Report

Treg versus Th17 lymphocyte lineages are cross-regulated by LIF versus IL-6

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Within the immune system there is an exquisite ability to discriminate between "self" and "non-self" that is orchestrated by T lymphocytes. Discriminatory pathways guide differentiation of these lymphocytes into either regulatory (Treg) or effector (Teff) T cells, influenced by cues from the naïve T cell's immediate micro-environment as it responds to cognate antigen. Reciprocal pathways may lead to commitment of naïve T cells into either the protective tolerance-promoting Treg, or to the pro-inflammatory Th17 effector phenotype. Primary activation of CD4+ lymphocytes stimulates their release of leukemia inhibitory factor (LIF), and Treg continue to release LIF in response to antigen, implying a role for LIF in tolerance. In contrast, interleukin-6 (IL-6), although very closely related to LIF, promotes maturation of Th17 cells. Here we show that LIF and IL-6 behave as polar opposites in promoting commitment to the Treg and Th17 lineages. Unlike IL6, LIF supported expression of Foxp3, the Treg lineage transcription factor, and LIF opposed IL6 by suppressing IL-6-induced IL-17A protein release. In striking contrast, we found that IL6 effectively inhibited LIF signalling, repressing transcription of the LIF receptor gp190, and strongly inducing axotrophin/MARCH-7, a novel E3 ubiquitin ligase that we discovered to be active in degradation of gp190 protein. In vivo, anti-LIF treatment reduced donor-specific Treg in recipients of foreign spleen cells. Conversely, a single dose of biodegradable LIF nanoparticles, targeted to CD4, successfully manipulated the LIF/IL6 axis towards development of donor-specific Foxp3+ Treg. The implications for therapy are profound, harnessing endogenous immune regulation by paracrine delivery of LIF to CD4+ cells in vivo.

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Introduction

In the periphery, naive T lymphocytes are sensitive to different fate determination pathways due to their intrinsic epigenetic plasticity. The immune synapse plays a critical role in such fate choice, integrating molecular information both according to T cell receptor affinity for presented antigen, and according to concurrent engagement of inhibitory and co-stimulatory receptors.[1] The overall result is heritable changes in chromatin structure and gene expression patterns for full phenotypic differentiation into aggressive effector T cells (Teff) of Th1-type, or Th2-type, that destroy target antigen via cellular, or humoral, mechanisms. Th17 is a recently recognised third Teff-type, generated in the presence of interleukin-6 (IL-6) and associated with chronic inflammatory conditions.[2-4]

The immune competent individual requires control mechanisms to protect against auto-immune disease. To this end, self-tolerance is regulated by a separate, tolerogenic class of T cells, Treg, that are able to actively suppress an aggressive response in an antigen-specific manner.[5] Expression of a single gene, *Foxp3*, is able to orchestrate differentiation of the naïve T lymphocyte towards Treg: thus Foxp3 represents a "master" switch for epigenetic profiling of Treg.[6-8] Given that Foxp3 is nodal for tolerance, exactly how Foxp3 itself is regulated is a burning question and, although it is recognised that a higher level of regulation, upstream of Foxp3, determines the Treg lineage,[9,10] the nature of the putative upstream regulator is unknown. Our previous work has demonstrated novel links between Foxp3 expression and the stem cell-related cytokine, leukemia inhibitory factor (LIF), suggesting that LIF plays a role in development and maintenance of Treg.[11] Moreover, since T cell-derived LIF is profoundly regulated by the RINGv E-3 ubiquitin ligase, axotrophin (alternatively known as MARCH-7,[12]), axotrophin is also implicated as a potential regulator of Foxp3.[13-15]

LIF belongs to the IL-6 family of cytokines,[16] yet IL-6 itself is strongly associated with immune aggression, driving differentiation of the Th17 lineage. Using an in vivo model of transplantation tolerance, we confirm opposing profiles in expression of each

cytokine in allo-tolerance. We then demonstrate polar opposite responses of CD4^{+} T cells to LIF versus IL-6, with LIF supporting Foxp3 expression. We discovered that IL-6 downregulates the LIF-receptor, implying a mechanism for cross-regulation at the LIF/IL-6 axis that may in turn link to regulation of Foxp3. We show for the first time that the LIF-receptor subunit gp190 is degraded by axotrophin, and axotrophin transcriptions massively induced by IL6. Finally, we demonstrate expansion of Foxp3^{+} cell numbers in fully immune competent mice following a single dose of LIF delivered to CD4^{+} T cells as biodegradable LIF-nanoparticles.

