus or spleen; however, Stat3 was required for IL-6-dependent down-regulation of Foxp3. Therefore, we conclude that Stat5a/b have an essential, non-redundant role in regulating Treg cells and that Stat3 and Stat5a/b appear to have opposing roles in the regulation of Foxp3.


Introduction

The development and differentiation of immune cells is carefully orchestrated by an array of cytokines. Signal transducers and activators of transcription (Stats) represent a small, but critical family of transcription factors that play important roles in transmitting cytokine signals. Consequently, Stats are critical for immunoregulation and the development of immune cells.\textsuperscript{1,2} Stat5a and Stat5b are two closely related proteins that have overlapping functions with respect to lymphoid development and differentiation.\textsuperscript{3,4} Gene targeting of Stat5a and Stat5b (collectively referred to as Stat5), results in impairment in the development of T, B and NK cells.\textsuperscript{5-7} In mice in which the amino termini of Stat5a and Stat5b are deleted (denoted as Stat5\textsuperscript{ΔN} mice) major disruption of various immune cell parameters was noted.\textsuperscript{8,9} However, residual Stat5 function permits T cell development, albeit suboptimally.\textsuperscript{10} This contrasts with the complete absence of Stat5a/b, which results in dramatic reduction in thymocyte numbers, in part due to effects on lymphoid stem cell function.\textsuperscript{5}

Regulatory T (Treg) cells comprise a population of cells enriched in CD4\textsuperscript{+}CD25\textsuperscript{+} T cells, which suppress T cell proliferation and function and attenuate immune responses against self or non-self antigens.\textsuperscript{11-13} Naturally arising Treg cells are produced in the thymus as a functionally distinct T cell subpopulation, whereas adaptive Treg cells are induced from naïve T cells after antigen exposure in the periphery.\textsuperscript{14-17} In classic studies, mice develop organ specific autoimmune disease following neonatal thymectomy, which is corrected by reconstitution with CD4\textsuperscript{+}CD25\textsuperscript{+} T cells.\textsuperscript{13} The essential role of Treg cells in maintaining tolerance has been confirmed by findings that defective function of this subset is a feature of many models of autoimmunity.\textsuperscript{18}
More recently, it was discovered independently by several groups that a subset of CD4+CD25+ T cells express the transcription factor Foxp3, which is necessary and sufficient for Treg cell development and function.\textsuperscript{19-22} Foxp3 is highly conserved in mice and humans. Mutation of Foxp3 in mice (scurfy) results in early autoimmune disease,\textsuperscript{23} whereas mutations of human Foxp3 are associated with a disorder termed immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX).\textsuperscript{24} In mice, Foxp3 is a reliable marker for the Treg lineage.

Multiple lines of evidence have indicated that IL-2 is an important growth factor for Treg development and maintenance. Mice lacking IL-2 or its receptor subunits, IL-2Rα (CD25) and IL-2Rβ (CD122), have deficits in CD4+CD25+ Treg cells and develop autoimmune disease similar to Foxp3 \textsuperscript{−/−} mice.\textsuperscript{25-27} However, IL-2 is dispensable for Treg cell development as some Foxp3-expressing cells are present in IL2\textsuperscript{−/−} and IL2r\textsubscript{α}−/− mice, suggesting the involvement of other cytokines.\textsuperscript{28} \textit{In vitro} culture of CD4+ T cells with transforming growth factor (TGF-β1) can promote the generation of Foxp3+ Treg cells from naïve CD4+ T cells. In contrast, \textit{in vitro} culture of CD4+ T cells with TGF-β1 and IL-6 promotes the differentiation of inflammatory Th17 cells and suppresses Treg cells.\textsuperscript{29}

A first step in IL-2 signaling is the activation of the Janus kinase, Jak3, which associates with the IL-2Rγ chain (CD132), also termed the common gamma chain (γc).\textsuperscript{30} Jak3 and γc are essential for Treg cell development and maintenance, as Jak3\textsuperscript{−/−} and Il2rg \textsuperscript{−/−} mice lack CD25 and Foxp3 expression in the thymus and spleen.\textsuperscript{28,31} Activation of Jaks results in phosphorylation of Stats and Stat5a/b are the most prominent Stats activated by IL-2.\textsuperscript{32} Stat5\textsuperscript{ΔN} mice, which express N-terminally truncated Stat5 proteins, have reduced numbers of CD25+CD4+ cells in the periphery and autoimmunity, although assessment of these mice has led to conflicting conclusions regarding the importance of Stat5 in Treg development.\textsuperscript{33-35} Interpretation of these studies is further complicated by the finding that
Stat5<sup>ΔN</sup> mice expressed truncated Stat5 proteins that are partially functional; as such these actually represent hypomorphic Stat5 alleles. As noted above, the residual function in Stat5<sup>ΔN</sup> mice was illustrated by comparing immune defects in these mice with those of a different model of Stat5a/b deletion in which both Stat5a and Stat5b were completely deleted. However, the role of Stat5 in regulating Foxp3 was not assessed, and other studies have argued for a role of Stat3 in regulating Foxp3. This suggests that Stat3 and Stat5 may play redundant roles in regulating this transcription factor. These findings prompted reassessment of the roles of Stat5 and Stat3 in Treg cell development and maintenance with focus on their effects on Foxp3 expression.

