# Leukaemia inhibitory factor (LIF) is functionally linked to axotrophin and both LIF and axotrophin are linked to regulatory immune tolerance

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**Abstract** **Axotrophin (*axot*) is a newly characterised stem cell gene and mice that lack axotrophin are viable and fertile, but show premature neural degeneration and defective development of the corpus callosum. By comparing *axot*$^{+/+}$, *axot*$^{+/-}$ and *axot*$^{-/-}$ littermates, we now show that axotrophin is also involved in immune regulation. Both T cell proliferation and T cell-derived leukaemia inhibitory factor (LIF) were suppressed by axotrophin in a gene-dose-dependent manner. Moreover, a role for axotrophin in the feedback regulation of LIF is implicated. This is the first evidence that fate determination mediated by LIF maybe qualified by axotrophin.**


*Keywords:* Leukaemia inhibitory factor; Axotrophin; Immune regulation

## 1. Introduction

The immune response is subject to fate determination signals that ensure protective tolerance towards self tissues whilst simultaneously being capable of aggressive attack towards foreign pathogens. Specificity of a given response is driven by antigen, whilst the phenotypic fate of the responding lymphocyte is guided by micro-environmental cues to result in either aggression, or tolerance. In self-tolerance, $CD4^+CD25^+$ regulatory T lymphocytes arise within the thymus and protect against auto-immunity in a forkhead transcription factor P3 (Foxp3)-dependent manner [1]. Mouse allograft models have shown that peripheral, naive $CD4^+$ lymphocytes may also be guided towards regulatory tolerance by therapeutic manipulation of signal transduction pathways at the time of initial antigen engagement [2–4]. Once established, a self-sustaining state of donor-specific tolerance occurs: this requires continuous presence of donor antigen and is capable of being adoptively transferred to fully immune competent recipients of a donor-type graft via "tolerant" lymphocytes [2]. Ex vivo analyses of tolerant splenocytes revealed leukaemia inhibitory factor (LIF) release in response to donor antigen [5]. LIF is a key neuropoietic cytokine [6] and regulator of stem cells [7]. Comparative gene array of graft-"tolerant" spleen cells versus graft-"rejected" splenocytes identified axotrophin, a newly characterised stem cell gene [9], as being specifically increased in tolerance [8]. Here, we ask if axotrophin plays a role in immune regulation and show not only that axotrophin plays a critical role in the regulation of T lymphocytes, but also that axotrophin is functionally linked to the regulation of LIF.

## 2. Materials and methods

### 2.1. Axot null mice

The axotrophin gene was identified in an in vitro preselection gene trap screen for novel genes involved in neural differentiation from embryonic stem (ES) cells [6] and the detailed methods for preparation of *axot* null mice are to be published elsewhere. Briefly, ROSA beta-geo [10] was inserted into the first intron of *axot* creating a null allele. ES cells heterozygous for the *axot* gene trap were injected into mouse blastocysts to create transgenic animals on a BALB/c background. In the experiments reported here, spleen, thymus and lymph node were obtained from 5 month old littermates of *axot* null, heterozygous, and wild type littermates and kept on ice prior to cell preparation for the phenotypic and functional analyses described below. The lymph node tissue yielded very few cells and was discarded. Spleen, thymus, kidney and lung from littermates of each *axot* genotype were also taken for histology.

### 2.2. FACS analyses

Splenic and thymic cell suspensions were depleted of erythrocytes and washed in FACS staining solution (0.2% BSA and 0.1% sodium azide in 1× PBS) prior to being mixed with the various monoclonal antibodies detailed below, these being either directly or indirectly conjugated with phycoerythrin (PE) or fluorescein isothiocyanate (FITC). PE-rat anti-mouse CD19 (557399), PE-hamster anti-mouse TCRα chain (553172) and rat anti-mouse dendritic cell clone 33D1 (551776) were from Pharmingen. Rat anti-mouse CD205-FITC (MCA949F), mouse anti-rat IgG2a heavy chain-FITC (MCA278F) and mouse anti-rat IgG2b chain-FITC were from Serotec Ltd., while rabbit anti-mouse CD25 (IL2Rα) and goat anti-rabbit IgG (H&L)-PE (4050–89) were from Santa Cruz Biotechnology and Southern Biotechnology Associates, respectively. Anti-CD4 (YTS177.9.6) and anti-CD8 (YTS 105.18.10) were a gift from Dr.

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*Abbreviations:* FoxP3, forkhead transcription factor P3; STAT3, signal transucer and regulator of transcription 3; SOCS3, suppressor of cytokine signalling 3; IL, interleukin

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Stephen Cobbold, University of Oxford. Analyses were performed on a Becton Dickinson FACSCalibur instrument equipped with CellQuest software.

