Immune self-tolerance is controlled by a subset of T lymphocytes that are regulatory (Treg) and epigenetically programmed to suppress autoreactive immune effector cells in vivo. Treg require expression of Foxp3, a transcription factor that not only represses the interleukin-2 gene promoter, but also sequesters key mediators of T-cell signal transduction by complexing with cytoplasmic NFAT and NFκB. We have discovered that expression of Foxp3 is linked to two stem cell-related factors, namely leukemia inhibitory factor (LIF) and axotrophin. Because both LIF and axotrophin each influence Foxp3, we now ask if reciprocal cross-talk occurs; for example, does Foxp3 in turn influence LIF and/or axotrophin? We compared the effect of wt-Foxp3 versus mutant ΔE251-Foxp3, which lacks transcriptional activity, on transcript levels of axotrophin, LIF, and suppressor of cytokine signaling–3 (SOCS-3; a feedback inhibitor of LIF) in the Jurkat human T-cell line. Unexpectedly, a 50-fold increase in SOCS-3 transcripts occurred in the ΔE251-Foxp3 cells, coincident with a dramatic decrease in LIF transcription. This implies that, either directly or indirectly, transcription of SOCS-3 is negatively regulated by wt-Foxp3. Suppression of SOCS-3 by Foxp3 would support a model wherein Foxp3 promotes LIF signaling in Treg and is further evidence of reciprocity between Foxp3, LIF, and axotrophin.

Keywords: LIF, Foxp3, SOCS-3, Immune regulation.

(Transplantation 2007;84: S6–S11)
cytes (7), implying that axotrophin is a nodal regulator of T-cell fate determination via LIF.

**Foxp3 Is Linked to Axotrophin**

We next looked for a direct effect of axotrophin on Foxp3. Comparison of transcripts in thymi from male BALB/c mouse litters that were null, heterozygous, or wild-type for axotrophin revealed a reduction of Foxp3 in the absence of axotrophin (5). Because the axotrophin null phenotype differs from the Foxp3 null phenotype, it appears that reduced thymic Foxp3 transcription does not prevent sufficient numbers of Treg developing in the periphery for self-tolerance, possibly due to homeostatic expansion of Treg in the axotrophin null mouse.

**Foxp3 Is Linked to LIF**

We then probed the relationship between Foxp3 and LIF, exploiting recent discoveries in the molecular biology of Foxp3 function (9). Foxp3 belongs to the forkhead (Fox) family of transcriptional regulators in which the Fox sub-group is characterized by a divergent DNA-binding winged helix domain (10, 11). Foxp3 further diverges from other Fox family members, having a truncated N-terminal domain and truncated C-terminal domain beyond the forkhead (FKH) binding domain (9). Foxp3 protein is able to form heterodimers with NFAT and NFκB (12), whereas homodimerization of the Foxp3 protein is required for Foxp3’s DNA binding activity (9). We have recently been shown that deletion of glutamic acid (ΔE250 mouse; ΔE251 human) in the leucine zipper domain of Foxp3 impairs both homodimerization and the suppressive function of Foxp3 in T cells (13). Here we have compared the effect of wt-Foxp3 versus ΔE251-Foxp3 on transcription of LIF, SOCS-3, and axotrophin. SOCS-3 was included because it is an inducible LIF response gene required for feedback regulation of LIF signaling pathways (14). Constructs of wt-Foxp3 and mutant-Foxp3, each under a doxycyclin-inducible promoter, were transfected into the Jurkat human T-cell line; control experiments demonstrated that both the wt-Foxp3 and mutant-Foxp3 constructs show similar induction in response to doxycycline, and that this induction results in equivalent levels of each respective protein (i.e., wt-Foxp3 and mutant-Foxp3 proteins) in the Jurkat cells (13).