In this report, we compared and contrasted Treg cell development in Stat5<sup>ΔN</sup> mice and Stat5a/b<sup>−/−</sup> (termed Stat5<sup>−/−</sup>) mice. Stat5 was demonstrated to be critical for both Treg cell development and maintenance and critical for Foxp3 expression. The intrinsic requirement for Stat5a/b in Treg cells was evident through the use of tissue specific Stat5 deletion and stem cell transplantation experiments. Moreover, Stat5 directly binds the foxp3 gene. In contrast, reduction of Stat3 in T cells did not reduce the numbers of Treg cells in the thymus or spleen. However, the ability of IL6 to downregulate Foxp3 expression was greatly attenuated in Stat3-deleted T cells. Thus, Stat5a/b have an essential and direct positive role in regulating Foxp3 and Treg cells. Although Stat3 is not essential for the development or maintenance of Treg cells, it does appear to have an important role in mediating IL-6 signals to attenuate Foxp3 expression.
Materials and Methods

Mice
Stat5a/b−/− (Stat5−/−), Stat5a/bfl−/−, CD4cre (Stat5fl−/− CD4cre), and Stat5∆N mice were described previously5−7 and housed at NIH under approved protocols. Stat5fl−/−, CD4cre, Yfp mice were generated by crossing Stat5fl−/−, CD4cre with Yfp indicator mice (ROSA26-stop-floxed-YFP reporter mice, Jackson Laboratory, Bar Harbor, Maine). Cre-mediated deletion was monitored by yellow fluorescent protein (YFP) expression from a ‘ROSA26-stop-floxed-YFP’ reporter. Stat3fl/fl mice were bred with mice expressing Cre under the control of the MMTV (MMTV-Cre) to produce Stat3fl/fl, MMTV-Cre mice. CD45.1 congenic Rag2−/−, Jak3−/− and IL2rg−/− (Y) mice were obtained from Taconic Farms (Hudson, NY). Animals were handled and housed in accordance with the guidelines of the NIH Animal Care and Use Committee.

Antibodies
Biotin-, FITC-, PE-, Per-CP-, PE-Cy5.5- and APC-conjugated antibodies to mouse CD4, CD25, CD8, CD62L, CD44, CD122 were purchased from BD Biosciences (San Jose, CA). Mouse Foxp3 staining kit was purchased from eBioscience (San Diego, CA).

Cell preparation, DNA, RNA and protein expression analysis
CD4+ single positive (SP) thymocytes and splenocytes from Stat5fl/fl mice and YFP*CD4+ SP thymocytes and splenocytes from Stat5fl−/−, CD4cre, Yfp mice were sorted using a Moflo cell sorter (Dako Cytomation, Denmark). The purity of the sorted cells was >99%. These sorted cells were subjected to DNA, RNA and protein analysis essentially as described before5. In brief, DNA was purified using DNA easy kit from Qiagen (Valencia, CA). RNA was prepared with Trizol reagent according to the manufacturer’s protocol (Invitrogen, Carlsbad, California). For real-time PCR, cDNA was generated
using a first strand cDNA synthesis kit (Applied Biosystems, Foster City, CA). The primers and probes for real-time PCR were purchased from Applied Biosystems (Applied Biosystems, Foster City, CA). RT-PCR was performed on ABI PRISM 7700 (Applied Biosystems, Foster City, CA).

**Cell culture**

CD25 CD4+ splenic T cells from Stat5^{fl/fl} mice, YFP+CD25 CD4+ splenic T cells from Stat5^{flcre}, CD4cre, Yfp mice or Stat3^{fl/fl} mice and Stat3^{fl/fl}, MMTVcre mice were isolated by Moflo cell sorter and cultured for 3 days with plate-bound anti-CD3 (5 µg/ml) and anti-CD28 (5 µg/ml) (BD PharMingen, San Diego, CA) plus TGF-β1 (5 ng/ml, PeproTech Inc), hIL-2 (100 unit/ml, provided by NCI-Frederick) and IL-6 (10 ng/ml, PeproTech Inc., Rocky Hill, NJ) as indicated, with or without anti-murine IL-2 antibody (20 µg/ml, R&D Systems, Minneapolis, MN) in complete RPMI 1640 medium containing 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin and 2 mM β-mercaptoethanol.