Results

In vivo, endogenous LIF and IL-6 correspond to different immune phenotypes. An in vivo/ex vivo murine model of transplantation tolerance, comparing allo-tolerance versus allo-rejection, has been designed to analyse the molecular biology of the immune response whilst retaining the complexity of the in vivo environment:[17] the ex vivo culture conditions are three dimensional for preservation of relationships between cellular and extracellular components including the laminins that may contribute to T cell responses.[18] Interdependent links between Foxp3, LIF and axotrophin have been revealed previously in this model[14,15] and here the model was extended to explore the differential between LIF and IL-6 during allo-primed responses to donor antigen. Figure 1A (upper) shows that LIF was expressed as a relatively late cytokine, first detected at 5 d, when levels were 10-fold greater in primed allo-tolerant hosts (320 pg/ml) relative to primed allo-rejecting recipients. Figure 1A (lower) shows IL-6 levels in the same samples as measured for LIF: IL-6 expression was strongly correlated with allo-rejection, reaching 400 pg ml^{-1} within 24 h, in marked contrast
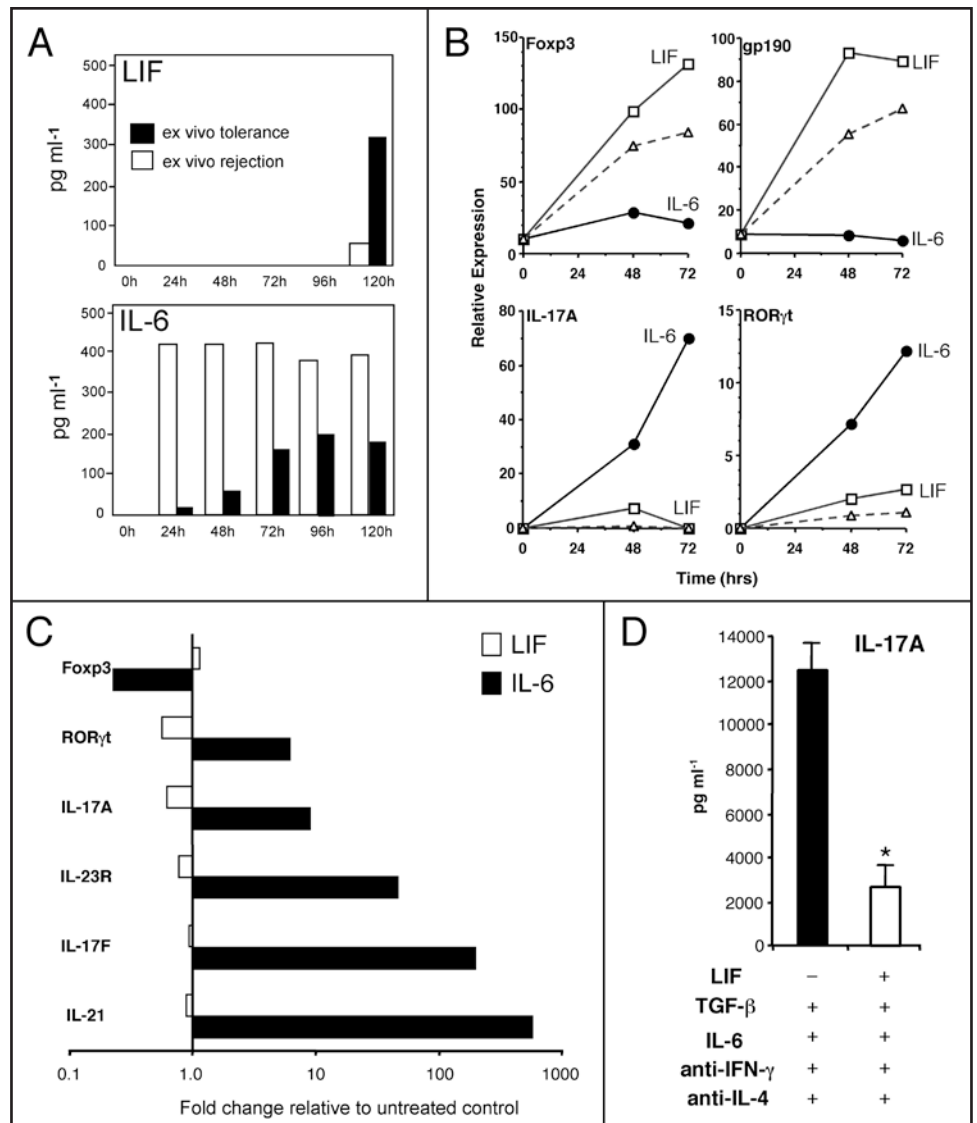

Figure 1. LIF is polar opposite to IL-6 in T cell regulation. (A) CBA recipients of a fully mismatched BALB/c vascularised heart were rendered tolerant to their graft by CD4 plus CD8 blockade as described previously.[11] In the same model, other recipients were allowed to reject their graft. Ex vivo stimulation of spleen cells from these in vivo primed allo-tolerant, or allo-rejected, mice used donor-type irradiated spleen cells, and LIF and IL-6 release were measured by ELISA. (B) FACS-sorted CD4^{+}GFP[+](Foxp3^{+}) nTreg cells from Foxp3-GFP knockin mice were stimulated with anti-CD3 and anti-CD28 microbeads in the presence of IL-2 and TGFβ alone (open triangle—broken line), or plus LIF (open square—solid line) or IL-6 (closed circle—heavy solid line). Expression of Foxp3, gp190, IL-17A and RORt mRNA relative to control GAPDH was determined at indicated time points by real-time PCR. (C) FACS-sorted CD4^{+}GFP^{-} cells were stimulated with plate-bound anti-CD3 and soluble anti-CD28 in the presence of TGFβ, TGFβ+LIF or TGFβ+IL-6 for 3 days. Fold changes in relative gene expression were derived by comparing LIF[-] (open bar) or IL-6 (closed bar) supplemented cultures to control (TGF only). (D) Amount of IL-17A secreted by Th17 cells in the absence (closed bar) or presence (open bar) of LIF. Representative ELISA data of three independent experiments (mean of duplicate wells ± s.d., * <0.01).

to IL-6 in tolerance which was 20-fold lower. This in vivo/ex vivo experiment confirmed the respective links between LIF and tolerance, and IL6 and rejection, in fully immune competent recipients of a vascularised heart allograft.

