# Chapter 5

# IL-2 Signals Maintain CD25$^+$CD4$^+$ Regulatory T Cell Homeostasis and Self-Tolerance

The work presented here is taken from:
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# 1. Introduction

CD25, the $\alpha$-chain of the IL-2R (IL-2R$\alpha$), defines a population of CD4$^+$ T cells that is present in the thymus and peripheral lymphoid organs and has the capacity to suppress the proliferation of conventional T cells and to block the development of autoimmune disease (Sakaguchi et al., 1995; Shevach et al., 2001). These cells are referred to as CD25$^+$CD4$^+$ regulatory T cells (T regs). CD25$^+$CD4$^+$ T regs are able to suppress systemic and tissue-specific, immune-mediated diseases and are thought to play a key role in maintaining self-olerance (Sakaguchi et al., 1995; Shevach et al., 2001). At the cellular level it has been shown that CD25$^+$CD4$^+$ T regs can block the proliferation and function of both CD4$^+$ and CD8$^+$ T cells (Piccirillo and Shevach, 2001; Sakaguchi et al., 1995; Shevach et al., 2001; Thornton and Shevach, 1998). A number of molecules have been implicated in CD25$^+$CD4$^+$ regulatory T cell function, most prominently cytokines such as IL-10 and the inhibitory surface receptor, CTLA-4 (Asseman et al., 1999; Nakamura et al., 2001; Powrie et al., 1996; Read et al., 2000; Takahashi et al., 2000).

As is the case for conventional T cell populations, CD25$^+$CD4$^+$ regulatory T cell development is dependent on the thymus. These cells start to appear in the peripheral lymphoid organs between days 3 and 10 of age in the mouse, suggesting that there is a burst of CD25$^+$CD4$^+$ T reg generation and dissemination that occurs during a defined developmental window (Asano et al., 1996; Itoh et al., 1999). It is becoming clear that Ag plays an important role in driving CD25$^+$CD4$^+$ regulatory T cell development. Recent studies have shown that TCR transgenic CD4$^+$ T cells can adopt regulatory cell phenotype and effector function when they encounter self-Ag in the thymus (Jordan et al., 2001; Jordan et al., 2000). Traditionally, Ag encounter in the thymus has been thought to induce the deletion of autoreactive T cells (Vonboehmer, 1990). The rules for when self-Ag triggers thymocytes to undergo apoptosis vs CD25$^+$CD4$^+$ T reg differentiation remain to be fully defined. However, consistent with the observation that self-Ag is a driving force in regulatory T cell development, peripheral populations of these cells are enriched for autoreactive cells (Romagnoli et al., 2002).

The generation and maintenance of CD25$^+$CD4$^+$ regulatory T cells also appears to be under the control of costimulatory molecules and growth factors. Genetic ablation of CD28 or B7 molecules that function as ligands for this receptor, leads to a marked decrease in CD25$^+$CD4$^+$ T reg numbers (Salomon et al., 2000). Although CD28 or B7 deficiency alone is not sufficient to promote autoimmunity, these mutations do accelerate disease in some mouse models of autoimmune disease (Salomon et al., 2000). Recent work has also shown that the CD40/CD40 ligand system is required to establish and maintain CD25$^+$CD4$^+$ T regs (Kumanogoh et al., 2001). The molecular mechanisms by which these costimulatory signals promote CD25$^+$CD4$^+$ T reg development remain unknown.

The seminal observation that the $\alpha$-chain of the IL-2R (Piccirillo and Shevach, 2001) is constitutively expressed on CD25$^+$CD4$^+$ T regs (Akashi et al., 1997; Asano et al., 1996; Hall et al., 1990; Wolf et al., 2001) suggested immediately that IL-2 might be an important growth factor for these cells. Recent studies have proven that functional $CD25^+CD4^+$ T regs are produced in the absence of IL-2, indicating that this cytokine is not required for the development of these cells (Almeida et al., 2002; Furtado et al., 2002). However, $CD25^+CD4^+$ T reg numbers are significantly reduced in the peripheral lymphoid organs of mice that lack IL-2 or an IL-2R compared with wild-type cells (Almeida et al., 2002; Furtado et al., 2002; Malek et al., 2002). Furthermore, adoptive transfer of wild-type $CD25^+CD4^+$ T regs can prevent the autoimmune disease and inflammatory bowel disease that spontaneously develop in the absence of IL-2 (Malek et al., 2002). These findings indicate that one mechanism by which IL-2 mediates its tolerogenic and immune-modulatory effects is by maintaining $CD25^+CD4^+$ T reg homeostasis.

The molecular basis of the IL-2 dependence of $CD25^+CD4^+$ T regs has not yet been addressed. In conventional T cells, the effects of IL-2 are elicited by at least two major signaling pathways. One leads to the activation of the serine/threonine kinase, AKT, and up-regulation of antiapoptotic molecules such as Bcl-2 and Bcl-$x_L$ and is required for T cell survival (Van Parijs et al., 1999b). The other leads to the activation of STAT5 and is required for T cell proliferation and differentiation; it may also stimulate the expression of antiapoptotic molecules (Moriggl et al., 1999a; Moriggl et al., 1999b; Van Parijs et al., 1999b). In this study we have investigated the roles of these IL-2 signaling pathways in $CD25^+CD4^+$ T reg biology. Using a variety of genetic approaches, we show that STAT5 activation is required to obtain normal numbers of $CD25^+CD4^+$ T regs and to prevent the development of autoimmunity, but that Bcl-2 expression is dispensable for these activities. Our findings uncover an essential function for STAT5 in CD25$^+$CD4$^+$ T reg homeostasis and the maintenance of self-tolerance.

## 2. Materials and Methods

### *Mice*

*Bcl-2* knockout, *Bcl-2* transgenic (strain 25), *IL-2* knockout, Janus kinase 3 (*Jak3*) knockout, and recombinase-activating gene 1 (*RAG1*) knockout mice (all on a C57BL/6 background) were purchased from The Jackson Laboratory (Bar Harbor, ME). *IL-2R$\beta$* knockout mice were a gift from Dr. Y. Refaeli (University of California, San Francisco, CA). *STAT5a/b* double knockout mice were generated by the laboratory of Dr. J. Ihle (St. Jude's Hospital, Nashville, TN), and were given to us by Dr. M. Socolovsky (Whitehead Institute, Cambridge, MA). We refer to these double knockout mice as STAT5 deficient in the text. To create *IL-2* knockout mice that constitutively expressed *Bcl-2* in T cells, *IL-2*$^{+/-}$ mice were bred with *Bcl-2* transgenic (Tg) mice to create *IL-2*$^{+/-}$x*Bcl-2* Tg mice. These were then interbred to obtain *IL-2*$^{-/-}$x*Bcl-2* Tg and control mice. Mice were genotyped using PCR protocols provided by The Jackson Laboratory. *STAT5a/b* heterozygous mice were bred for two generations with *RAG1*$^{-/-}$ mice to generate *Stat5a/b*$^{+/-}$x*RAG1*$^{-/-}$ and were then interbred to obtain *Stat5a/b*$^{-/-}$x*RAG1*$^{-/-}$ and control mice. All mice were housed and bred under specific pathogen-free conditions at Massachusetts Institute of Technology and were between 6 and 9 wk old when used in the experiments described here.