**Chromatin immunoprecipitation**

Chromatin immunoprecipitation assays were performed as previously described.38 CD4+CD25+ and CD4+CD25− cells were sorted from thymi and spleens and stimulated with IL-2 (100 U/ml) for 1h. Formaldehyde (final concentration 1%) was then added to crosslink proteins and DNA. The cell lysates were sonicated, and immunoprecipitated with normal rabbit serum (Upstate Biotechnology, Charlottesville, VA), α-Stat5 (R&D systems, Minneapolis, MN) and α-Stat3 (Santa Cruz). The immunoprecipitated DNA was eluted and amplified by real time PCR using an ABI 7700 (Applied Biosystems, Foster City, CA). Values were normalized to corresponding input control and are expressed as fold enrichment relative to normal rabbit serum for each experiment. The sequences
specific primers and probes used for amplification of the \textit{foxp3} gene surrounding putative Stat binding sites were as follows: site in I - 5’-CCTCCTGGGAAACCTGTGTCAC-3’, 5’-AACTTGTCAGAGAGGGCA-3’, 5’-6FAM-TACCCCTCATTTCCTTATC-3’; site in II - 5’-CTTCTGGGAGCCAGCCATT-3’, 5’-GCTGTACTCCCCACAAATT-3’, 5’-6FAM-TGAGACTCTCTGTATTCTGT-3’; sites in III - 5’-ACAACAGGGCCCCAGATGATA-3’, 5’-GGAGGTTGTTTCTGGGACATAGA-3’, 5’-6FAM-CCCAGATAGGAAAACA-3’. The primers and probe used for irrelevant IV are 5’-CACCCAAAGGCTGGAAGCCT-3’, 5’-CAGACGAGCCTCCACAGAGTT-3’, 5’-6FAM-CCGTGCCTTGTCAGG-3’.

**Stem cell transplants**

Single cell suspensions were generated from E14.5 \textit{Stat5}+/+ or \textit{Stat5}−/− fetal livers, and cells (2x10^6) were injected into tail veins of lethally irradiated (900 rads) Rag2−/− CD45.1 congenic recipient mice housed under pathogen-free conditions with acidified water as previously described.\textsuperscript{5} Seven to eight weeks later, tissues were harvested. Thymi and spleens were analyzed by flow cytometry for donor-derived CD45.2+ cells.
Results

Stat5a/b are critical for thymic development of regulatory T cells.

Previous studies employing Stat5<sup>∆N</sup> mice have reached conflicting conclusions regarding requirement for Stat5a/b in thymic Treg cell development, but because of the aforementioned limitations with this animal model we sought to revisit this issue. As shown in Figure 1, the proportion of CD4<sup>+</sup> CD25<sup>+</sup> cells in thymi from Stat5<sup>∆N</sup> mice was substantially reduced compared to wild type (WT) littermates (Figure 1A). This is consistent with the well-documented effect of Stat5 on CD25 expression. Additionally, the proportion of CD4<sup>+</sup> Foxp3<sup>+</sup> thymocytes in Stat5<sup>∆N</sup> mice was reduced (Figure 1A, lower panels). Furthermore, the level Foxp3 expression, as assessed by mean fluorescence intensity (MFI) was also reduced in Stat5<sup>∆N</sup> by approximately 20% compared to controls. The specificity of Foxp3 staining was documented by comparison with isotype control (supplementary figure1). As the cellularity of the thymus in adult Stat5<sup>∆N</sup> mice is roughly comparable to wild-type mice, the absolute numbers of Foxp3<sup>+</sup> CD4 single positive (SP) and CD25<sup>+</sup> CD4 SP thymocytes were also reduced. In interpreting data using Stat5<sup>∆N</sup> mice, one needs to bear in mind that truncated Stat5a/b proteins are expressed. However, Stat5<sup>−/−</sup> mice in which the entire Stat5a and Stat5b loci were deleted die perinatally, but a small numbers of mice (approximately 2%) survive for 6-8 weeks after birth. Stat5<sup>−/−</sup> viable mice exhibited marked reductions in the proportions and absolute numbers of CD25-and Foxp3- expressing CD4 SP thymocytes (Figure 1B). In fact, the reductions in CD25- and Foxp3 - expressing cells from Stat5<sup>−/−</sup> mice were comparable to that observed in Jak3<sup>−/−</sup> and IL2rg<sup>−/−</sup>(Y) mice (Figure 1C). The presence of Treg cells in Stat5<sup>∆N</sup> mice is likely due to the residual activity of the truncated Stat5 protein, rather than Stat5-independent development.
Stat5a/b are critical for peripheral Foxp3 expression and Treg cell maintenance

