Regulatory Transplantation Tolerance and "Stemness": Evidence That Foxp3 May Play a Regulatory Role in SOCS-3 Gene Transcription

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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.

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Leukemia inhibitory factor (LIF) is a critical regulator of stem cells (*1*) and acts in concert with epigenetic factors to determine cell fate and genomic function by the control of dynamic changes in the local and global organization of chromatin in a heritable manner. The expression pattern of inducible genes becomes established as the result of microenvironmental cues during development down a particular cell lineage, and these patterns are controlled in a tissue-specific manner to allow expression of relevant tissue-specific genes while silencing those that are irrelevant. For the immune system, T lymphocytes develop in response to microenvironmental fate determination signals in a manner that ensures protective tolerance towards self tissues while simultaneously being capable of aggressive attack towards foreign pathogens. Self-tolerance is regulated at several levels, including apoptotic deletion of high-affinity self-reactive T cells upon engagement of antigen presented by thymic stromal cells (*2*), a process that requires induction of the proapoptic gene Bim (*3*). Regulatory T cells (Treg) also regulate self-tolerance and Foxp3 is a transcriptional repressor required for lymphocyte development down the Treg lineage (*4*); we have recently discovered that expression of Foxp3 is linked to the expression of two stem cell genes, LIF and axotrophin (*5*). Axotrophin was first described as a neural stem cell gene (Gary Lyons, University of Wisconsin, personal communication) and the only recognizable encoded domain is a RINGvariant E3-ligase domain, suggesting that axotrophin functions in ubiquitin-mediated degradation of specific target protein(s).

Here we summarize the links between regulatory transplantation tolerance and stemness, and provide new data in support of functional interactive relationships between Foxp3, LIF, and axotrophin.

Axotrophin Is a Regulator of T-Cell-Derived LIF

A link between axotrophin and immune tolerance was discovered when comparing genetically identical, but phenotypically distinct, "tolerant" spleen cells versus "rejected" spleen cells. Subtractive kinetic studies of gene expression employed full arrays of 36,000 genes and used a four-way analysis between progressive time points, thus imposing a high level of analytical stringency: 129 genes showed phenotype-related behavior, of which 10 increased only in the tolerant phenotype (*6*). One such gene was axotrophin, and this led to the discovery that axotrophin plays a critical role in T, but not B, lymphocyte regulation (*7*). Notably, the effect of lack of axotrophin only became manifest after T-cell stimulation, with eightfold hyperproliferation and fivefold excess release of T-cell-derived LIF (*7*). The effect on LIF release was of particular interest because we had already shown that transplantation tolerance is linked to LIF (*8*). We also found that LIF expression is functionally coupled to axotrophin expression, with axotrophin playing a role in coordinating the positive and negative regulation of LIF release in T lympho-

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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 littermates 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 Foxp subgroup is characterized by a divergent DNA-binding winged helix domain (*10*, *11*). Foxp3 further diverges from other Foxp 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 NFkB (*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 inductive 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% CO_2 and aliquots of 2 mL (i.e., 10^7 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 $2^{-\Delta\Delta CT}$ 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 0h, the calibrator control. Primer pairs used for QTR-PCR were: *β-actin:* forward: 5′-CCAACCGCGAGAAGATGACC-3′, reverse: 5′CCCCTCGTAGATGGGCACAG-3′; *Axotrophin*: forward: 5′-AAAAGTGCGCCTTCAAGAGA-3′, reverse: 5′-TGCACTTGCATGGCTCTATC-3′; *LIF*: forward: 5′-CTGTTGGTTCTGCACTGGAA-3′, reverse: 5′-CCCCTGGGCTGTGTAATAGA-3′; *Foxp3:* forward: 5′-GCAAATGGTGTCTGCAAGTG-3′, reverse 5′-CAC AGA TGA AGC CTT GGT CA *SOCS-3*: forward 5′-TGCGCAAGCTGCAGGAGAGC, reverse: 5′-GCGTGCTTCGGGGGTCACTC.