### *Abs and Flow Cytometry*

The following Abs used in this study were purchased from BD PharMingen (San Diego, CA): anti-CD4, -CD8a, -CD25, -CD62L, and -Bcl-2, as well as the Fc-blocking reagent anti-CD16/CD32. Magnetic beads against CD4, CD8, and B220 were purchased from Miltenyi Biotec (Auburn, CA).

For surface staining experiments, cells were blocked with anti-CD16/CD32 for 10 min on ice and washed once with PBS. After blocking, cells were stained with anti-CD4, anti-CD25, or anti-CD62L Abs for 20 min on ice and washed with PBS before analysis. Data were analyzed using CellQuest software (BD Biosciences, Mountain View, CA). Intracellular Bcl-2 was determined using the Cytofix/Cytoperm Plus Kit (with GolgiStop) from BD PharMingen (San Diego, CA) according to the protocol provided by the company.

### *T Reg Purification and Functional Assays*

$CD25^+$ and $CD25^-$ $CD4^+$ cell populations were purified using a two-step protocol. Lymph node and spleen cell suspensions were enriched for $CD4^+$ T cells by incubating with magnetic anti-CD8a, -CD11b, -CD11c, and -B220 microbeads (Miltenyi Biotec) and passing them through an AUTO-MACS magnetic column (Miltenyi Biotec). The resulting population was stained for CD4 and CD25. $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ populations were then sorted using a MoFlo cell sorter (Cytomation, Fort Collins, CO). The resulting populations were >95% enriched for the desired cell type.

To measure regulatory T cell activity, increasing numbers of $CD25^+CD4^+$ cells were cultured with $1 \times 10^5$ wild-type $CD25^-CD4^+$ cells in the presence of 1 μg/ml anti-CD3 and $2 \times 10^5$ irradiated syngeneic spleen cells in a 200-μl volume. Proliferation was assayed after 72 h of culture by $[^3H]$thymidine incorporation. In adoptive transfer experiments, between $2 \times 10^5$ and $5 \times 10^5$ T regs purified from wild-type C57BL/6 mice were injected i.p. into 3-day-old *STAT5a/b*$^{+/-}$ and *STAT5a/b*$^{-/-}$ mice. These mice were analyzed between 8 and 12 wk of age.

### *Generation of Inducible STAT5 Transgenic Mice*

A conditional allele of *STAT5* (STAT5ER) was created by fusing a constitutively active allele of *STAT5* (STAT5CA) (Onishi et al., 1998) in-frame with a mutated version of the human estrogen receptor, a gift from G. Evan (University of California, San Francisco, CA). *STAT5ER* was subsequently introduced into a green fluorescence protein (GFP)-expressing bicistronic retroviral vector (MIG) (Van Parijs et al., 1999b). Both control (MIG) and *STAT5ER*-expressing retroviruses were generated by transient transfection of 293.T cells as described previously (Van Parijs et al., 1999b). To test the activity of STAT5ER, we infected activated $CD4^+$ T cells with MIG, MIG-STAT5ER, or MIG-STAT5CA, a retrovirus that expresses a constitutively active form of *STAT5* (Van Parijs et al., 1999b). Infected T cells were cultured for 48 h in the presence or the absence of 100 nM 4-hydroxytamoxifen (OHT; Sigma-Aldrich, St. Louis, MO) (Kelly et al., 2002). Active STAT5 was detected by gel-shift assay (data not shown) (Van Parijs et al., 1999b). STAT5 transcriptional activity was detected by assaying *cis* levels by Western blot (data not shown) (Moriggl et al., 1999b). STAT5 biological activity was assayed by measuring proliferation by [$^3$H]thymidine incorporation (Kelly et al., 2002).

To generate inducible STAT5 mice, bone marrow cells derived from cohorts of 5–10 wild-type or *IL-2*-deficient mice treated with 5 mg of 5-fluorouracil were cultured in the presence of IL-3 (20 ng/ml), IL-6 (50 ng/ml), and SCF (50 ng/ml) and infected with retrovirus. The efficiency of bone marrow infection in the three separate experiments performed was 34, 59, and 48% for MIG and 43, 69, and 31% for STAT5ER. Infected bone marrow cells were used to reconstitute the immune systems of 6-wk-old lethally irradiated (1200 rad in two doses separated by 4 h) female C57BL/6 mice. Bone marrow chimeras were used in experiments at least 8 wk after injection to allow full reconstitution of the immune system. To induce STAT5 activity, mice were treated every 2–3 days with 1 mg of OHT. OHT was dissolved in ethanol to produce a 100 mg/ml solution, which was then diluted to 10 mg/ml in autoclaved sunflower oil, followed by 30 min of sonication (Metzger and Chambon, 2001). This emulsion was introduced by i.p. injection. In the first two experiments we injected mice seven times over a 21-day period, and in the third experiment we injected the mice eight times over a 16-day period. Longer treatments were associated with excessive toxicity (data not shown).

### *Statistical Analysis*

Statistical analysis was performed using one-sided, unpaired *t* test, and $p < 0.05$ was considered significant.

## 3. Results

### *IL-2 Signals Maintain CD25$^+$CD4$^+$ T Reg Homeostasis and Self-Tolerance*

Previous studies have shown that IL-2 maintains self tolerance by increasing the numbers of CD25$^+$CD4$^+$ T regs present in peripheral lymphoid organs (Hall et al., 1990; Papiernik et al., 1998; Sakaguchi et al., 1995; Shevach et al., 2001; Wolf et al., 2001). The goal of our study was to define the signaling pathways responsible for this function of IL-2. We started by testing whether IL-2 signaling was necessary to obtain normal CD25$^+$CD4$^+$ T reg numbers. IL-2, like other cytokines that exert their activities by binding to receptors that use the common γ-chain, is dependent on the Jak3 kinase to initiate signals (Thomis and Berg, 1997). We, therefore, examined whether mice that were deficient in Jak3 showed defects in the CD25$^+$CD4$^+$ T reg compartment and in their ability to maintain self-tolerance. Consistent with an essential role for IL-2 signaling in this process, we found that the frequency of CD25$^+$CD4$^+$ T regs in the spleen of *Jak3* knockout mice was similar to that in *IL-2* and *IL-2R*β knockout mice and was reduced compared with that in wild-type mice (Figures 5-1*A* and 5-2*A*). Furthermore, *Jak3* knockout mice exhibited symptoms of autoimmunity, including the accumulation of activated CD4$^+$ T cells that were enriched for autoreactive cells (Figures 5-1*B* and 5-2*B*) (Thomis et al., 1995). These findings indicated that Jak3 signals were required to obtain normal numbers of CD25$^+$CD4$^+$ T regs in peripheral lymphoid organs and to maintain self-tolerance.