LIF and IL-6 have polar opposite effects on CD4^{+} T cells. The differential phenotypic associations of LIF and IL-6 in the complex

model of in vivo tolerance versus rejection raised several questions: firstly, does LIF play an active role in immune tolerance? Secondly, do the opposing effects of LIF and IL-6 influence commitment to T cell phenotypes, and more specifically, to the Foxp3^{+} Treg cell? We therefore used purified populations of freshly isolated natural CD4^{+}Foxp3^{+} Treg (nTreg) from Foxp3-GFP knockin mice[3] to test
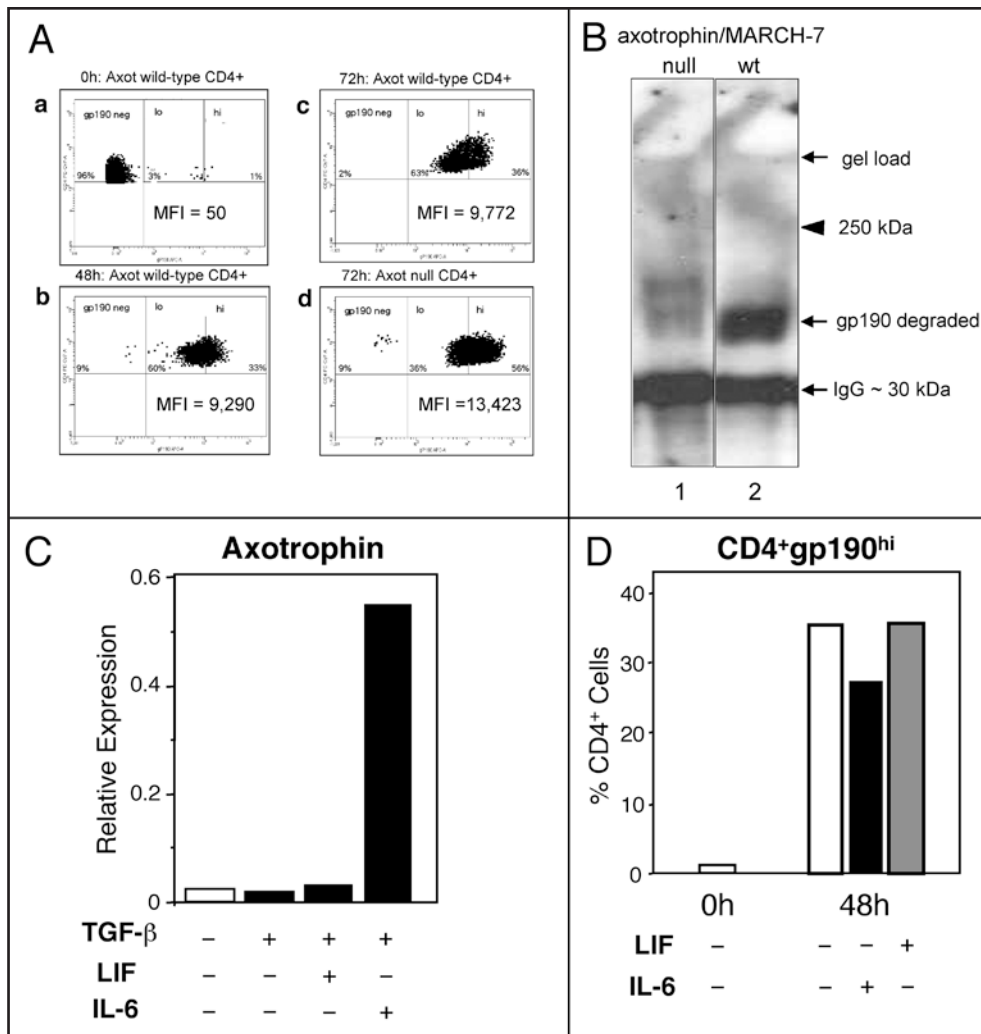
Figure 2. Both gp190 and IL-6 are linked to axotrophin. (A) Probability plots of CD4⁺ cells analysed by flow cytometry for expression of gp190 before and after stimulation of mouse spleen cells with plate-bound anti-CD3 and soluble anti-CD28. Domains of gp190 negative, gp190 low, and gp190 high expression are delineated. (a–c) show wild-type mouse CD4⁺ cells. (a) 0 h, (b) 48 h, (c) 72 h. (d) shows *axotrophin* null mouse spleen cells at 72 h. At 48 h the *axotrophin* null CD4⁺ cells showed equivalent expression of gp190 to those in (c). (B) IP western of activated mouse spleen cells from axotrophin null and wild-type littermates. The two lanes are directly comparable and differ only in the absence of axotrophin in lane 1. The scan is from the same multi-lane blot; the cropped lanes were treated identically and no image enhancement used. Gel load indicates the loading well; gp190 degr. indicates gp190 degradation products; IgG indicates reactivity of the secondary goat anti-rabbit HRP antibody with rabbit anti-gp190 in the sample. (C) FACS-sorted CD4⁺GFP⁻ cells were stimulated with plate-bound anti-CD3 and soluble anti-CD28 for 3 days in the presence or absence of TGFβ, LIF or IL-6 as indicated: the effect of cytokine on axotrophin transcript levels was measured relative to GAPDH. (D) FACS analysis of gp190 hi CD4⁺ mouse spleen cells after stimulation with anti-CD3/anti-CD28, in the presence or absence of either LIF, or IL-6.

