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Leukemia Inhibitory Factor Inhibits T Helper 17 Cell Differentiation and Confers Treatment Effects of Neural Progenitor Cell Therapy in Autoimmune Disease

Wei Cao,[1,6] Yiqing Yang,[1,6] Zhengyi Wang,[1,6] Ailian Liu,[2] Lei Fang,[2] Fenglan Wu,[2] Jian Hong,[3] Yufang Shi,[4] Stewart Leung,[2] Chen Dong,[5] and Jingwu Z. Zhang[1,2,*]

[1]Institute of Health Sciences, Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences and Shanghai JiaoTong University School of Medicine, Shanghai 200025, China

[2]Department of Neuroimmunology, GlaxoSmithKline Research and Development Center, Shanghai 201203, China

[3]Department of Neurology, Baylor College of Medicine, Houston, TX 77030, USA

[4]Key Laboratory of Stem Cell Biology of the Chinese Academy of Sciences, Shanghai 200025, China

[5]Department of Immunology and Center for Inflammation and Cancer, M.D. Anderson Cancer Center, Houston, TX 77054, USA

[6]These authors contributed equally to this work

*Correspondence: jingwu.z.zang@gsk.com

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SUMMARY

Neural progenitor cell (NPC) therapy is considered a promising treatment modality for multiple sclerosis (MS), potentially acting through neural repair. Here, we showed that intravenous administration of NPCs ameliorated experimental autoimmune encephalomyelitis (EAE) by selectively inhibiting pathogenic T helper 17 (Th17) cell differentiation. Leukemia inhibitory factor (LIF) produced by NPCs was responsible for the observed EAE suppression. Through the inducible LIF receptor expression, LIF inhibited the differentiation of Th17 cells in EAE mice and that from MS subjects. At the molecular level, LIF exerted an opposing effect on interleukin 6 (IL-6)-induced signal transducer and activator of transcription 3 (STAT3) phosphorylation required for Th17 cell differentiation by triggering a signaling cascade that activated extracellular signal-regulated MAP kinase (ERK) and upregulated suppressor of cytokine signaling 3 (SOCS3) expression. This study reveals a critical role for LIF in regulating Th17 cell differentiation and provides insights into the mechanisms of action of NPC therapy in MS.

INTRODUCTION

Although the etiology and pathogenesis of multiple sclerosis (MS) remain elusive, it is generally considered an autoimmune disease of the central nervous system (CNS) that is characterized by multifocal inflammation, demyelination, and axonal loss and degeneration (Sospedra and Martin, 2005). Increasing evidence indicates that T helper cells with pathogenic potential (mainly interferon-γ [IFN-γ]-producing T helper 1 [Th1] cells and interleukin-17 [IL-17]-producing Th17 cells) play a key role in the inflammatory and demyelinating pathology, whereas regulatory T (Treg) cells function to keep the autoimmune response in check (Bettelli et al., 2006; Jäger et al., 2009).

Th17 cells have been found in the brain lesions of patients with MS, and elevated amounts of IL-17 have been reported in their serum and cerebrospinal fluid (Kebir et al., 2007; Tzartos et al., 2008). The critical role of Th17 cells in the disease process has been elegantly demonstrated in experimental autoimmune encephalomyelitis (EAE), an animal model of MS (Hemmer et al., 2006). Genetic deficiency of IL-17 leads to resistance to EAE, as shown by delayed onset and decreased disease severity, whereas adoptive transfer of Th17 cells derived from EAE mice into naive recipients leads to the development of the disease (El-behi et al., 2010; Furuzawa-Carballeda et al., 2007; Langrish et al., 2005). There is growing evidence for the role of Th17 cells in the pathogenesis of autoimmune disease (Dong, 2008; Komiyama et al., 2006). Th17 cell differentiation is induced by IL-6 and IL-21 in the presence of transforming growth factor-β (TGF-β) through the activation of STAT3 and retinoic-acid-receptor-related orphan receptors α and γt (RORα and RORγt, respectively) (Ivanov et al., 2006; Manel et al., 2008; Stockinger and Veldhoen, 2007; Yang et al., 2008b). The survival and expansion of committed Th17 cells require IL-23 through STAT3 and IL-7 through STAT5 (Chen et al., 2007; Harris et al., 2007; Liu et al., 2010; Mathur et al., 2007). SOCS3 serves as an important negative regulator of Th17 cells; *Socs3*$^{-/-}$ T cells exhibited greatly increased Th17 cell differentiation and cytokine expression (Chen et al., 2006).