After Foxp3 induction, the transcript levels of Foxp3, LIF, SOCS-3, and axotrophin were measured by quantitative polymerase chain reaction (Q-PCR) as detailed in the legend to Figure 1. When wt-Foxp3 was induced, there was concurrent induction of SOCS-3, axotrophin, and LIF transcripts, with LIF and SOCS-3 being of similar magnitude, and with axotrophin plateauing after 12 hours (Fig. 1A–C). In marked contrast, when ΔE251-Foxp3 was induced, there was a major dichotomy in transcription of the other genes, with an increase in both level and kinetics of SOCS-3 induction, and a decrease in LIF; axotrophin, normally expressed constitutively at a steady state level, also decreased in the presence of ΔE251-Foxp3 induction (Fig. 1D–F). These findings were interpreted as follows: firstly, that homodimerization and DNA-binding of wt-Foxp3 is required to repress SOCS-3 transcription, evidenced by the 50-fold increase in SOCS-3 in the ΔE251-Foxp3 cells (compare Fig. 1A to 1D). Foxp3-mediated suppression might be direct, acting on the regulatory sites of the SOCS-3 gene, or indirect, acting, for example, to repress transcription of a factor required to promote SOCS-3 transcription. Secondly, we deduce that consequential downstream effects of loss of functional Foxp3 homodimers, and the associated dysregulation of SOCS-3 gene expression, includes a reduction in axotrophin and loss of LIF. Here, the loss of LIF is likely to be the result of excessive feedback inhibition by SOCS-3 and, given that LIF is inducive for axotrophin (5), this in turn leads to the subsequent reduction in axotrophin transcription.

The experiment illustrated in Figure 1 is representative of a total of six experiments that gave similar results. The plasmids and cell culture details were as previously reported (5). Wt-Foxp3-Jurkat or ΔE251-Foxp3-Jurkat clones in early log phase of growth were used experimentally and induction was with either 0.2, 0.5 (data shown), or 1.0 μg/mL doxycycline. The flasks were incubated at 37°C in 5% CO2 and aliquots of 2 mL (i.e., 107 cells per aliquot) were harvested from each flask at 0, 12, 24, and 36 hours after doxycycline addition. Prewarmed growth medium (2 mL) containing the relevant concentration of doxycycline was added back to each flask immediately after cell harvest at each time point. Total RNA was isolated using Nucleospin RNA II kit (Macherey-Nagel GmbH) with on-column DNase treatment. First-strand cDNA was synthesized using oligo(dT)15 primers (Invitrogen) with BD Sprint PowerScript reverse transcriptase (BD Biosciences). Axotrophin, Foxp3, LIF, and SOCS-3 transcript levels were measured in duplicate relative to actin by reverse-transcription Q-PCR. Primers were designed to span an exon-exon boundary to eliminate possible influence of contaminating genomic DNA. Platinum Taq DNA polymerase and SYBR Green dye (Invitrogen) were used for reverse-transcription Q-PCR analysis on a Stratagene MX3000 PCR machine. A melt curve analysis was performed after each polymerase chain reaction cycle by a temperature gradient from 60°C to 95°C and reverse-transcription Q-PCR amplified sample products for each primer pair were separated on an agarose gel to confirm product specificity and lack of primer dimers. All reverse-transcription Q-PCR measurements were made in triplicate: only triplicates that differed by less than 0.5 cycles were accepted. The 2−ΔΔCt method was used to determine the relative expression of target transcript levels at each time point. The transcript expression of each target gene was measured relative to that of actin, which functioned as the endogenous control within each sample. The changes in relative expression levels at different time points were determined in comparison to oh, the calibrator control. Primer pairs used for QTR-PCR were: β-actin: forward 5′-CCCAACGGGAGAAGATGACC-3′, reverse 5′-CCCCCTCTGTAAGATGGGCACAG-3′; Axotrophin: forward 5′-AAAAGTGCGCCTTCAAGAGA-3′, reverse 5′-TCGACATTGCTGGGGCTTC-3′; SOCS-3: forward 5′-CGGAGAGGAGAGC-3′, reverse 5′-TGA AGC CTT GGT CA-3′.

**A Mechanistic Model for the Interrelationships Between Foxp3, LIF, and Axotrophin**

The discovery that Foxp3 is required to control SOCS-3 transcription (either directly of indirectly) added a new di-
mension to our working model of a three-way reciprocity between Foxp3, LIF, and axotrophin. The new data implies that SOCS-3 is a direct or indirect target gene for suppression by Foxp3. Thus we propose a mechanism linking Foxp3 to LIF via SOCS-3, indirectly regulating LIF by influencing the strength of the LIF/STAT3 signaling pathway (Fig. 2). The data in support of this model is as follows. Because axotrophin and LIF are interdependent (7) and LIF is inductive for axotrophin (15), then reduced LIF activity due to high SOCS-3 in the absence of wt-Foxp3 would bias the interactions towards decreased axotrophin (Fig. 2A), as found experimentally. Foxp3 would therefore play a major integrative