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-

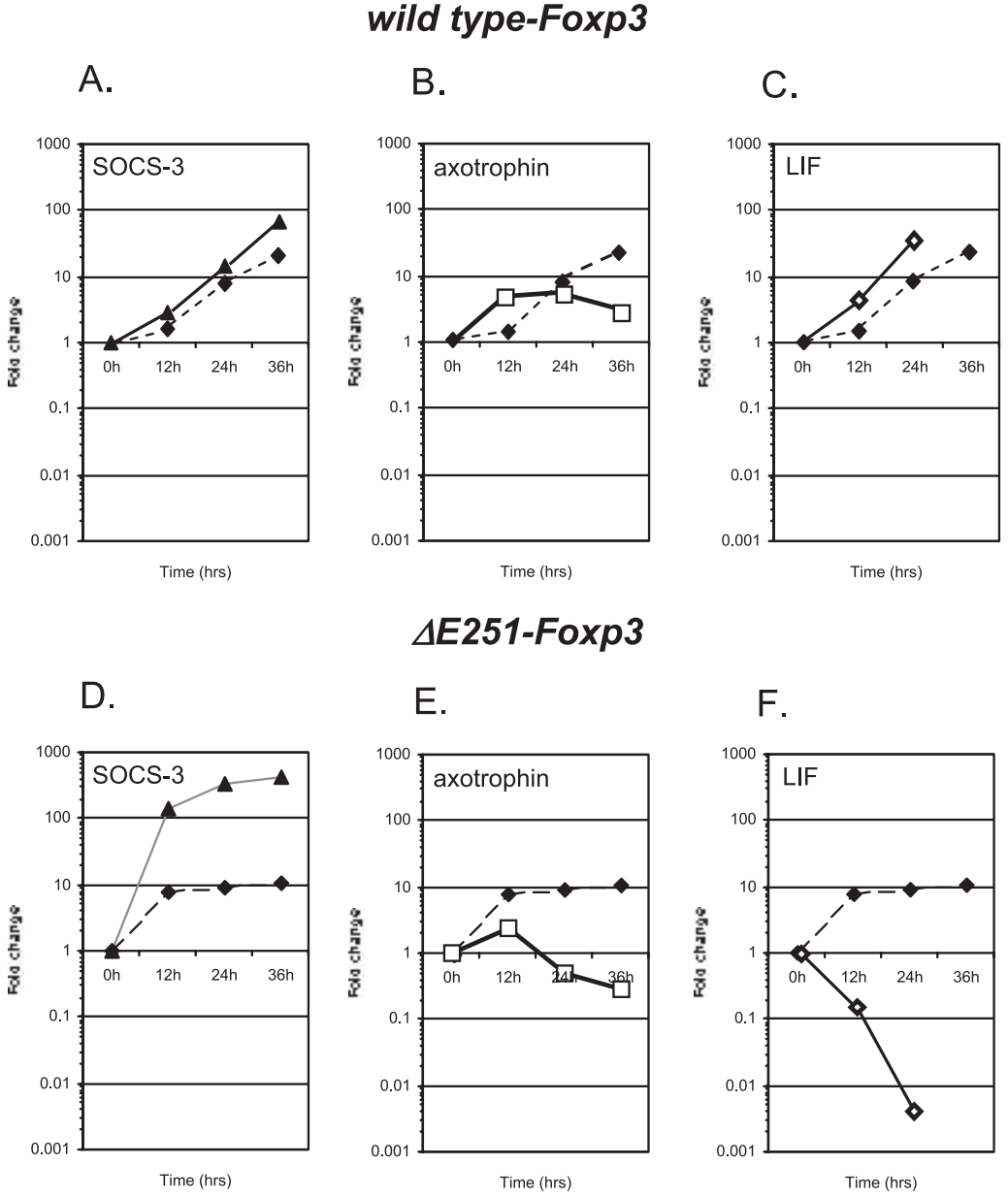

FIGURE 1. SOCS-3 transcription is dysregulated in ΔE251-Foxp3-transfected Jurkat T cells. Induction of wt-Foxp3 in Jurkat T cells is associated with coinduction of SOCS-3 (A), axotrophin (B), and LIF (C) in terms of transcript levels. For both SOCS-3 and LIF, this is progressive, whereas for axotrophin 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 (F) 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: SOCS3 showed the lowest basal level prior to Foxp3 induction. The abscissa of all graphs is the log-fold change in the target gene/βactin transcripts for each sample, where the 0-hour value of target gene/βactin 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.