Previous studies using Stat5\textsuperscript{ΔN} mice noted a substantial reduction of CD25\textsuperscript{+}CD4\textsuperscript{+} T cells in the periphery; however, expression of Foxp3 was not examined\textsuperscript{33,34}. As shown in Figure 1D, the proportion of Foxp3-expressing CD4\textsuperscript{+} T cells was not reduced in 4-5 week old Stat5\textsuperscript{ΔN} mice, despite the dramatic reduction in CD25-expressing cells. Since the cellularity of spleens from Stat5\textsuperscript{ΔN} mice is comparable to that of wild-type mice at this young age,\textsuperscript{8} the absolute number of Foxp3\textsuperscript{+} Treg cells was normal in Stat5\textsuperscript{ΔN} mice, which might lead one to conclude that peripheral Foxp3 expression does not require Stat5a/b. In contrast though, the proportions of Foxp3-expressing and CD25-expressing splenic CD4 T cells were both markedly reduced in Stat5\textsuperscript{−/−} mice (Figure 1E). This is consistent with the notion that Stat5a/b are essential for Treg cell maintenance, and the expression of Foxp3 in Stat5\textsuperscript{ΔN} mice is the result of residual Stat5 expression. The loss of peripheral Foxp3 expression was again comparable to that seen in spleens from Jak3\textsuperscript{−/−} and Il2rg\textsuperscript{−/−} mice (Figure. 1F).

Intrinsic requirement for Stat5a/b but not Stat3 for Treg cells

While the data above indicate a requirement for Stat5a/b in Foxp3 expression in the thymus and periphery, Stat5a/b are lacking in all tissues from Stat5\textsuperscript{−/−} viable mice (data not shown), and an intrinsic requirement for Stat5a/b in T cells cannot be inferred. We therefore approached this problem in two ways: (1) stem cell transplantation using Stat5\textsuperscript{−/−} precursors and (2) tissue-specific deletion of Stat5 using transgenic expression of Cre.

Because Stat5 deficiency is usually lethal (approximately 98%) and affects multiple cell lineages and pathways, we first reconstituted irradiated Rag2\textsuperscript{−/−} recipient mice with Stat5\textsuperscript{−/−} fetal liver cells to analyze Treg cell development. As noted above, reconstitution with Stat5\textsuperscript{−/−} precursors is inefficient, due to defective hematopoietic/lymphoid stem cell
functions. Nonetheless, T cell development with production of SP thymocytes does occur as previously published and data not shown. However, no CD25+ or Foxp3+ cells were detected in the CD4+ population of thymocytes from transplanted mice (Figure 2A right panel). In contrast, these cells were readily detected when normal stem cells were used to transplant \textit{Rag2}−/− recipients (Figure 2A left panel). CD25+ or Foxp3+ CD4+ T cells were also absent in spleens of \textit{Rag2}−/− recipient mice transplanted with \textit{Stat5}−/− precursors (Figure 2B), consistent with what we observed in viable \textit{Stat5}−/− mice.