Recently, neural progenitor cell (NPC) transplantation has been extensively studied as a novel therapeutic approach for MS because NPCs are capable of differentiating into oligodendrocytes and repairing injured myelin tissue (Pluchino et al., 2003). Prompted by this therapeutic potential, researchers have conducted experiments in EAE mice to evaluate the efficacy of NPC treatment. It has been shown that NPCs were indeed capable of differentiating into oligodendrocytes in lesioned areas of the brain and spinal cord in EAE mice after direct CNS delivery and, to some degree, intravenous delivery (Ben-Hur et al., 2003; Pluchino et al., 2003). However, there is also evidence suggesting that, in addition to their capacity for myelin repair, NPCs have anti-inflammatory properties in the

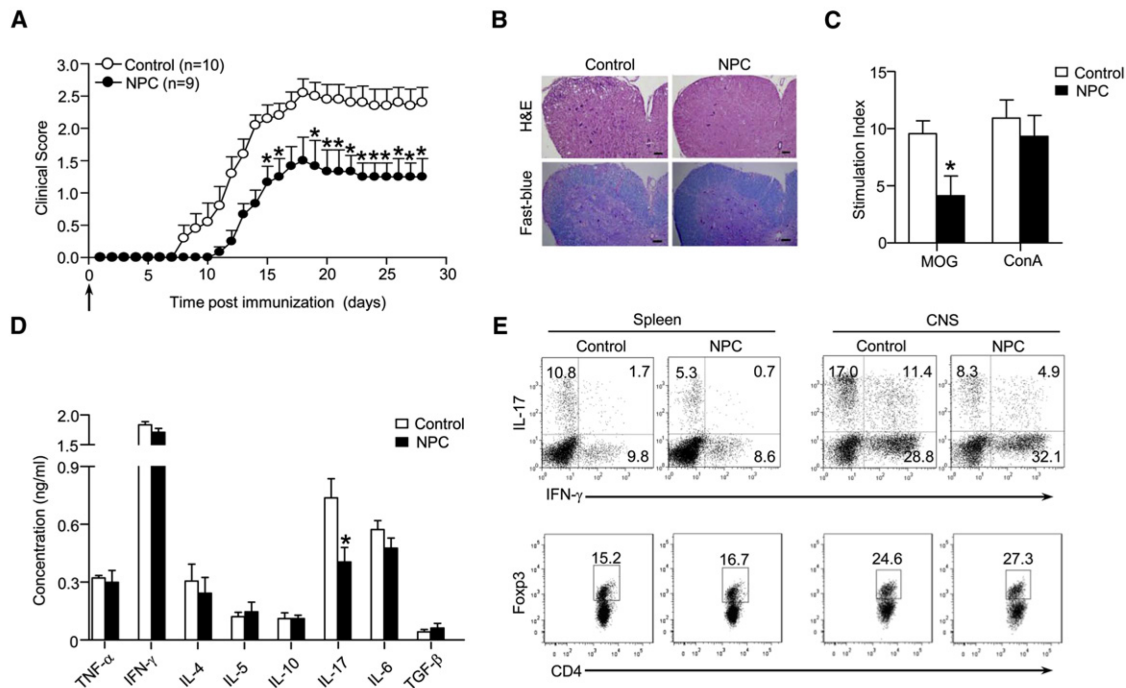


Figure 1. Amelioration of EAE by Intravenous Administration of NPCs

(A) Clinical scores of EAE mice treated with NPCs (n = 9) or PBS (n = 10) intravenously at the day of immunization as indicated by the arrow.

(B) Representative H&E-stained or Luxol fast blue-stained spinal cord sections from treated or control EAE mice harvested on day 28 postimmunization. Scale bars represent 100 μm.

(C) Splenocytes derived from treated or control EAE mice were restimulated with MOG_{35-55} (20 μg/ml) or ConA (2 μg/ml) for 72 hr. Proliferation was examined by [^{3}H]-thymidine incorporation. Stimulation index shown on the *y*-axis is calculated as C.P.M. of experiment group/C.P.M. of medium control.

(D) Cytokines production by splenocytes restimulated with MOG_{35-55} (20 μg/ml) were determined by ELISAs.