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

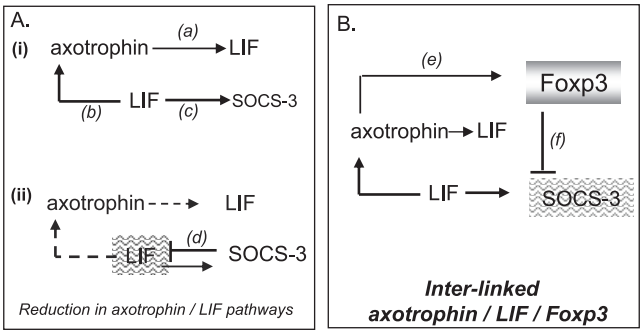

FIGURE 2. Mechanistic model for the interrelationships between Foxp3, LIF, and axotrophin. We propose a model of a regulatory "cassette" involving reciprocal interactions between Foxp3, LIF, and axotrophin where, in the absence of Foxp3, the axotrophin/LIF axis of the cassette is reduced due to SOCS-3 activity. In the presence of Foxp3, SOCS-3 is transcriptionally repressed and the axotrophin/LIF axis is maintained. Because both axotrophin and LIF are stem cell-associated, with LIF contributing to maintenance of the undifferentiated state, the axotrophin/LIF axis is in accord with a model of "stemness" in regulatory immune tolerance (*20*). (A) The evidence for the interrelationships between axotrophin and LIF are as follows: (i) (a) in the axotrophin null mouse T cell-derived LIF is dysregulated (*7*); (b) exogenous LIF added to an ex vivo alloimmune response enhances axotrophin transcription (*15*); (c) SOCS-3 is known to be a target gene of LIF/STAT3 signaling (*19*); and (ii) (d) SOCS-3 is a feedback inhibitor of the LIF/STAT3 signaling pathway (*19*). (B) The proposed model is based on further evidence that (e) axotrophin is required for normal thymic Foxp3 expression (*5*); and (f) the current data showing that SOCS-3 is a putative target gene for repression by Foxp3 homodimers. This model provides a mechanism by which concordant activities within the Foxp3/LIF/axotrophin cassette in T lymphocytes regulate SOCS-3 levels to perpetuate the Treg phenotype.

role in the axotrophin/LIF axis, acting to contain SOCS-3 transcription within the permissive range for axotrophin expression and function (Fig. 2B). The model is in accord with our findings that allotolerance is specifically associated with increased Foxp3, axotrophin, LIF, and *low* SOCS-3 (*15*). Thus, we propose that Foxp3 is involved in a compound interdependency with LIF and axotrophin via SOCS-3.

The mechanistic model in Figure 2 also explains why axotrophin has specific regulatory effects on T lymphocytes, controlling proliferation and LIF release in vitro (*7*). Despite axotrophin being expressed in most cell types, functional interdependence with Foxp3 would endow specificity for cells that express Foxp3, such as T lymphocytes and stromal cells of the thymus (*16*). We propose that tissue-specific Foxp3 contributes to a regulatory cassette of Foxp3/axotrophin/LIF in T lymphocytes. We speculate that similar regulatory cassettes may operate in a tissue-specific manner in other cell types, possibly with Fox-family proteins endowing the tissue-specific component including, for example, in stem cells and precursor cells. These concepts are compatible with novel approaches to therapy and it has been proposed that modulation of SOCS-3 might facilitate the endogenous repair of central nervous system injury (*17*); our model of a regulatory cassette involving SOCS-3, LIF, and axotrophin would provide access to such modulation, exploiting natural mechanisms not only in immunity but in other biological systems.