We also approached the issue of an intrinsic requirement for Stat5 by selectively reducing Stat5a/b levels in T cells by breeding \textit{Stat5}fl/− mice with \textit{CD4 cre} mice. This approach had advantages, but also had some significant limitations in that Stat5 levels were reduced but not totally absent. To monitor Cre-mediated deletion, we also introduced YFP into the mice by breeding \textit{Stat5}fl/−, \textit{CD4cre} mice with indicator mice in which the gene encoding yellow fluorescent protein was inserted into the Rosa locus (ROSA26-stop-floxed-YFP reporter mice). Flow cytometric analysis of thymocytes from 4-5 weeks old \textit{Stat5}fl/−, \textit{CD4cre}, Yfp+ mice showed normal proportions of CD4 and CD8 thymocytes. Cre-mediated gene deletion as assessed by YFP expression was apparent in 90% of CD4+CD8+ double positive (DP) thymocytes (data not shown), and in 98% of CD4+ single positive (SP) thymocytes (supplementary Figure 2A). In sorted YFP+ CD4 SP thymocytes, genomic deletion of the Stat5a/b loci was observed as previously published. In YFP+ CD4+ cells, the levels of Stat5a/b mRNA were reduced to approximately 10% and the levels of Stat5 protein were reduced to 20-30% (supplementary Figure 2B, 2C); not surprisingly, in YFP− cells, the levels of Stat5 were even greater. Thus, the presence of residual Stat5 protein is a caveat that needs to be considered in interpreting experiments using these cells.
Examination of thymi from these mice revealed that the percentages of Foxp3- and CD25-expressing CD4 SP thymocytes were reduced by about 50% compared to Stat5$^{fl/fl}$ or Stat5$^{fl/-}$ littermates (5.18% and 5.65% versus 2.52% and 2.81% for Foxp3$^+$CD4$^+$ and CD25$^+$CD4$^+$ cells respectively, Figure 3A, 3C). The absolute numbers of Foxp3$^+$CD4 SP thymocytes were also reduced by 50% compared to WT (Figure 3E), since the numbers of total thymocytes and proportions of CD4 SP T cells were normal in Stat5$^{fl/-}$, CD4cre, Yfp mice (Figure 3D and 3B). In the spleen, 97% of the T cells were YFP positive, and Stat5 protein levels were reduced by approximately 70% (supplementary Figure 2A and 2C). Flow cytometric analysis of the splenic CD4$^+$ T cells showed that the percentage of CD25$^+$CD4$^+$ T cells was reduced (Figure 4A) and the proportion of Foxp3-expressing cells from Stat5$^{fl/-}$, CD4cre, Yfp mice was modestly and consistently reduced compared to that in Stat5$^{fl/fl}$ or Stat5$^{fl/-}$ mice (Figure 4A, 4C). The reduction in CD25-expressing cells was more dramatic than that of Foxp3-expressing cells, and in fact, the majority of the Foxp3-expressing CD4$^+$ T cells from Stat5$^{fl/-}$, CD4cre, Yfp mice were CD25$^{dim}$ (Figure 4A, bottom panel). The total numbers of splenic CD4$^+$ T cells in Stat5$^{fl/-}$, CD4cre, Yfp mice were roughly half of that seen in control mice (Figure 4B, 4D); as a result, the absolute number of Foxp3$^+$CD4$^+$ T cells in Stat5$^{fl/-}$, CD4cre, Yfp mice was reduced by approximately 60% (Figure 4E). These results contrast with the near absence of Treg cells found in Stat5$^{-/-}$ mice. While this could be interpreted to suggest that extrinsic Stat5 expression contributes to the loss of Treg cells, in view of the stem cell transplant experiments, we would argue that the modest reductions of Treg cells in Stat5$^{fl/-}$, CD4cre, Yfp mice are more likely due to persistence of Stat5 expression in this model of tissue-specific deletion. Thus, these results further demonstrate the importance of Stat5 in both Treg cell development and maintenance. Consistent with the reduction in Foxp3$^+$CD4$^+$ T cells, we noted that systemic autoimmune disease was evident in Stat5$^{fl/-}$, CD4cre, Yfp mice (supplementary Figure 3), similar to what has been observed in Stat5$^{dn}$ mice.33
Recent studies using transient transfection have suggested that Stat3 and Stat5a/b may both positively regulate Foxp3. The reduction in Foxp3+ CD4+ T cells in the thymus and periphery of Stat5−/− mice argues against an essential role for Stat3 in regulating Foxp3 under normal circumstances. Nonetheless, it was important to formally document whether Stat3 was a significant contributor to the regulation of Foxp3. In Stat3fl/fl, MMTVCre mice, Stat3 mRNA levels were reduced to approximately 16% of normal levels in CD4+ T cells (supplementary Figure 4A). Examination of thymi and spleens from these mice revealed that the proportion and absolute numbers of Foxp3+ CD4+ T cells were normal (Figure 5A, 5B, 5C, 5D).

**Stat5 and Stat3 have opposing effects on cytokine-dependent FoxP3 regulation**

Despite the persistence of residual Stat5 protein expression in Stat5fl−/, CD4cre, Yfp mice, this system was more amenable to analyzing T cells with reduced Stat5 levels. Previous studies have shown that Foxp3 can be induced in vitro by addition of exogenous TGF-β1. We therefore assessed the in vitro induction of Foxp3 in YFP+ CD25− CD4+ T cells from Stat5fl−/, CD4cre, Yfp mice. As shown in Figure 6A, CD25− CD4+ T cells expressed little Foxp3 prior to stimulation. Stimulation with anti-CD3, anti-CD28 and TGF-β1 was a potent inducer of Foxp3 in wild type cells (Figure 6A panel 3). When endogenous IL-2 production was neutralized with anti-murine IL-2 antibody, the percentage of Foxp3+ CD4+ T cells induced by TGF-β1 was markedly reduced (Figure 6 panel 4). Conversely, addition of exogenous human IL-2 enhanced the generation of Foxp3+ CD4+ T cells (Figure 6 panel 5). However, under all conditions, the proportion of Foxp3+ cells was markedly reduced when cells from Stat5 fl−/, CD4cre, Yfp mice were used, and few Foxp3 expressing cells were induced even with addition of exogenous IL-2. Moreover, the level of induction of Foxp3 indicated by MFI was also lower, only 35% of that in wild type cells, even under optimal conditions (Figure 6A panel 3 and 5). Thus, despite the limitations of residual, low-level Stat5 expression in T cells from Stat5 fl−/,
CD4cre, Yfp mice, this system clearly supports the importance of Stat5a/b in Foxp3 regulation.