the effect of exogenous LIF, versus exogenous IL-6, on expression of the respective lineage-specific transcription factors, *Foxp3* (Treg) and *RORγt* (Th17).[19] We used IL-6 to provide a reference comparator for known IL-6 effects (e.g., induction of *RORγt*)[19] in addition to probing for cross-regulation between IL-6 and LIF. Figure 1B shows transcript levels and revealed that LIF increased *Foxp3* transcription in nTreg, whilst IL-6 both profoundly repressed *Foxp3* and induced *RORγt*. For the LIF-specific receptor subunit, gp190, the response to LIF versus IL-6 was the polar opposite: gp190 transcripts were increased by LIF but strongly inhibited by IL-6 (Fig. 1B), a result that indicated a potential mechanism for cross-regulation between the two cytokines, wherein IL-6 would reduce LIF signaling by repressing synthesis of the LIF receptor.

We next asked if induction of the T cell lineage to either Foxp3⁺ Treg, or Th17, can be influenced by LIF. Figure 1C shows that exogenous LIF did not oppose expression of Foxp3 during induction of CD4⁺Foxp3⁻ naïve T cells to the Treg lineage by TGFβ. Moreover, genes linked to the Th17 lineage (*RORγt, IL-17A, IL-23R* and *IL-21*) tended to be suppressed by LIF. Conversely, when these cells were induced in the presence of IL-6, a profound repression of *Foxp3* and strong induction of the Th17 lineage genes

RORγt, IL-17A, IL-23R, IL-17F and *IL-21* was found. Given that the sole difference between these TGFβ-stimulated naïve CD4⁺ cells was LIF versus IL-6, this data confirms an opposing influence of each cytokine on Treg versus Th17 lineage induction. Moreover, at the protein level there was evidence of cross-regulation between LIF and IL-6, since addition of LIF to IL-6-driven Th17 polarization suppressed release of IL-17A (Fig. 1D).

LIF-receptor protein gp190 is regulated by the E-3 ligase axotrophin/MARCH-7. The IL-6 receptor is composed of gp130/gp130 homodimers whilst the LIF receptor is qualified by the presence of gp190, forming gp130/gp190 heterodimers.[16] The differential effects of LIF and IL-6 on T cell differentiation will involve signaling through their respective receptors and we focussed our attention on the regulation of the LIF-specific gp190 receptor subunit. Since LIF becomes dysregulated in T cells that lack axotrophin,[14,15] we suspected axotrophin's E-3 ubiquitin ligase activity may target gp190 for degradation through the ubiquitin-proteasome pathway.

Figure 2A (a and b) shows that expression of gp190 on the surface of CD4⁺ T cells is activation dependent, increasing from 5% to over 80% when stimulated with anti-CD3/anti-CD28.

Axotrophin null cells also show activation-dependent expression of gp190 (data not shown). Figure 2A (c and d) reveal that lack of axotrophin is associated with increased cell surface expression of gp190 protein, demonstrating that axotrophin, either directly or indirectly, is required for feedback control of gp190 levels. We therefore used immuno-precipitation of gp190 followed by western blot to look for degradation products derived from gp190, with and without axotrophin. Following 72 h activation a strong band of approximately 80–90 kDa was found in axotrophin wild-type spleen cells (Fig. 2B, lane 2): In marked contrast, axotrophin-null cells showed only a faint doublet around 120 kDa and 90 kDa (Fig. 2B, lane 1). We conclude that axotrophin ER-ligase activity regulates LIF signalling by controlling expression of the gp190 subunit of the LIF receptor. This is in accord with the excessive LIF activity found in activated T cells that lack axotrophin.[14] Of note, the cytoplasmic domain of gp190 includes a membrane-proximal lysine that may represent a putative ubiquitin target site for axotrophin.

At the level of transcription we found that IL-6 suppresses gp190 (Fig. 1B). We next asked, does IL-6 influence axotrophin? Figure 2C shows that IL-6 causes a 30-fold induction of axotrophin in activated CD4^{+} T cells. In marked contrast, LIF induced only a two-fold increase in axotrophin, this being in accord with the two-fold increase seen in ex vivo allo-tolerance.[13] If axotrophin is indeed downregulating gp190 expression, we reasoned the IL-6-mediated increased axotrophin transcription—assuming an associated increase in axotrophin translation—would correlate with decreased cell surface gp190 protein: we found that it did (Fig. 2D).