(E) Intracellular staining of IL-17, IFN-γ, and Foxp3 in $CD4^+$ T cells derived from the spleen (left) or CNS (right) of treated or control EAE mice.

*$p < 0.05$ (two-tailed Student's t test). Data are representative of three independent experiments (mean and SEM in A, C, and D).

periphery or in the CNS (Einstein et al., 2003, 2006, 2007; Pluchino et al., 2005). NPCs produce a variety of cytokines and neurotrophins, including tumor necrosis factor-α (TNF-α), brain-derived neurotrophic factor (BDNF), bone morphogenetic protein 4 (BMP-4), and leukemia inhibitory factor (LIF), some of which exhibit immunoregulatory activity by inhibiting inflammatory responses (Makar et al., 2008; Pluchino et al., 2009). However, the exact mechanism underlying the treatment efficacy of NPCs in EAE is not completely understood. In particular, the extent to which the immunoregulatory properties of NPCs contribute to their treatment efficacy in EAE is unknown.

This study was prompted by our observations that NPCs did not accumulate in the CNS upon intravenous administration and that the same EAE efficacy could be achieved with irradiated NPCs or NPC-derived supernatant. We addressed this molecular mechanism starting from the critical finding that NPCs specifically inhibited pathogenic Th17 cell differentiation through secreted factor(s). By focusing on the critical factor(s) responsible for the immunoregulatory properties of NPCs both in vitro and in vivo, we provided compelling evidence identifying LIF as the key factor directly accountable for the treatment efficacy of NPCs in EAE through the inhibition of Th17 cell differentiation. In addition, we elucidated signaling mechanism wherein LIF antagonized IL-6-induced Th17 cell differentiation, through the activation of ERK and SOCS3 expression and the subsequent inhibition of STAT3 phosphorylation. This study has important implications in the understanding of LIF biology and the development of MS therapy.

RESULTS

Amelioration of EAE and Selective Reduction of Th17 Cells by NPC Treatment

First, we validated the potential of mouse embryonic stem cell (ESC)-derived NPCs to differentiate into the three neural cell lineages, i.e., neurons, oligodendrocytes, and astrocytes (see Figure S1A available online). A single injection of mouse NPCs (2×10^6 cells per mouse) was intravenously administered to mice on the day of immunization. The treatment resulted in a substantial delay of the disease onset and a marked reduction in EAE severity accompanied by decreased inflammation and demyelination in the affected spinal cord compared to control mice (Figures 1A and 1B). Administration of NPCs via a therapeutic protocol was also efficacious in the amelioration of EAE (Figure S1B). To track the distribution pattern and kinetics of transplanted NPCs, we injected NPCs labeled with fluorescent Q-dot nanocrystals (QDs) and enumerated the labeled NPCs in the indicated tissues throughout the course of EAE (days 2, 7, 14, 21, and 30). Detailed fluorescence microscopic analyses of

serial sections spanning the entire CNS showed that QD-positive cells were rarely detectable in the CNS sections. In contrast, labeled NPCs were detected predominantly in the spleen and lymph nodes and the cell number declined over a period of 15 to 20 days (Figure S1C).

The lack of injected NPCs in CNS prompted us to investigate the possibility of a peripheral anti-inflammatory mechanism in the observed therapeutic efficacy of NPCs. We observed that MOG_{35-55}-stimulated T cell reactivity was markedly inhibited in the treated EAE mice, whereas the T cell response to ConA was not affected (Figure 1C). Furthermore, we found that the overall activation, proliferation, and survival of $CD4^+$ T cells and the function of antigen-presenting cells (APCs) were not affected in NPC-treated EAE mice (Figure S1D). Among a panel of pro- and anti-inflammatory cytokines tested, the production of IL-17, but not other cytokines, by MOG_{35-55}-stimulated T cells was significantly reduced in NPC-treated compared with control EAE mice (0.41 ± 0.07 ng/ml versus 0.74 ± 0.09 ng/ml, $p < 0.05$; Figure 1D). In parallel, the frequency of effector or infiltrating Th17 cells was markedly decreased in both the spleen and the CNS of the treated versus control EAE mice (spleen: 5.40% ± 2.32% versus 10.22% ± 2.46%, $p < 0.01$; spinal cord: 8.15% ± 2.53% versus 15.87% ± 4.07%, $p < 0.01$), whereas the percentage of Th1 cells (spleen: 8.54% ± 2.53% versus 9.88% ± 3.70%, $p > 0.05$; spinal cord: 31.07% ± 4.03% versus 28.23% ± 5.93%, $p > 0.05$) or Treg cells (spleen: 16.28% ± 3.54% versus 15.72% ± 1.91%, $p > 0.05$; spinal cord: 26.80% ± 6.42% versus 23.83% ± 4.92%, $p > 0.05$) was not affected (Figure 1E). In addition, there was no difference in the regulatory function of Treg cells in NPC-treated and control EAE mice (Figure S1E). These results indicate that intravenously administered NPCs predominantly reside in the peripheral lymphoid tissues but not in the CNS and that they ameliorate EAE by inhibiting Th17 cell differentiation.