TGF-β1 in combination with IL-2 promotes in vitro differentiation of Treg cells and enhances Foxp3 expression, whereas IL-6 inhibits Foxp3 expression. To determine the role of Stat3 in mediating this effect of IL-6, we next analyzed Treg cell differentiation in CD25 CD4+ cells from Stat3fl/fl, MMTVcre mice. As shown in Figure 6B, Foxp3 levels were induced to comparable levels in CD25 CD4+ T cells from Stat3fl/fl, MMTVcre mice and wild type mice. However, when IL-6 was added to cultures of wild type T cells, the induction of Foxp3 was blocked, consistent with previously published data. In contrast, addition of IL-6 to cultures of T cells from Stat3fl/fl, MMTVcre mice failed to block the upregulation of Foxp3 (Figure 6B and supplementary figure 4). We interpret these data to indicate that Stat5a/b are critical in vivo and in vitro for enhancing Foxp3 expression. In contrast, Stat3 is not required for induction of Foxp3; however, it does appear to be critical for mediating IL-6-dependent negative regulation of this key transcription factor.

**Foxp3 is a direct target of Stat5**

The present data suggest that Stat5a/b are critical for promoting Foxp3 expression in vivo and in vitro, likely through their role in mediating IL-2 signals, but it was possible that actions of Stat5a/b may not be direct. However, multiple consensus Stat binding sites are present in the mouse foxp3 gene promoter region (I, II) and the first intron (III). The most highly conserved sites are in the first intron (Figure 7). Stat5 binding to the native foxp3 gene in murine primary cells was assessed using sorted CD25+CD4+ and CD25− CD4+ SP thymocytes and chromatin immunoprecipitation with anti-Stat5 antibodies. Subsequent real-time PCR amplification of the foxp3 gene surrounding three putative Stat binding sites showed significant IL-2-inducible Stat5 binding. As a control, we also
assessed a segment that does not contain consensus Stat binding sites but found that this segment was not amplified with anti-Stat5 immunoprecipitation (Figure 7C). Stat5 binding to the foxp3 gene was also not detected in CD25 CD4+ SP cells Stat5 binding but was demonstrable in CD25+CD4+ splenocytes (data not shown). These data suggest that Stat5 may play a direct role in regulating foxp3 transcription.

Because IL-6 mediated inhibition of Foxp3 expression was Stat3 dependent, we also assessed whether we could also detect direct binding of Stat3 to the foxp3 locus. However, IL-6-dependent binding of Stat3 to the regions of foxp3 gene to which Stat5 binds was marginal (supplementary figure 4C). That is, in contrast to Stat5 which enriched the binding regions by more than 20 fold, the same regions were enriched by less than 4 fold with anti-Stat3 antibody compared to normal rabbit serum. This is of interest because the binding sites for Stat3 and Stat5 binding are thought to be quite similar44. It is notable therefore that Stat5 evidently binds the foxp3 gene well, whereas Stat3 binds this locus poorly despite the clear Stat3-dependent functional effects on Foxp3 in T cells.
Discussion

In this study, we explored the role of Stat5a/b in regulating Foxp3 expression and Treg cells. Our data indicate that Stat5a/b are critical for both the development and maintenance of Treg cells. This appears to be an intrinsic requirement for Stat5a/b in Treg cells and is likely mediated through direct effects of Stat5a/b on the transcription of the foxp3 gene, which has multiple Stat binding sites.

Stat5a/b have long been recognized to mediate IL-2 signals, and the importance of IL-2 in Treg cell development and maintenance has been established by a number of approaches. IL-2 signaling not only leads to the activation of Jaks and Stats, but also Ras-MAPK and PI3K-AKT pathways. Jak3 and γc are essential for Treg cells, but previous studies using CD25 as a marker in Stat5ΔN mice led to the conclusion that Stat5 is required for peripheral Treg maintenance but not for Treg development. This would suggest that Stat5 might not be the key factor regulating Foxp3 expression or it was redundant with other factors. However, it is now clear that Stat5ΔN mice have residual Stat5 function that may support Foxp3+ CD4+ T cell development. Indeed, analysis of mice completely deficient in Stat5 showed that Foxp3+ cells were severely reduced (Figures 1 and 3) documenting the criticality of this transcription factor for Foxp3 expression. Of note, we found reduced but not absent Treg cells in both thymi and spleens of Stat5fl/-; CD4cre, Yfp mice, but like cells from Stat5ΔN mice, some residual Stat5 protein is also present in T cells from Stat5fl/-; CD4cre, Yfp mice. We interpret these data to suggest that low levels of Stat5 in Stat5ΔN and Stat5fl/-; CD4cre mice permit Foxp3 expression, even in circumstances where CD25 levels are dramatically reduced. We have found other circumstances where selective pressure for Stat5 expression allows for escape of cells in which the Stat5 genes are not deleted. We believe that this is simply a
limitation of this system and needs to be carefully considered in assessing phenotypes using Cre-mediated deletion.