We concluded that gp190 is differentially regulated by LIF, versus IL-6, at both the transcript and the surface protein levels, and that this is linked to axotrophin E3-ligase activity. Overall the data is compatible with a mechanistic model where IL-6, by reducing gp190 expression, subverts the LIF signaling pathway. Accordingly, LIF-associated gene activity—including that of Foxp3 and gp190 (Fig. 1B)—is reduced due to loss of LIF receptor, permitting dominant expression of Th17 lineage genes to become established. We speculate that axotrophin functions at the hub of the LIF/IL-6 axis.

LIF therapy promotes Treg. The functional link between LIF and Treg raised the question, does LIF provide a potential therapeutic for induction of antigen-specific tolerance? The immediate barrier for LIF as a therapy is its rapid breakdown by serum proteases and thus we explored the option of delivering LIF within a biodegradable nanoparticulate carrier[20] (LIF-nano): the LIF-nano would be constructed to provide a sustained low-level release of LIF to the immediate environment of the CD4^{+} T cell in a paracrine fashion. This technological approach, to be published in detail elsewhere, was based on previous work demonstrating that delivery of IL-2 has profound effects on proliferation of targeted T cells in vitro using nanodelivery systems.[21] In the current study, LIF-nano were decorated with avidin and coupled to biotinylated antibody against CD4, as illustrated in Figure 3A. When tested in vitro, LIF-nano treatment almost doubled Foxp3^{+} T cell numbers in response to TGFβ: empty nanoparticles had little effect whilst IL-6-nano reduced Foxp3^{+} Treg induction (Fig. 3B).

We next asked, can LIF-nano enhance numbers of donor-specific Foxp3^{+} Treg in vivo? This was tested in a tolerance promoting donor-specific transfusion (DST) model, wherein transfer of DBA/2 splenocytes (H-2[d], Mls[a]) into BALB/c mice (H-2[d], Mls[b]) causes specific expansion of Vβ6^{+} but not Vβ8^{+} CD4^{+} T cells, due to the minor lymphocyte stimulatory superantigen Mls-1a encoded in the mouse genome of the DBA/2 strain.[22] Figure 3C shows that co-infusion of donor spleen cells with a single dose of anti-CD4-conjugated LIF-nano resulted in 5-fold increase in donor-specific Foxp3^{+} Treg after 5 d. The LIF-nano effect was significantly greater than in controls receiving empty-nano. Non-specific activity was very small although slightly increased by nanoparticle treatment per se.

We argued that, if LIF biases the Treg/Th17 axis towards Treg, then removal of endogenous LIF in vivo should in turn reduce Foxp3^{+} Treg expansion in response to donor antigen. This was tested in the DST model. Recipient treatment with 3 daily doses of anti-LIF antibody resulted in a significant reduction in donor-specific Treg after 5 days, both in the spleen and in the draining lymph nodes (Fig. 4). Again, the effect was donor-antigen specific.

Discussion

We have identified LIF as a nodal regulator of the Foxp3^{+} Treg lineage, acting to oppose IL-6, the nodal regulator of the Th17 lineage. This discovery, represented schematically in Figure 5, is intimately linked to our unexpected finding that counter-regulation between LIF and IL-6 is based on rheostat-style control of the LIF receptor, g190, and includes the E3 ligase, axotrophin.

Both LIF and IL-6 signal through signal transducer and activator of transcription 3 (STAT-3). STAT-3 is common to many signaling pathways and the importance of epigenetic profiles that qualify the genes accessible to STAT3 activation is emphasised by our data in Figure 1B, where polar opposite responses to LIF and IL-6 in terms of gene expression were discovered. Unmasking of epigenetically silenced genes in mouse spleen cells by histone deacetylase inhibition has been shown to increase gp190 expression,[23] and we note the promotion of Treg in such conditions.[24] In vivo, the adjacent cellular environment will contribute to the T cell's micro-environment as it responds to antigenic stimulation, including local mast cells that are a rich source of LIF and known to support Foxp3^{+} Treg.[25]