### *Bcl-2 Is Not a Target of IL-2 Signals that Establish Normal CD25$^+$CD4$^+$ T Reg Numbers in the Periphery and Maintain Self-Tolerance*

Since Jak3 is used by many different cytokines to induce signals that regulate the development and function of multiple types of immune cells, our next goal was to define IL-2-specific signals that were necessary to maintain CD25$^+$CD4$^+$ T reg homeostasis. Since other members of the IL-2 cytokine family, specifically IL-7 and IL-15, had previously been shown to promote the development and maintenance of T cell populations by up-regulating Bcl-2 (Akashi et al., 1997; Maraskovsky et al., 1997; Wu et al., 2002), we tested whether induction of this anti-apoptotic molecule by IL-2 was required to establish a normal CD25$^+$CD4$^+$ T reg compartment. To accomplish this we created a strain of IL-2-deficient mice that constitutively expressed Bcl-2 in T cells as a transgene. In these experiments we used a Bcl-2 transgene that had previously been shown to rescue the development of T cells in IL-7R-deficient mice (Maraskovsky et al., 1997). We found that the numbers of CD25$^+$CD4$^+$ T regs present in the spleen of IL-2-deficient mice were not increased by Bcl-2 expression, nor did expression of this molecule prevent the accumulation of activated CD4$^+$ T cells seen in the absence of IL-2 (Figure 5-3A) or affect the onset and severity of the autoimmune disease (Figure 5-3, B and C). Indeed, when we compared expression of Bcl-2 in mature CD25$^+$CD4$^+$ T regs from wild-type and IL-2-deficient mice by intracellular staining with fluorescent Abs, we detected comparable levels of this protein in both cell populations (Figure 5-3A).

Further supporting the idea that Bcl-2 was not involved in establishing the $CD25^+CD4^+$ T reg compartment or maintaining self tolerance, we found that *Bcl-2* knockout mice with intact IL-2 activity had normal numbers of $CD25^+CD4^+$ T regs in the periphery (Figure 5-2*A* and Table 5-1) and exhibited no signs of autoimmunity (Figures. 5-2*B* and 5-3*C*).

### ***$CD25^+CD4^+$ T Reg Numbers and Function Are Defective in STAT5-Deficient Mice, Leading to Deregulated Lymphoid Homeostasis***

Having excluded a role for Bcl-2 as a downstream target of IL-2 signals that regulate the $CD25^+CD4^+$ T reg compartment, we next examined whether activation of STAT5 was required in this process. First, we determined the numbers of $CD25^+CD4^+$ T regs that were present in the spleens of *STAT5*-deficient mice and found that these were significantly reduced compared with wild-type controls (Figure 5-2*A* and Table 5-1). In the thymus, however, *STAT5* knockout mice did not have statistically lower numbers of $CD25^+CD4^+$ T regs than those found in littermate controls (Table 1). We had previously seen this pattern of normal thymic T reg levels but reduced numbers of peripheral T regs in *IL-2* and *IL-2Rβ*-deficient mice. Our findings suggested that STAT5 might be a key target of IL-2 signals that control $CD25^+CD4^+$ T reg homeostasis.

We and others have noted that *STAT5*-deficient mice, like *IL-2*- and *IL-2Rβ*-deficient mice, exhibited defects in lymphoid homeostasis, reflected by the accumulation of activated $CD4^+$ T cells and the development of splenomegaly (Figure 5-4, *A* and *B*) (Teglund et al., 1998). These symptoms may arise due to a defect in the $CD25^+CD4^+$ T reg compartment, although previous studies have also provided compelling evidence that the accumulation of cells in the spleen of *STAT5*-deficient mice might arise from a defect in erythropoiesis (Socolovsky et al., 1999; Socolovsky et al., 2001). To directly test the contribution of the lymphoid compartment to the splenomegaly that develops in *STAT5*- and *IL-2*-deficient mice, we bred mice that lacked these genes with mice that carried a null allele of *RAG1*, which prevents the generation of B and T cells (Mombaerts et al., 1992; Spanopoulou et al., 1994). We found that in the absence of lymphocytes, both *IL-2* and *STAT5* knockout mice had similar numbers of splenocytes compared with wild-type (*RAG1*-deficient) mice (Figure 5-4*B*). The laboratory of Dr. J. Ihle has reported similar results in a recent review article (Ihle, 2001).

To formally test whether the deregulation of lymphoid homeostasis seen in *STAT5*-deficient mice was the result of a defect in regulatory T cells, we injected between 0.2 and 0.5 x $10^6$ wild-type $CD25^+CD4^+$ T regs into neonatal *STAT5* knockout mice and monitored the development of disease in adult mice. In *IL-2R$\beta$*-deficient mice, this treatment has been found to result in the homeostatic expansion of the injected $CD25^+CD4^+$ T regs and to block the development of autoimmune disease (Malek et al., 2002). We found that adoptive transfer of wild-type $CD25^+CD4^+$ T regs into *STAT5*-deficient mice was sufficient to prevent the development of splenomegaly and the accumulation of activated T cells (Figure 5-4, *A* and *B*), suggesting that these disease symptoms arose due to a defect in the regulatory T cells compartment.

### ***STAT5 Activation Increases CD25$^+$CD4$^+$ T Reg Numbers in the Absence of IL-2***

The results of our experiments with *STAT5*-deficient mice were consistent with a role for this transcription factor as a target of IL-2 signals that regulate the T reg compartment. To test this directly, we examined whether STAT5 activation was sufficient to increase CD25$^+$CD4$^+$ T reg numbers in the absence of IL-2. To accomplish this we introduced an active form of *STAT5* (Onishi et al., 1998) together with GFP as a marker gene in bone marrow stem cells derived from *IL-2*-deficient mice using a retrovirus-based expression vector (Van Parijs et al., 1999b). These cells were used to reconstitute the immune system of lethally irradiated recipient mice (Van Parijs et al., 1999a).