Because of its role in binding IL-2, CD25 is a critical factor in regulating Foxp3. Thus it was possible that the importance of Stat5 for Treg cells was primarily due to its requirement in regulating CD25 expression rather than directly regulating Foxp3. However, we found that in vitro induction of Foxp3 was poor in Stat5-deficient cells, despite levels of IL-2 that would obviate the need for CD25. While regulation of CD25 may contribute to the poor expression of Foxp3, we believe our data also argue for a more direct role independent of effects on CD25 expression. Additionally, analysis of Il2γc and Il2ra(CD25)γc mice showed that the impairment of Foxp3 expression is less severe than what was observed in mice lacking Jak3, Il2rg or Stat5. This also suggests that other γc cytokines, which signal predominantly through Stat5, may also play a role in promoting Foxp3 expression and Treg cell development.

Previous studies have noted two consensus Stat-binding elements located in intron 1 of the human FOXP3 gene. Gain-of-function Stat5 and Stat3 alleles were also found to trans-activate a FOXP3 reporter construct in transient transfection assays. This led the authors to conclude that IL-2 induced Foxp3 expression is mediated through both Stat5 and Stat3, and both of the Stats might play positive roles in regulating Foxp3 expression. Similarly, constitutive Stat3 activation was observed in malignant T cells that expressed the NPM/ALK fusion protein. These T cells express IL-10, TGF-β and Foxp3 and have immunosuppressive properties reminiscent of Treg cells. The authors attributed this phenotype to constitutive activation of Stat3, but Stat5 is also constitutively activated in this setting. However, we found that reduction of Stat3 levels in T cells did not have a major effect on Foxp3 expression in Stat3M/T, MMTVcre mice, indicating that Stat3 and Stat5 do not have redundant roles in Foxp3 regulation in normal mice. Indeed, IL-6
dependent Stat3 activation was suggested to inhibit Treg development during allergic airway inflammation. Recently, it was shown that in vitro stimulation of CD4+ T cells with IL-6 and TGFβ-1 down-regulated Foxp3 expression. Our data demonstrate that the inhibitory effect of IL-6 on Foxp3 expression is dependent on Stat3 by using Stat3 deficient T cells.

In addition, our data clearly indicate that Stat5a/b bind to the murine foxp3 gene, implying a direct role of Stat5 in regulating Foxp3 transcription. In contrast, Stat3 binding to these sites in the foxp3 locus was marginal. Given that IL-6 inhibits Foxp3 expression in Stat3-dependent manner, Stat3 must either bind to the foxp3 locus at sites distinct from the Stat5 binding sites or it must act indirectly. Regardless, it is clear that Stat5a/b and Stat3 function distinctly in regulating Foxp3 and it will be important to elucidate precisely how they mediate cytokine effects. It will be of great interest to define how they interact with other transcription factors and co-activators and to determine their ability to influence epigenetic modifications of the foxp3 locus.
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Z.Y. was the principal participant designing and performing research, analyzed data and wrote the manuscript. Y.K., G. S., M. K., L.D. performed research and analyzed data. Y.K. and W.W. performed research and preparation of the manuscript. A.L., R.G. and L.H provided materials. E.S., R.M. analyzed data and participated in preparation of the manuscript. J. O. and C. W. were responsible for the overall study and wrote the manuscript.
References

Figure Legends

Figure 1. Stat5a/b are critical for the generation of thymic Foxp3+ CD4+ T cells and maintenance of peripheral Foxp3+ CD4 T+ cells.

CD25 and Foxp3 expression were analyzed by flow cytometry in CD4 SP thymocytes (A-C) and splenocytes (D-F) from 4-6 weeks old Stat5+/+ and Stat5∆N mice (A,D), Stat5-/- mice (B,E), Jak3-/- and ll2rg-/- mice (C, F).

Figure 2. Intrinsic requirement of Stat5a/b for generation of Foxp3+ CD4 T cells.