Our findings are of universal importance, LIF being recognised as a critical regulator of both embryonic stem cells and adult stem cells in vivo in addition to being a pleiotrophic cytokine including in the nervous system. We propose that development of the Foxp3^{+} Treg cell mirrors pathways that regulate epigenetic plasticity, controlling fate determination including in stem cells and precursor cells. Specifically, we suggest that signaling qualified by the LIF-receptor gp190 is coupled to regulation of Foxp3 expression. Since Foxp3 is able to suppress transcription of suppressor of cytokine signaling-3 (SOCS-3, the suppressor of the LIF/STAT-3 signaling pathway),[26] we anticipate that LIF and Foxp3 will promote a reciprocal positive feed back loop that maintains the profile of accessible STAT-3 responsive genes required for the Foxp3^{+} Treg phenotype. Thus, rather than a linear
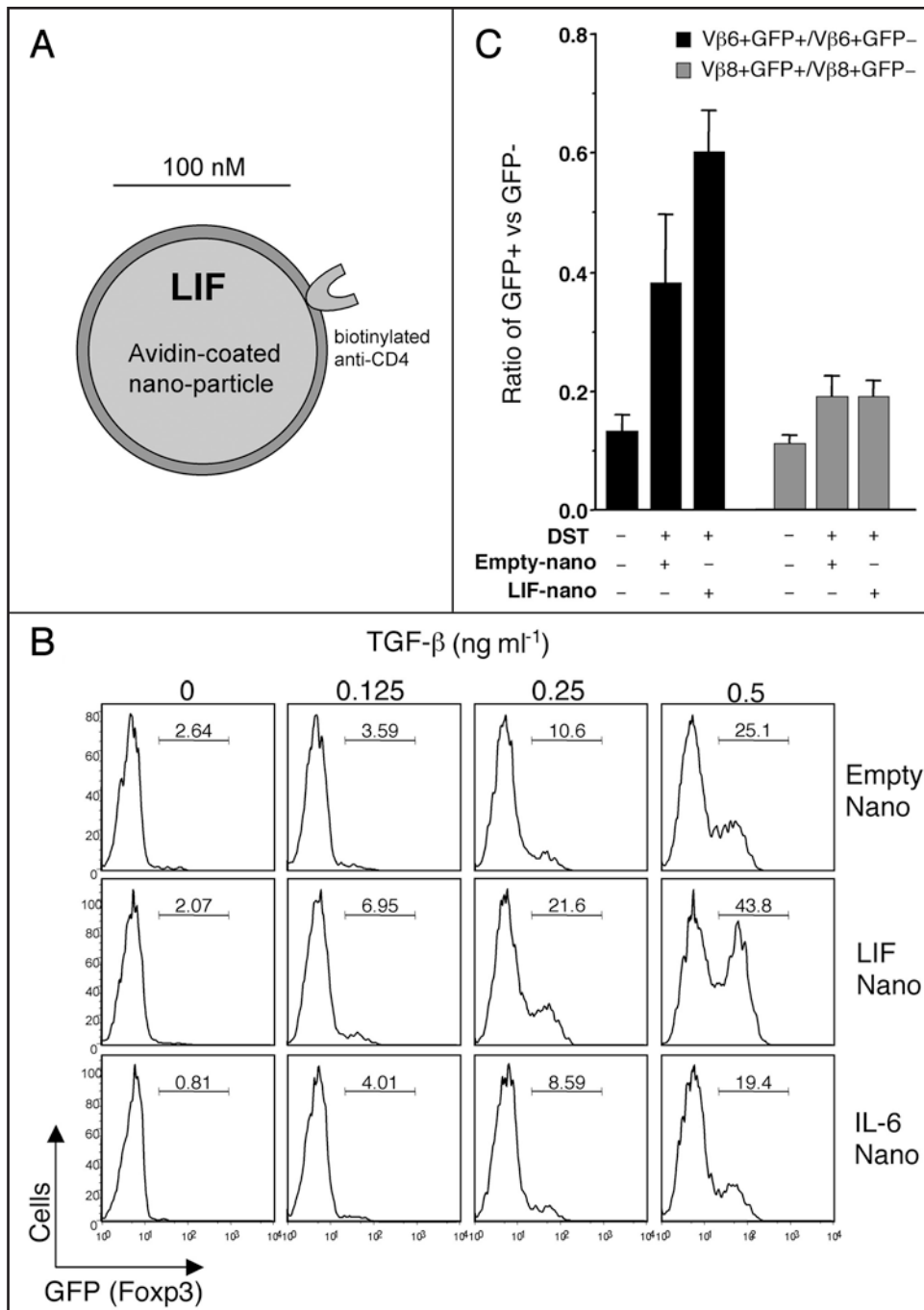
Figure 3. LIF expands the Foxp3+ cell population. (A) Schematic of the nanoparticle construction0. Soluble LIF is trapped within the biodegradable matrix of poly (lactic-co-glycolic acid) (PLGA) prepared from FDA-approved materials. Avidin is incorporated at the particle surface, permitting attachment of biotinylated antibody and thus antibody-mediated targeting. The matrix is impermeable to enzyme, and degrades slowly providing a vehicle for sustained paracrine delivery of LIF. (B) FACS-sorted CD4+GFP- cells were stimulated with plate-bound anti-CD3, soluble anti-CD28 and increasing doses of TGFβ in the presence of empty nanobeads or nanobeads that were loaded with either LIF, or IL-6 (50 μg nanobeads per 0.5 ml culture medium). Induced expression of Foxp3-GFP at 72 h is shown on the x-axis. (C) In vivo local delivery of LIF expands antigen-specific nTreg cells. DBA/2 splenocytes (DST) were incubated for 15 minutes with anti-CD4 conjugated empty-, or LIF-nanobeads, and infused (10^7 cells/mouse, i.v.) into BALB/c Foxp3-GFP mice (n = 3 per group). Host lymph node cells were harvested 5 days later, and ratios of GFP+ vs. GFP- cells were calculated in the donor specific Vβ6+ (black) or Vβ8+ (grey) CD4+ T cell compartments (mean ± s.d.).

upstream-downstream regulation, we envisage a self-regulatory node involving Foxp3 and LIF that is controlled by axotrophin via gp190 expression. Early exposure to IL-6, by repressing gp190, will favor escape from the node, permitting differentiation to proceed towards the Th17 lineage accompanied by masking of Treg-linked gene expression.