Complicating these experiments, we found that constitutive expression of an active form of *STAT5* in hemopoietic stem cells resulted in the development of tumors in reconstituted mice (data not shown). To overcome this problem, we engineered an inducible form of STAT5 by fusing an active allele of this molecule with a modified version of the binding domain of the estrogen receptor (Littlewood et al., 1995). This conditional allele of *STAT5* (STAT5ER) was only active in the presence of OHT, an estrogen analog (Littlewood et al., 1995) (Figure 5-5*A*), and allowed us to create *IL-2*-deficient mice in which we could activate STAT5 transiently. We found that the numbers of CD25$^+$CD4$^+$ cells found in the spleen of chimeric mice was significantly increased after 2 wk of STAT5 activation (Figure 5-5*B*), and that these cells possessed regulatory activity (Figure 5-5*C*). This effect of STAT5 was cell intrinsic, since we only observed an increase in

$GFP^+$ (retrovirus-infected) $CD25^+CD4^+$ T regs in these experiments (Figure 5-5*B*). Thus, activation of STAT5 was sufficient to promote $CD25^+CD4^+$ T reg numbers in the absence of IL-2. Not all $CD4^+$ T cells adopted a T reg fate upon STAT5 activation (Figure 5-5*B*), consistent with the idea that other factors play important roles in T reg development and the maintenance of these cells (Fontenot et al., 2003; Khattri et al., 2003).

## 4. Discussion

Recent studies have established that IL-2 functions to maintain $CD25^+CD4^+$ T reg homeostasis (Almeida et al., 2002; Furtado et al., 2002; Malek et al., 2002). In this study we have used genetic approaches to investigate the contributions of two key IL-2 signaling molecules, Bcl-2 and STAT5, to this process. Our findings demonstrate that $CD25^+CD4^+$ T reg homeostasis is not dependent on Bcl-2, but that STAT5 activation is required to establish normal T reg numbers in the peripheral lymphoid organs of mice. This function of STAT5 is necessary to prevent the accumulation of activated $CD4^+$ T cells and to block the development of splenomegaly. Our study identifies a key molecular component of the IL-2 signaling pathway that controls T reg homeostasis and the maintenance of self tolerance.

IL-2 and related cytokines, such as IL-7 and IL-15, activate signaling pathways that result in cellular proliferation, survival, and differentiation (Nelson and Willerford, 1998). Biochemical and genetic analysis of IL-2 signaling in T cell lines and primary T cells suggests that activation of STAT5 and the expression of c-Myc are required to induce proliferation, while up-regulation of Bcl-2 family molecules is required to promote survival (Nelson and Willerford, 1998). Both IL-7 and IL-15 have been implicated in the development and homeostasis of specific lymphocyte populations, namely immature T cells (and B cells) in the case of IL-7 (Peschon et al., 1994; Vonfreedenjeffry et al., 1995), and CD8$^+$ T cells in the case of IL-15 (Lodolce et al., 1998). The essential function of these cytokines appears to be to promote survival, since the expression of Bcl-2 is reduced in the affected T cell populations in the absence of cytokine, *Bcl-2* deficiency leads to a decrease specifically in CD8$^+$ T cell numbers, and ectopic *Bcl-2* expression is sufficient to rescue T cells in IL-7R-deficient mice (Akashi et al., 1997; Maraskovsky et al., 1997; Wu et al., 2002). Our results indicate that IL-2 does not appear to function in the same manner in CD25$^+$CD4$^+$ T regs. Bcl-2 is not necessary to obtain a normal CD25$^+$CD4$^+$ T reg compartment, and transgenic expression of *Bcl-2* does not rescue these cells in *IL-2*-deficient mice.

Instead, our study demonstrates that CD25$^+$CD4$^+$ T reg homeostasis is dependent on the activation of STAT5. *STAT5*-deficient mice show reduced numbers of these cells, and transient activation of STAT5 in *IL-2*-deficient mice increases the numbers of CD25$^+$CD4$^+$ T regs in the periphery. How STAT5 acts in CD25$^+$CD4$^+$ T regs remains to be determined. In conventional T cells, STAT5 is predominantly responsible for inducing proliferation (Moriggl et al., 1999a; Moriggl et al., 1999b). However, this transcription factor may also function during lymphocyte development and is necessary to obtain NK cells (Moriggl et al., 1999b). STAT5 has been reported to promote the survival of hemopoietic cells both by stimulating the expression of Bcl-2 family proteins and by triggering Bcl-2- and Bcl-x-independent survival pathways (Nelson and Willerford, 1998; Socolovsky et al., 1999). Our findings suggest that it is unlikely that STAT5 functions only to activate a survival signal in CD25$^+$CD4$^+$ T regs.

Recent gene expression profiling experiments have uncovered that STAT5 target genes, such as members of the SOCS family of proteins, SOCS1 and -3, and *cis*, are upregulated in CD25$^+$CD4$^+$ T regs compared with conventional T cells (McHugh et al., 2002). SOCS molecules function to inhibit the proliferative effects of cytokines and can antagonize STAT5 activity (McHugh et al., 2002; Moriggl et al., 1999a). Whether they also contribute to the anergic state that characterizes CD25$^+$CD4$^+$ T regs is not known. STAT5 activation is also necessary for high-level expression of CD25 on T cells (McHugh et al., 2002; Moriggl et al., 1999a). This raises the possibility that STAT5 may be activated by other cytokines, such as IL-7 (Lin and Leonard, 2000), and function to render CD25$^+$CD4$^+$ T regs responsive to IL-2. Our experiments demonstrate that activation of STAT5 in the absence of IL-2 leads to an increase in the number of CD25$^+$CD4$^+$ T cells with regulatory activity, but do not formally exclude an important role for STAT5 upstream of IL-2. Indeed, Tg mice bearing a mutant IL-2R $\beta$-chain that fails to activate STAT5 do not develop autoimmunity (Fujii et al., 1998). Although the status of CD25$^+$CD4$^+$ T regs was not assessed in these mice, this finding does suggest that STAT5 may also act downstream of cytokines other than IL-2 to regulate the CD25$^+$CD4$^+$ T reg compartment and T cell tolerance.