Inhibition of Th17 Cell Differentiation by Soluble Factor(s) Secreted by NPCs

To elucidate the mechanism of action of NPCs, we injected irradiated NPCs intravenously into EAE mice. Irradiated NPCs maintained their ability to secrete cytokines and neurotrophins but were unable to undergo lineage-specific differentiation (data not shown). NPC-derived supernatant was intraperitoneally administered into EAE mice. Both treatments achieved the same therapeutic efficacy as that of nonirradiated NPCs in inhibiting EAE severity as well as in reducing the frequency of Th17 cells in the spleen and the CNS (Figures 2A and 2B). The findings further support the possibility that the therapeutic efficacy of NPCs is mediated by secreted factor(s) that modulate peripheral immunoregulation rather than a direct effect on myelin repair.

Next, we examined the effect of NPC supernatant on the differentiation of Th1, Th17, and Treg cells from purified $CD4^+CD25^-$ T cells. Addition of NPC supernatant markedly suppressed the differentiation of Th17 cells in a dose-dependent manner (Figure 2C) but did not affect the differentiation of Th1 cells or Treg cells (Figure 2D). Consistently, injection of NPC supernatant resulted in the inhibition of Th17 cell differentiation in a Th17 cell-driven EAE model with *Ifng*$^{-/-}$ (Figure 2E) or *Stat1*$^{-/-}$ mice (data not shown). These findings strongly support a peripheral mechanism of NPC therapy that acts through secreted factor(s) to selectively inhibit Th17 cell differentiation, conferring EAE treatment efficacy.

Role of LIF in NPC-Mediated Suppression of Th17 Cell Differentiation and EAE Efficacy

We then focused on identifying the secreted factor(s) responsible for the observed effects of NPCs. To this end, a panel of cytokines and neurotrophins known to be produced by NPCs were tested for their ability to suppress in vitro Th17 cell differentiation. The results led to the discovery of LIF as the primary factor for this property. First, we revealed that fully differentiated NPCs produced a substantial amount of LIF (0.84 ± 0.05 ng/ml) during differentiation from ESCs. Second, the inhibitory effect of NPC supernatant on Th17 cell differentiation in vitro was almost completely abolished by addition of a LIF-neutralizing antibody (Figure 3A). Pretreatment of NPC supernatant with the LIF-neutralizing antibody abrogated the therapeutic efficacy in EAE mice, with an associated increase in the percentage of Th17 cells in the spleen and CNS (Figure 3B). Correspondingly, the administration of a LIF neutralizing antibody into NPC-treated EAE mice completely abolished EAE efficacy of NPC treatment (Figure S2A).

Furthermore, direct administration of recombinant mouse LIF led to markedly reduced EAE severity (Figure 3C) and decreased percentage and absolute number of Th17 cells in the spleen and CNS of the treated mice (Figure 3D), whereas it had no effect on Th1 or Treg cells. The effect of LIF in EAE mice was also seen with a therapeutic protocol (Figure S2B). Consistently, LIF treatment selectively inhibited in vitro differentiation of Th17 cells in a dose-dependent manner (Figure S2C) but not the differentiation of Th1 or Treg cells (Figure 3E). We then assessed the expression of the LIF receptor (LIFR) and its dynamics in the context of Th17 cell differentiation in vivo and in vitro. The results showed that LIFR was not expressed on resting $CD4^+$ T cells in naive mice and its expression rose from less than 1% on resting T cells to more than 80% on Th17 cells both in EAE mice and during in vitro Th17 cell differentiation (Figure 3F). Taken together, these data indicate that LIF is responsible for both the inhibition of Th17 cell differentiation and the observed efficacy of NPCs in EAE.