### 2.3. Proliferation assays

Splenocytes and thymocytes were collected in sterile growth medium, RPMI-1640 (Gibco™ Invitrogen Co.) supplemented with 10% FCS. Cultures of 100 μl containing $5 \times 10^5$ nucleated cells per well were incubated at 37 °C, 5% $CO_2$ for 48 or 72 h LPS (Sigma Chemical Co.) at 50 μg/mL and conA (ICN Biochemicals, USA) at 10 μg/ml unless otherwise indicated. All experiments were performed in triplicate and are expressed as a mean: there was less than 10% variation in all cases. Immediately prior to harvest, supernatants were collected for ELISA analysis and the cells were labelled for 2 h with methyl-[$^3$H]thymidine (TRK686, specific activity 80 Ci/mmol, Amersham Biosciences) at a final concentration of 1 μCi/mL. To determine the effect of LIF on conA-stimulated proliferation, BALB/c *axot*$^{+/+}$ splenic and thymic cells were incubated in the presence of conA (2 or 10 μg/mL) with or without 500 pg/mL, or 1000 pg/mL, rmLIF (Santa Cruz Biotechnology, SC-4378). To measure the relationship between LIF release and T cell proliferation in *axot*$^{+/+}$ spleen cells, a range of conA concentrations were used to stimulate different levels of proliferation.

### 2.4. Cytokine release

ELISA assays were performed on culture supernatants using the DuoSet ELISA for IFNγ (DY485), IL2 (DY402), IL4 (DY404), IL10 (DY417) and Quantikine M Immunoassay for LIF (MLF00), from R&D Systems. Goodness of fit was measured for the standard curves: this approached 1.0 in all assays. Sample replicates showed high reproducibility.

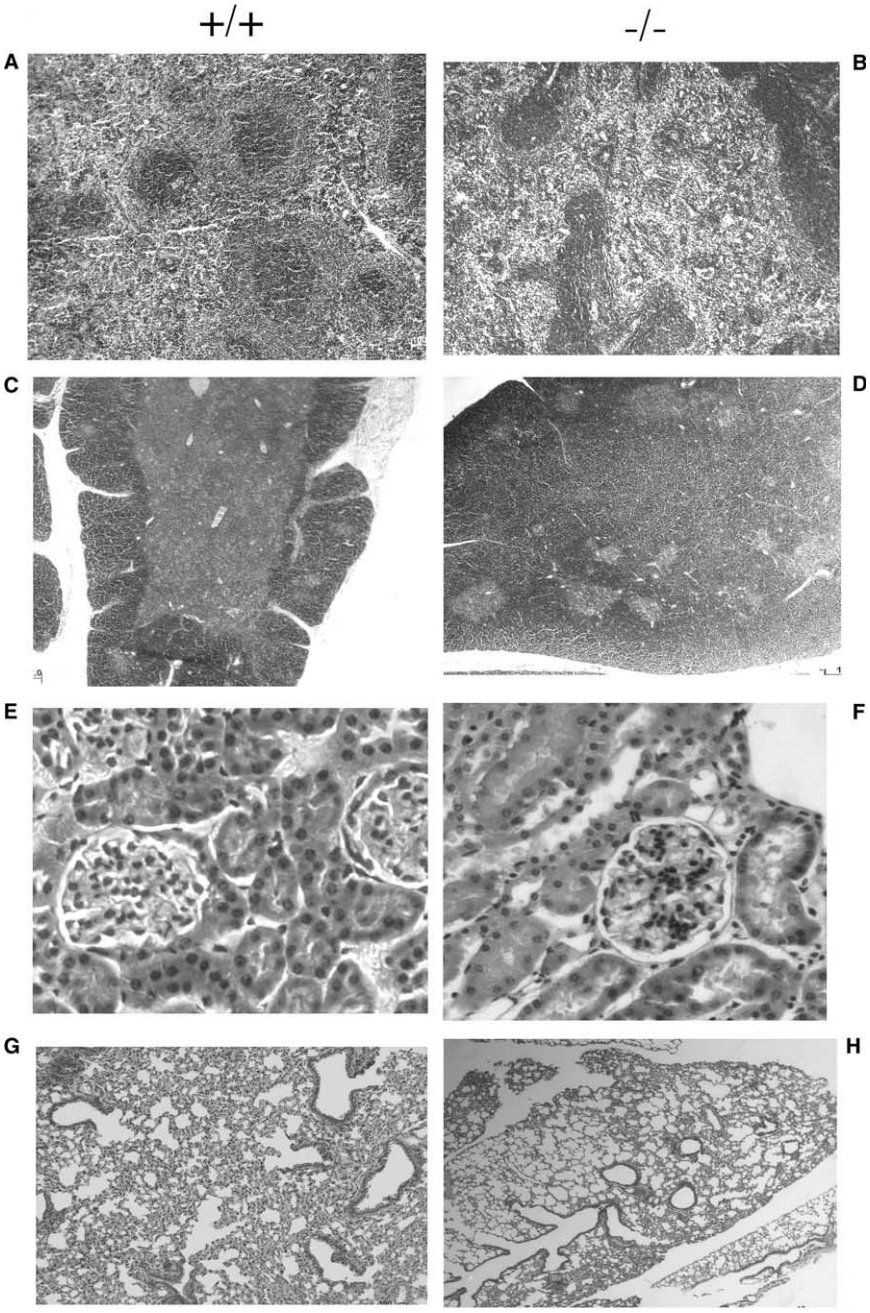


Fig. 1. Axotrophin null mice show normal spleen, thymic, kidney and lung development. H&E stained sections of spleen (A and B) and thymus (C and D) comparing *axot* null mice (−/−) with wild type (+/+). Kidney (E and F) and lung (G and H).