The cellular mechanisms by which IL-2 signals and STAT5 promote $CD25^+CD4^+$ T reg activity in the mouse remain to be established. In principal, they could act to promote the following events: 1) development of $CD25^+CD4^+$ T cells, 2) activity of $CD25^+CD4^+$ T cells, or 3) expansion and homeostasis of $CD25^+CD4^+$ T cells. Supporting a role for IL-2 signaling during the development of $CD25^+CD4^+$ T regs, a recent report demonstrates that the expression of IL-2R in the thymus is sufficient to obtain a normal $CD25^+CD4^+$ T reg compartment and to prevent autoimmune disease (Malek et al., 2002). However, functional $CD25^+CD4^+$ T regs have been detected in mice that lack IL-2 or a functional IL-2R, demonstrating that this cytokine is not essential for the development or activity of $CD25^+CD4^+$ T regs (Almeida et al., 2002; Furtado et al., 2002). Recent adoptive transfer experiments suggest that IL-2 may also promote the persistence of $CD25^+CD4^+$ T regs in peripheral lymphoid tissues. Future studies of the cellular consequences of IL-2 and STAT5 signaling in these cells should help determine how the $CD25^+CD4^+$ T reg compartment is established and maintained.

**Table 5-1**: CD25+CD4+ regulator T cell numbers in mouse strains with defects in IL-2 and key IL-2 signaling molecules[a]

| Genotype | No. of Splenic T Regs ($x10^{-6}$) | No. of Thymic T Regs ($x10^{-6}$) |
|---|---|---|
| Wild-type | 2.3 ± 1.0 (n=6) | 0.26 ± 0.02 (n=3) |
| IL-2 KO | 0.4 ± 0.2 (n=4) | 0.25 ± 0.06 (n=3) |
| STAT5 KO | 0.7 ± 0.5 (n=6) | 0.32 ± 0.08 (n=6) |
| Bcl-2 KO | 1.7 ± 0.5 (n=3) | N.D. |

[a]: The numbers of CD25+CD4+ T regs present in the spleen and thymus of 6- to 8 wk-old wild-type, IL-2 knockout (IL-2 KO), STAT5 knockout (STAT5 KO), and Bcl-2 knockout (Bcl-2 KO) C57BL/6 mice was determined by counting and by flow cytometry. Average CD25+CD4+ T reg numbers and SD from three to six mice are shown. N. D., not determined.

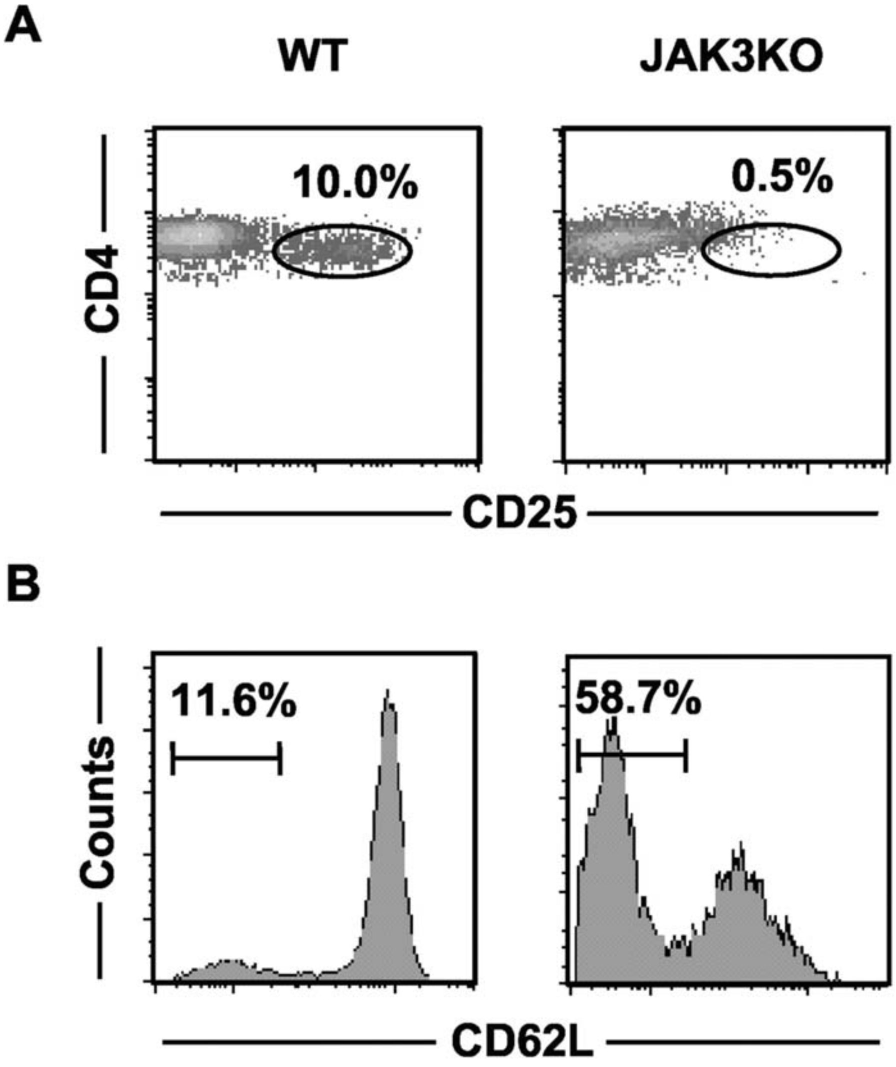

Figure 5-1

**Figure 5-1:** Jak3 is required to establish normal CD25$^+$CD4$^+$ T reg numbers in the periphery and to prevent the accumulation of activated CD4$^+$ T cells. The frequency of CD25$^+$CD4$^+$ T regs and activated CD4$^+$ T cells present in the spleen of wild-type (WT) and *Jak3* knockout (JAK3KO) mice ($n$ = 3) was assayed by staining and flow cytometry. In these experiments we used forward/side scatter profiles to identify live cells and gated on CD4$^+$ cells. (A) Frequency of CD25$^+$CD4$^+$ T regs. Spleen cells were stained with anti-CD4 and anti-CD25. The percentages indicate the fraction of CD4$^+$ T cells that were also CD25$^+$. (B) Frequency of activated CD4$^+$ T cells. Spleen cells were stained with anti-CD4 and anti-CD62L. Activated CD4$^+$ T cells were identified as CD4$^+$ CD62L$^{low}$.