Inhibition of Th17 Cell Differentiation by LIF through Upregulation of ERK and SOCS3 and Downregulation of STAT3 Phosphorylation

To delineate the molecular mechanism underlying the observed inhibitory effect of LIF on Th17 cell differentiation, we assessed the signaling activity of the JAK-STAT pathways, which are critically involved in the differentiation of T helper subsets, in $CD4^+$ T cells derived from LIF-treated EAE mice. Among the signaling molecules tested, the phosphorylation of JAK-2 and STAT3 was inhibited in $CD4^+$ T cells, whereas the phosphorylation of other STATs involved in Th1 or Th2 cell differentiation was not altered (Figure 4A). This finding was confirmed by in vitro experiments performed on $CD4^+$ T cells under Th1, Th17, or Th2 cell differentiation conditions (Figures 4B).

Next, we investigated the upstream signaling events involved in the alteration of STAT3 phosphorylation by LIF. Upon LIF treatment, SOCS3 expression was elevated in $CD4^+$ T cells both in vivo and in vitro (Figure 4C). The causal relationship between

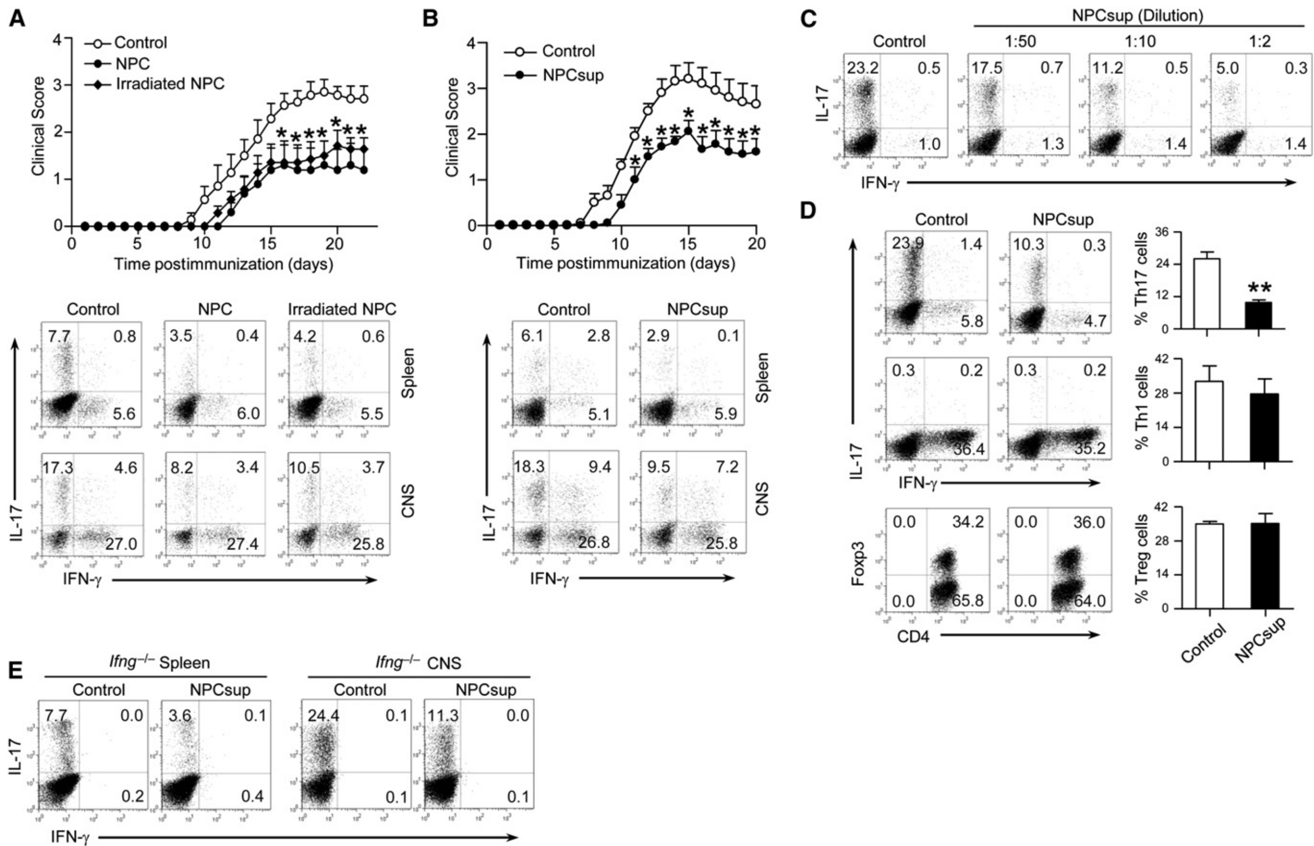


Figure 2. Selective Inhibition of Th17 Cells by Factor(s) Secreted by NPCs

(A) Clinical scores (top) and intracellular staining of IL-17 and IFN-γ in CD4+ T cells (bottom) from the spleen or CNS of EAE mice treated with NPCs (n = 7), irradiated NPCs (n = 7), or PBS (n = 8) on the day of immunization.

(B) Clinical scores (top) and intracellular staining of IL-17 and IFN-γ in CD4+ T cells (bottom) from the spleen or CNS of EAE mice treated with NPC supernatant (NPCsup, n = 7) or control medium (n = 10) every other day, starting from the day of immunization.

(C) Purified $CD4^+CD25^-$ T cells from naive mice were cultured under Th17 cell differentiation conditions in the presence of NPC supernatant at the indicated dilutions. Intracellular staining of IL-17 and IFN-γ were investigated by flow cytometry.

(D) $CD4^+CD25^-$ T cells cultured under Th17, Th1, and Treg cell differentiation conditions in the absence or presence of NPC supernatant (1:10), followed by intracellular staining of IL-17, IFN-γ, and Foxp3. Averages are presented graphically on the right.

(E) Intracellular staining of IL-17 and IFN-γ in $CD4^+$ T cells derived from the spleen or CNS of *Ifng*$^{-/-}$ mice immunized with $MOG_{35–55}$ and treated with NPC supernatant or control medium.