These experiments focus on Foxp3 as a transcriptional suppressor. One possibility is that SOCS-3, either at the gene promoter or at a more distant regulatory site for the SOCS-3 gene, is subject to regulation by Foxp3 homodimers. The known sites within the SOCS-3 promoter for transcriptional regulation include a STAT-responsive element (*18*). This functions in feedback of STAT3 signaling pathways induced by LIF-family members, namely LIF, interleukin (IL)-6, IL-11, oncostatin M, ciliary neurotrophic factor, and cardiotrophin. A second regulatory site is a 5′-upstream GC-rich element that lies downstream of the STAT-responsive element: this GC-rich element binds Sp3 (specificity protein 3) (*19*). Sp3 acts as an enhancer of both basal and induced SOCS-3 expression, requiring Sp3 lysine-483, a potential target for acetylation (*19*). We are currently investigating other SOCS-3 regulatory sites that may interact directly with Foxp3.

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-derived LIF 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

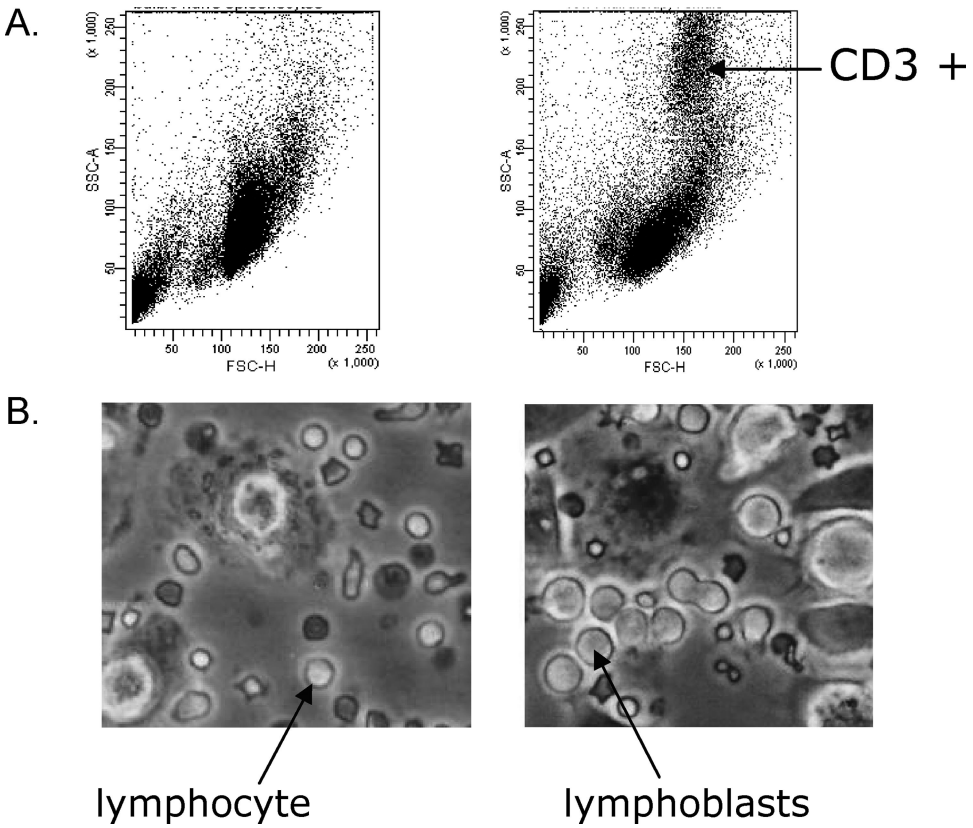

FIGURE 3. Spleen cells isolated after graft rejection by wild-type, or axot-null, recipients. Freshly isolated spleen cells from BALB/c axotrophin-null mouse control (right panels) and an axotrophin-null mouse recipient mice that had rejected a CBA heart graft (left panels) were observed ex vivo by flow 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*).

cells, in which LIF release might both suppress aggressive immunity and act as a neural growth factor.

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