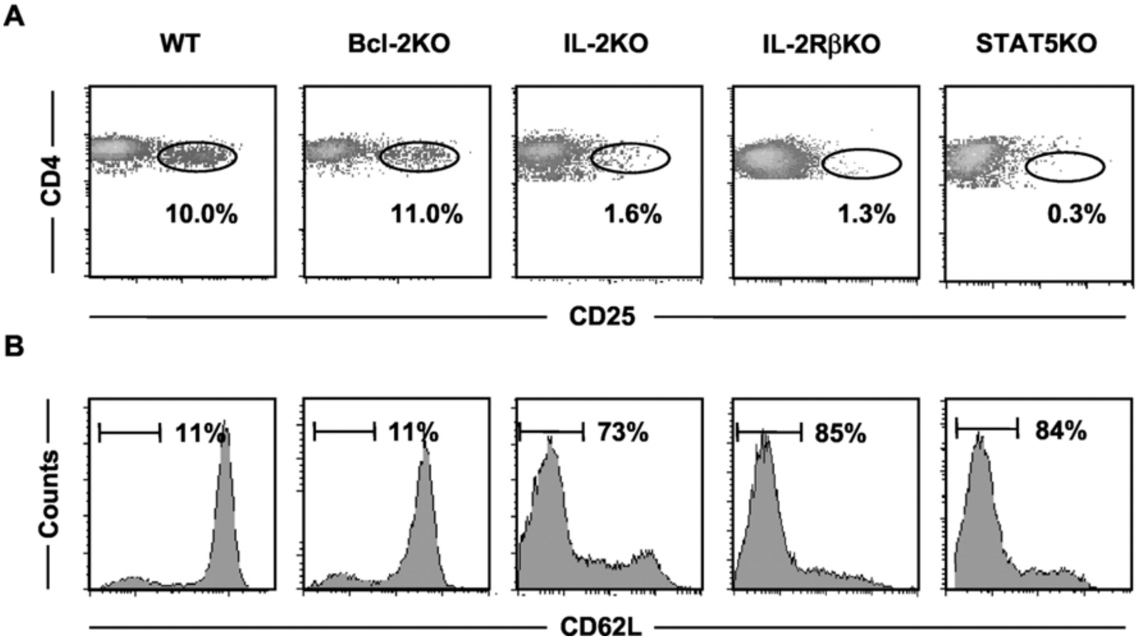
A
WT
Bcl-2KO
IL-2KO
IL-2RβKO
STAT5KO
CD4
10.0%
11.0%
1.6%
1.3%
0.3%
CD25
B
Counts
11%
11%
73%
85%
84%
CD62L

Figure 5-2

**Figure 5-2:** IL-2 and STAT5, but not Bcl-2, are required to establish normal CD25$^+$CD4$^+$ T reg numbers in the periphery and to prevent the accumulation of activated CD4$^+$ T cells. The frequency of CD25$^+$CD4$^+$ T regs and activated CD4$^+$ T cells present in the spleen of wild-type (WT; *n* = 25) *Bcl-2* knockout (Bcl-2 KO; *n* = 4), *IL-2* knockout (IL-2 KO; *n* = 25), *IL-2R$\beta$* knockout (IL-2R$\beta$ KO; *n* = 3), and *STAT5* knockout (STAT5 KO; *n* = 7) mice was assayed by staining and flow cytometry. In these experiments we used forward/side scatter profiles to identify live cells and gated on CD4$^+$ cells. (A) Frequency of CD25$^+$CD4$^+$ T regs. Spleen cells were stained with Abs against CD4 and CD25. The percentages indicate the fraction of CD4$^+$ T cells that were also CD25$^+$. (B) Frequency of activated CD4$^+$ T cells. Spleen cells were stained with Abs against CD4 and CD62L. Activated CD4$^+$ T cells were identified as CD4$^+$CD62L$^{low}$.

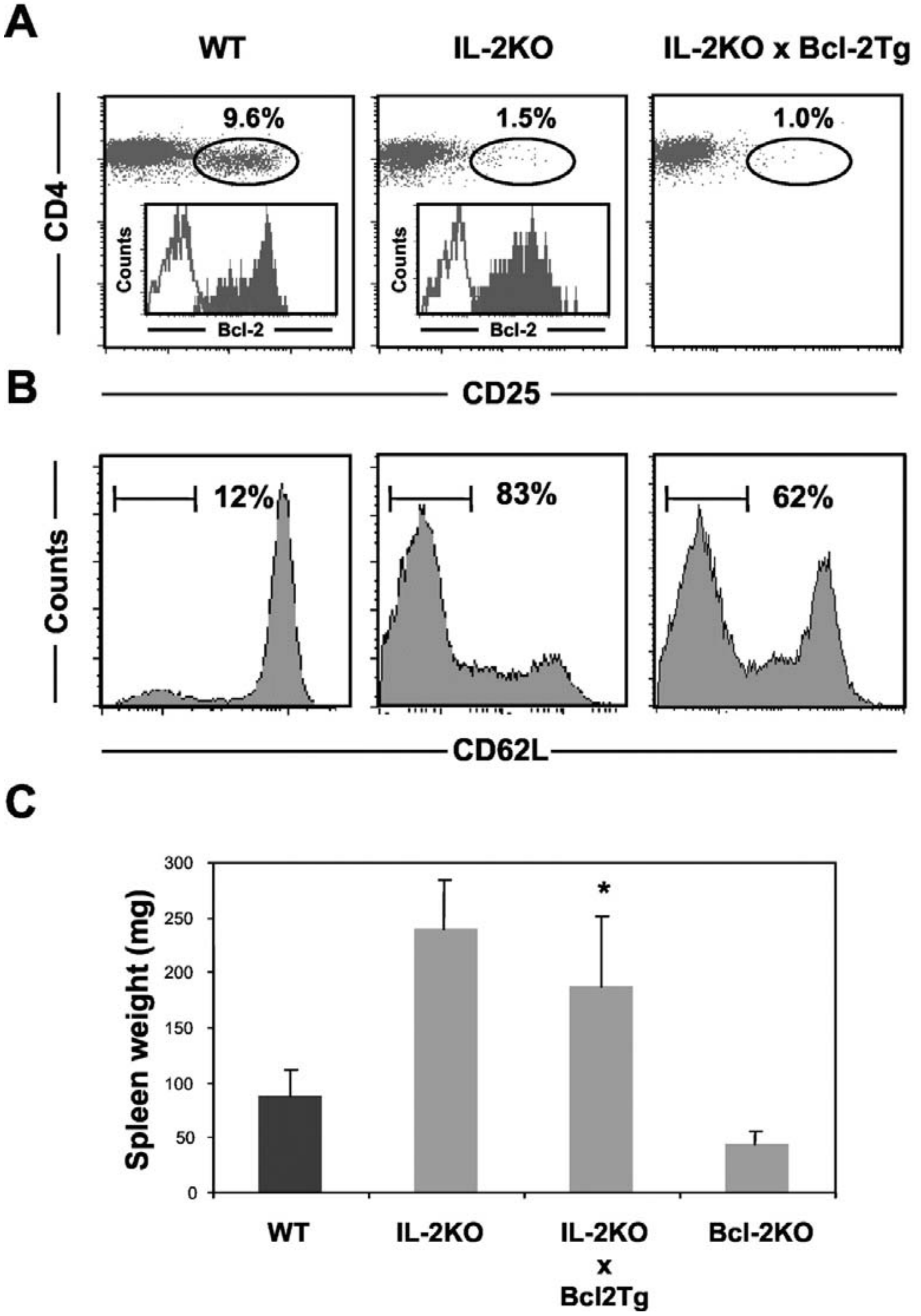
A
WT
IL-2KO
IL-2KO x Bcl-2Tg
CD4
9.6%
1.5%
1.0%
Counts
Bcl-2
Counts
Bcl-2
B
CD25
Counts
12%
83%
62%
CD62L
C
Spleen weight (mg)
300
250
200
150
100
50
0
*
WT
IL-2KO
IL-2KO
x
Bcl2Tg
Bcl-2KO