FIGURE 1. SOCS-3 transcription is dysregulated in ΔE251-Foxp3-transfected Jurkat T cells. Induction of wt-Foxp3 in Jurkat T cells is associated with coinduction of SOCS-3 (A), axotrophin (B), and LIF (C) in terms of transcript levels. For both SOCS-3 and LIF, this is progressive, whereas for axotrophin the initial induction appears to level off within 24 hours. In marked contrast, induction of mutant ΔE251-Foxp3, encoding Foxp3 protein that is unable to form Foxp3 homodimers, is associated with dysregulated SOCS-3 expression, revealed as some 50-fold increase in transcript levels at 12 hours (D). Where SOCS-3 is overexpressed, then LIF transcript levels decrease (E) and axotrophin also becomes reduced (E) compared to that in wt-Foxp3-induced Jurkat cells. These results were highly reproducible within a series of six experiments. The basal transcript level of each individual gene was consistent between experiments: SOCS-3 showed the lowest basal level prior to Foxp3 induction. The abscissa of all graphs is the log-fold change in the target gene/β-actin transcripts for each sample, where the 0-hour value of target gene/β-actin transcripts was assigned to a value of 1. In each graph, the relevant Foxp3 transcript levels are shown for comparison; for each gene, the following symbols are used: closed triangle, SOCS3; closed diamond, Foxp3; open square, axotrophin; open diamond, LIF.
Axotrophin Downregulates Alloreactive T-Cell Proliferation In Vivo

Axotrophin’s relationship to regulatory transplantation tolerance was revealed in ex vivo analyses of fully immune competent mice bearing a vascularized heart allograft. A highly pertinent question arose: can tolerance be induced in the axotrophin null mouse? Using identical protocols for tolerance induction (i.e., 21 days of alternate day therapy with blocking CD4 and CD8 monoclonal antibodies [20]), we discovered that both wild-type and axotrophin-null littermates became tolerant to their graft. In untreated axotrophin null control recipients, lack of axotrophin was associated with slight prolongation in graft survival, from 7 days to around 12 days. These results would be in accord with excessive T cell alloactivation due to the absence of axotrophin, this in turn playing a protective role for graft survival, a hypothesis that is now being tested in LIF-null mice.

Despite the apparent lack of effect of axotrophin in graft survival, a remarkable effect was observed ex vivo, where a fourfold splenomegaly was associated with the presence of large numbers of splenic lymphoblasts (Fig. 3), demonstrating that axotrophin normally plays a key role in downregulation of the activated T-cell response. Flow cytometry showed that this axotrophin-null alloactivated spleen cell population is CD3 positive and a full phenotypic characterization is being undertaken.

In summary, having discovered that certain features of immune tolerance are in common with the regulation of stem cell fate, it is proposed that “stemness” signals play a role in peripheral tolerance, possibly by suppressing terminal differentiation of immune effector cells [21]. Secondly, the possibility arises that allogeneic stem cells might bias the alloimmune response towards allo-tolerance by signaling for stemness, so favoring successful therapeutic engraftment [21]. Thus, in addition to tolerance in transplant recipients, any links between regulatory immune tolerance and stem cell biology should benefit regenerative medicine and the successful outcome of implanted stem cell allografts, in particular neuronal stem
cells, in which LIF release might both suppress aggressive immunity and act as a neural growth factor.

ACKNOWLEDGMENTS

We are indebted to the British Heart Foundation Cambridge-Yale Cardiovascular Research Program for facilitating the collaborative study on Foxp3/SOCS-3.

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FIGURE 3. Spleen cells isolated after graft rejection by wild-type, or axot-null, recipients. Freshly isolated spleen cells from BALB/c axotrophin-null mouse control (right panels) and an axotrophin-null mouse recipient mice that had rejected a CBA heart graft (left panels) were observed ex vivo by flow cytometry (A) and by microscopy (B). The rejected spleen showed approximately fourfold splenomegaly and flow cytometry revealed a subpopulation of lymphoblasts that stained positively for CD3 (not shown); these lymphoblasts (20- to 30-μm diameter) include dividing cells. In marked contrast, axotrophin-null spleen cells from the nontransplanted mouse lack the blastoid cell subpopulation, as previously reported (7).