Our discoveries identify novel drug targets to modulate endogenous pathways that guide the immune response in vivo. Proof of concept is provided using targeted LIF-nanotherapy to enhance antigen-specific Treg cells in vivo by delivering paracrine LIF activity to CD4+ T cells.

Materials and Methods

Mice. Foxp3-GFP knockin mice were generated on the C57BL/6 background as described.[3] They were further backcrossed for 8 generations onto the BALB/c background. DBA/2 mice were purchased from the Jackson Laboratory (Bar Harbor, ME).

In vitro T cell stimulation. Natural Treg cells (CD4+GFP+) from C57BL/6 Foxp3-GFP knockin mice were FACS-sorted, and stimulated at 1.5×10^5 cells/U-bottom well with biotinylated anti-CD3 and anti-CD28 coated onto anti-biotin microbeads (Myltenyi) in the presence of human IL-2 (50 ng ml^{-1}) and TGFβ1 (1 ng ml^{-1}) alone, or plus mouse IL-6 (20 ng ml^{-1}) (all R&D Systems) or LIF (20 ng ml^{-1}, Santa Cruz SC-4378 or Millepore ESG-1106) for 3 days. FACS-sorted CD4+GFP- cells (1×10^6 ml^{-1}) were stimulated with plate-bound anti-CD3 (10 μg ml^{-1}, 145-2C11) and soluble anti-CD28 (1 μg ml^{-1}, 37.51) in 48-well plate for 3 days. For Foxp3+ Treg induction, cultures were supplemented with TGFβ1 (1 ng ml^{-1}). For Th17 cell induction, TGFβ1 (1 ng ml^{-1}) and IL-6 (20 ng ml^{-1}) were supplemented.

Real-time PCR. Standard protocols for expression of gene-of-interest utilised specific primers and probes obtained from Applied Biosystems. The amplification step used ABI 7900
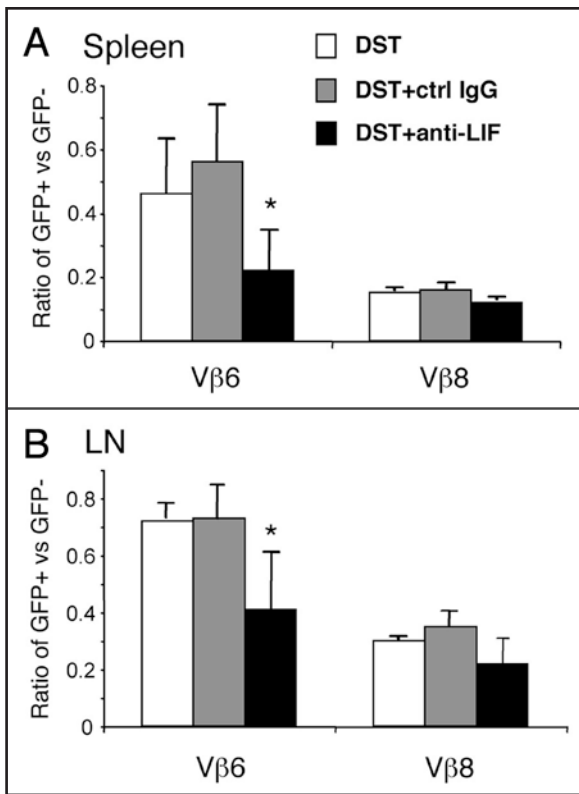
Figure 4. Blocking LIF in vivo reduced donor-specific Foxp3^{+} cells. Using the DST model, anti-LIF antibody was given i.p. to the BALB/c Foxp3-GFP recipients (n = 3 per group) at a dose of 150 µg on days 0, 1, 2 post grafting. Five days after DST, host lymphocytes were harvested and CD4^{+}GFP^{+} cells enumerated by flow cytometry. The anti-LIF therapy resulted in specific inhibition of the expansion of antigen-specific Vβ6^{+} Treg cells in spleen (A) and lymph nodes (B). * <0.05, compared to DST alone or DST plus control IgG.

Figure 5. Schematic model of LIF versus IL-6 cross-regulation for Treg versus Th17 lineage differentiation. Naive CD4^{+} T cells, when stimulated by cognate antigen through the T cell receptor (TCR), release endogenous LIF. This model predicts that, where TCR stimulation is weak or attenuated (A) released LIF induces further LIF transcription in addition to gp190 transcription, setting up an autocrine loop for LIF signaling where feedback regulation includes low level proteasomal degradation of gp190 protein primed by the E-3 ubiquitin ligase axotrophin. LIF signaling augments expression of Foxp3 leading to Treg-type epigenetic profiling for stable antigen-specific tolerance. (B) proposes that, where TCR stimulation occurs in the presence of IL-6, then suppression of gp190 by IL-6, acting to inhibit gp190 transcription and also massively induce axotrophin transcription, prevents a LIF autocrine loop becoming established, resulting in failure to establish a Foxp3-linked Treg epigenetic profile. Instead, IL-6 induces RORγt to drive Th17 lineage development. (C) predicts that, by providing exogenous LIF to the T cell micro-environment, LIF-induced gp190 expression is sufficiently maintained as to permit sustained LIF signaling and so supports Treg expansion. The model anticipates counter-balancing effects that create a rheostat control mechanism, sensitive to micro-environmental cues including inflammatory mediators (e.g., IL-6), or conversely to sources of LIF (e.g., local mast cells).