Figure 5-3

**Figure 5-3:** Tg expression of Bcl-2 does not rescue $CD25^+CD4^+$ T reg numbers or prevent the accumulation of activated $CD4^+$ T cells and the development of splenomegaly in *IL-2*-deficient mice. The frequency of $CD25^+CD4^+$ T regs present in the spleen of wild-type (WT; $n = 3$), *IL-2* knockout (IL-2 KO; $n = 3$), and *IL-2* knockout mice expressing a *Bcl-2* transgene (IL-2 KOxBcl-2Tg; $n = 3$) was assayed. In these experiments we used forward/side scatter profiles to identify live cells and gated on $CD4^+$ cells. (A) Frequency of $CD25^+CD4^+$ T regs. Spleen cells were stained with Abs against CD4 and CD25. The percentages indicate the fraction of $CD4^+$ T cells that were also $CD25^+$. (B) Frequency of activated $CD4^+$ T cells. Spleen cells were stained with Abs against CD4 and CD62L. Activated $CD4^+$ T cells were identified as $CD4^+CD62L^{low}$. (C) Development of splenomegaly. The average weight of spleens from three to five WT, IL-2KO, IL-2KOxBcl-2Tg, and *Bcl-2* knockout (Bcl-2KO) mice was determined. *, $p < 0.05$ vs WT control and $p > 0.1$ vs IL-2KO.

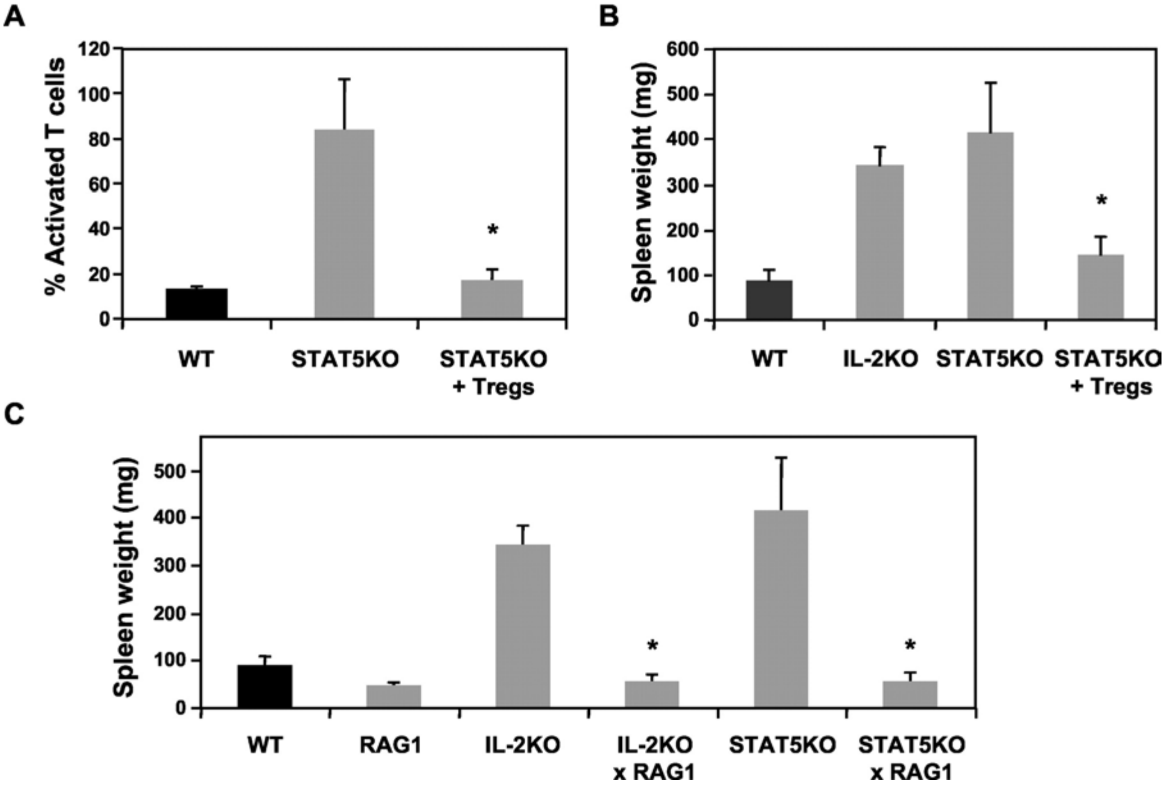
A
% Activated T cells
0
20
40
60
80
100
120
WT
STAT5KO
STAT5KO + Tregs
*
B
Spleen weight (mg)
0
100
200
300
400
500
600
WT
IL-2KO
STAT5KO
STAT5KO + Tregs
*
C
Spleen weight (mg)
0
100
200
300
400
500
WT
RAG1
IL-2KO
IL-2KO x RAG1
STAT5KO
STAT5KO x RAG1
*
*

**Figure 5-4**

**Figure 5-4:** Adoptive transfer of $CD25^+CD4^+$ T regs prevents the development of autoimmunity in *STAT5*-deficient mice. The development of autoimmune symptoms was assayed in adult wild-type (WT; *n* = 6), *STAT5* knockout (STAT5KO; *n* = 6), IL-2KO (*n* = 4), RAG1KO (*n* = 4), IL-2KOxRAG1KO (*n* = 4), STAT5KOxRAG1KO (*n* = 6), and *STAT5* knockout mice injected neonatally with wild-type $CD25^+CD4^+$ T regs (*n* = 6). (A) Frequency of activated T cells present in the spleens of WT, STAT5 KO, and STAT5KO injected with wild-type $CD25^+CD4^+$ T regs identified by flow cytometry as $CD4^+CD62L^{low}$ cells. (B) and (C) Spleen weight. *, $p < 0.001$ vs STAT5 KO (A and B), $p < 0.001$ vs STAT5KO or IL-2KO (C).