Irradiated C57BL/6 Rag2-/- CD45.1+ congenic mice were reconstituted with 2 x 10⁶ total fetal liver cells from CD45.2+ wild-type (left panels) or Stat5-/- (right panels) mice. Eight weeks after reconstitution, cell populations in the thymus (A) and spleen (B) were analyzed. Donor-derived CD4 SP T cells were analyzed for CD25 and Foxp3 expression.

Figure 3. Reduction of thymic Foxp3+ CD4+ T cells with tissue-specific diminution of Stat5a/b levels.

(A) CD25 and Foxp3 expression were assessed on sorted CD4 SP thymocytes from Stat5fl/fl mice and YFP+CD4 SP thymocytes from Stat5fl/−, CD4Cre, Yfp mice. The average proportion of CD4 SP thymocytes is shown in (B) and the mean percentage of Foxp3+ CD4+ T cells is depicted in (C). The average total numbers of thymocytes are depicted in (D), and the absolute numbers of Foxp3+ CD4+ T cells are shown in panel E. Means ± SE (n=6) are shown and statistical significance (p<0.01) as determined by Student’s t-test is depicted by an asterisk.
Figure 4. Reduction of peripheral Foxp3+ CD4+ T cells with tissue-specific decrease of Stat5a/b levels.

(A) CD25 and Foxp3 expression were analyzed in sorted CD4+ SP splenocytes from Stat5fl/fl mice and YFP+CD4+ SP splenocytes from Stat5fl/−, CD4Cre, Yfp mice. The average proportion of CD4+ SP splenocytes is shown in (B) and the mean percentage of Foxp3+ CD4+ T cells is depicted in (C). The average total numbers of splenocytes are depicted in (D), and the absolute numbers of Foxp3+ CD4 T cells are shown in (E). Means ± SE (n = 6) are shown (p<0.01).

Figure 5. Reduction of Stat3 levels in CD4 T cells has no effect on Foxp3 expression from both thymi and spleens.

(A) Foxp3 expression was analyzed in CD4 SP thymocytes by flow cytometry. (B)The mean proportion of CD4 SP thymocytes, the mean percentage of Foxp3+ CD4+ T cells, the average total numbers of thymocytes and the absolute numbers of Foxp3+ CD4 T cells in thymocytes are shown. (C) Foxp3 expression was analyzed in CD4+ splenocytes by flow cytometry. (D) The average proportion of CD4+ SP splenocytes, the mean percentage of Foxp3+ CD4+ T cells, the average total numbers of splenocytes and the absolute numbers of Foxp3+ CD4 T cells are shown. Means ± SE (n = 5) are shown.

Figure 6. Stat5a/b are important for the in vitro induction of Foxp3.

(A) Sorted splenic CD25− CD4+ T cells from Stat50/0 and YFP+ CD25− CD4+ T cells from Stat50/−, CD4Cre, Yfp+ mice were cultured with plate-bound anti-CD3 and anti-CD28 combined with or without TGFβ1, IL-2 (100 unit/ml) and/or anti-IL-2 antibody as indicated. This is a representative of 3 experiments. (B) Sorted splenic CD25-CD4+ T cells from Stat30/0 and Stat30/−, MMTVcre mice were cultured with plate-bound anti-CD3 and anti-CD28 combined with or without TGFβ1, IL-6 as indicated.
Figure 7. Stat5 binds the foxp3 gene

(A) Schematic of the mouse foxp3 gene. Vertical lines depict potential Stat binding sites in the first intron and the putative promoter (I, II, III). Site IV does not contain a Stat binding site and was used as a control. (B) Stat binding sites in the mouse foxp3 gene are underlined and aligned with sequences from other species. Site I is located between 006010267 and 006010275 in the mouse genome (http://genome.ucsc.edu/cgi-bin/hgGateway?hgsid=83436504&clade=vertebrate&org=Mouse&db=mm7). Site II is located between 006012112 and 006012120. Intronic sites designated III are located between 006017209 and 006017217, 006017406 and 006017414, 006017523 and 006017531. All sequences are from the sense strand; note that the previous Stat binding site sequences identified by Zorn et al 36 were from the anti-sense strand, although the same sites were interrogated in our analysis. (C) Sorted thymic CD25+CD4 SP (black bar) and CD25-CD4 SP (gray bar) T cells were treated with IL-2 for 1 hr. Proteins and DNA were cross-linked with formaldehyde, cells were lysed and DNA was sheared. Chromatin immunoprecipitation was performed using either normal rabbit serum or anti-Stat5 antibody. Quantification of immunoprecipitated DNA fragments by real time PCR using primers and probes for sites I, II, III and the irrelevant site IV. Values were normalized to corresponding input control and are expressed as fold enrichment relative to normal rabbit serum for each experiment.
Figure 1.
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Figure 6
Nonredundant roles for Stat5a/b in directly regulating Foxp3


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