Sequence Detection system (Applied Biosystems). Expression was normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Gp190 immune precipitation and western blotting. Spleen cells from axotrophin wildtype, or null, littermates were stimulated for 72 h with anti-CD3 plus anti-CD28, with inclusion of the proteasome inhibitor NG132 (Sigma) for the last 18 h. Cells were harvested, lysed and probed for gp190 using rabbit polyclonal anti-gp190 (sc-659) to prepare gp190-affinity beads by standard methods. Pelleted gp190 beads were washed and subject to SDS-PAGE using a 4%–15% gel: the membranes were probed for gp190.

Donor specific transfusion (DST). A control group of 10–13 week old BALB/c Foxp3-GFP mice each received 10^7 DBA/2 splenocytes i.v. on day 0. Experimental groups received 10^7 DBA/2 splenocytes i.v. on day 0 and were treated with 3 doses of anti-LIF antibody (R&D Systems) at 150 µg per dose given i.p. on days 0, 1 and 2. On day 5, recipient spleen and lymph node cells were analysed by flow cytometry for Foxp3-GFP and Vβ6, or Vβ8 expression using antibodies from BD Bioscience.

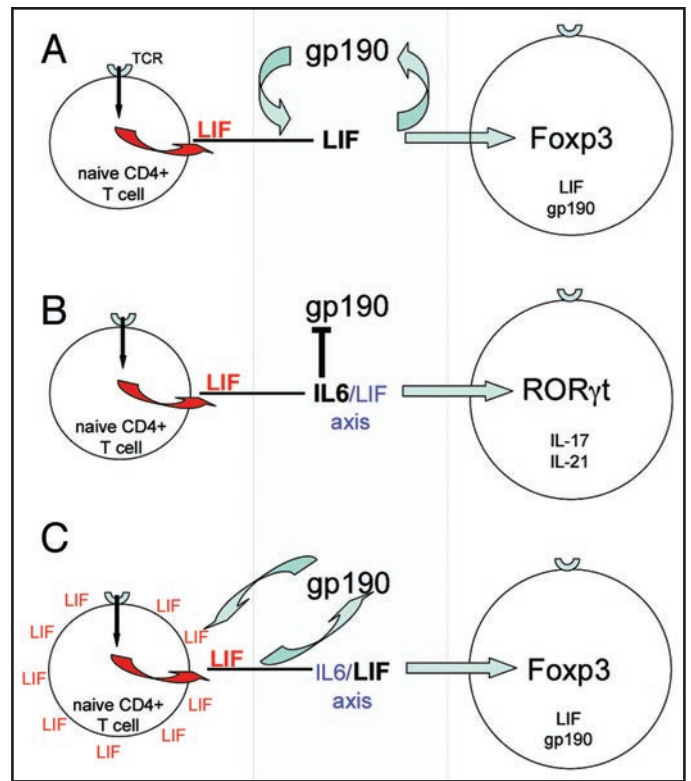**In vivo/ex vivo model of allo-tolerance versus allo-rejection.** Brief blockade of CD4 and CD8 using non-depleting antibodies was used to generate donor-specific allo-tolerant CBA (H2[k]) recipients of a full mismatch vascularised BALB/c (H2[d]) heart graft as described in detail previously.[11,17] After 100 d, spleen cells from the tolerant mice have been shown to be able to adoptively transfer donor-specific tolerance sequentially over 15 generations of naive recipients of a donor-type heart graft without further antibody therapy.[11] Adoptive transfer of tolerance required CD4^{+} T cells and was dominant in vivo over infused primed allo-aggressive spleen cells.[27] For comparison, recipients primed to reject a vascularised heart graft used the same model but without CD4/CD8 antibody therapy. Preparations of total

spleen cell populations from the in vivo allo-primed tolerant, or in vivo allo-primed rejected, recipients were challenged ex vivo with irradiated BALB/c spleen cells using tilted flasks to obtain 3D culture conditions containing 4×10^6 responders plus 6×10^6 irradiated stimulators per ml growth medium (RPMI 1640 containing 10% FCS). No growth factors were added and cultures were undisturbed until time of harvest. Supernatant was collected daily over 5 d for measurement of secreted LIF and IL6 using R&D Systems ELISA kits for LIF (MLF00) and R&D Duo Set kit DY406 for IL6.

Author contributions: S.M.M., T.B.S. and W.G. were responsible for experimental design and project progression; W.G., L.T., Q.Z. and P.P. performed experiments and analyses led by W.G. and S.M.M.; and T.M.F. both designed and prepared the cytokine-loaded nanoparticles. S.M.M. and W.G. co-wrote the manuscript.

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