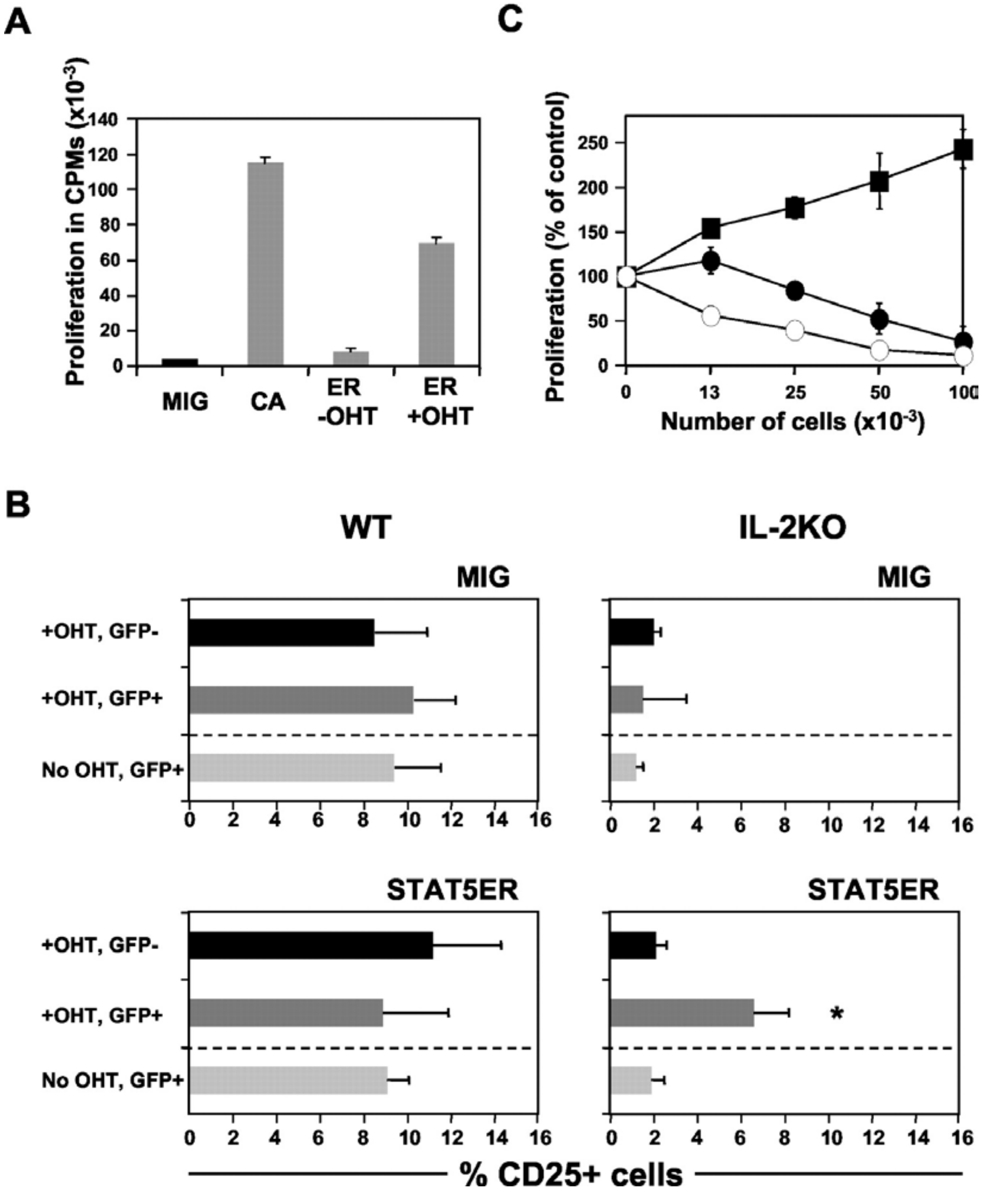
A
Proliferation in CPMs ($x10^{-3}$)
0
20
40
60
80
100
120
140
MIG
CA
ER -OHT
ER +OHT
C
Proliferation (% of control)
0
50
100
150
200
250
0
13
25
50
100
Number of cells ($x10^{-3}$)
B
WT
IL-2KO
MIG
STAT5ER
+OHT, GFP-
+OHT, GFP+
No OHT, GFP+
*
0
2
4
6
8
10
12
14
16
% CD25+ cells

Figure 5-5

**Figure 5-5:** Transient activation of STAT5 increases $CD25^+CD4^+$ T reg numbers in *IL-2*-deficient mice. The effect of STAT5 signaling on the development of $CD25^+CD4^+$ T regs in wild-type (WT) and *IL-2* knockout (IL-2KO) mice was assayed by expressing a conditionally active allele of STAT5 in hemopoietic cells of bone marrow chimeras using a retrovirus-based expression system. (A) Creation of a conditionally active allele of *STAT5*. Activated $CD4^+$ T cells from WT mice were infected with a retrovirus (MIG) engineered to express a constitutively active allele of *STAT5* (CA) or a conditionally active allele of *STAT5* (ER) and were cultured in the presence or the absence of OHT. Proliferation was assayed after 48 h by [$^3$H]thymidine incorporation. (B) Frequency of $CD25^+CD4^+$ T cells. WT or IL-2KO bone marrow chimeras expressing a control virus (MIG) or STAT5ER in 30–50% of all hemopoietic cells were treated for 2–3 wk with OHT or vehicle alone (No OHT). As a specificity control, we compared the frequencies of $CD25^+CD4^+$ T regs within the infected ($GFP^+$) and noninfected ($GFP^-$) $CD4^+$ T cell populations. Each data point represents the mean of a group of three mice ($n = 3$). (C) Regulatory activity of *STAT5*-expressing $CD25^+CD4^+$ T regs from *IL-2*-deficient mice. $CD4^+CD25^+GFP^+$ cells (○ and ●) or $CD4^+CD25^-GFP^+$ cells (■) were purified from the spleens of OHT-treated IL-2KO mice (○) or wild-type mice (■) and cultured at increasing cell numbers with 100,000 conventional $CD4^+$ ($CD25^-$) T cells in the presence of anti-CD3 and irradiated whole splenocytes. Proliferation was assayed after 72 h by [$^3$H]thymidine incorporation. *, $p < 0.025$ vs $GFP^-$ control.

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