*$p < 0.05$ and **$p < 0.01$ (two-tailed Student's t test). Data are representative of three independent experiments (mean and SEM in A, B, and D).

increased SOCS3 expression and altered STAT3 phosphorylation in the context of LIF treatment was verified by employing specific siRNAs. Specific gene targeting of *Socs3* in $CD4^+$ T cells increased the differentiation of Th17 cells in the absence of LIF. It was evident that LIF treatment had little effect on Th17 cell differentiation and STAT3 phosphorylation when *Socs3* was targeted in $CD4^+$ T cells, confirming a SOCS3-dependent mechanism (Figure 4D). To further investigate the pathway(s) involved in LIF-induced upregulation of SOCS3 expression, we pretreated $CD4^+$ T cells with pathway-specific inhibitors, including PD98059 (a MEK1 inhibitor) and Stattic (a STAT3 inhibitor), followed by culture under Th17 cell differentiation conditions in the presence of LIF. The results showed that the MEK1-specific inhibitor abrogated the effect of LIF on increased SOCS3 expression in $CD4^+$ T cells whereas the addition of the STAT3 inhibitor did not (Figure 4E). This was further validated with a genetic approach where LIF treatment increased SOCS3 expression in *Stat3*$^{-/-}$ and wild-type $CD4^+$ T cells (Figure S3). Consistently, we found that pretreatment of $CD4^+$ T cells with a MEK1 inhibitor led to a loss of the inhibitory effect of LIF on STAT3 phosphorylation and Th17 cell differentiation, indicating that the regulation of SOCS3 by LIF involves the ERK signaling pathway (Figure 4E). In addition, inhibition of Th17 cell differentiation and the associated changes in signaling activities (ERK, SOCS3, and STAT3) were abrogated when LIFR expression was silenced with specific siRNAs (Figures 4F and 4G). These results strongly suggest that LIF signals through LIFRs to trigger an inhibitory signaling cascade, opposing IL-6-induced STAT3 phosphorylation critical for Th17 cell differentiation. In addition, treatment of NPC supernatant had similar effects on upregulation of ERK phosphorylation and increased SOCS3 expression, which correlated with impaired STAT3 phosphorylation and Th17 cell differentiation and reversed by the addition of LIF-neutralizing antibody (Figures 5A and 5B).

Figure 3. A Critical Role of LIF in the Inhibition of Th17 Cells

(A) Intracellular staining of IL-17 and IFN-γ in CD4⁺ T cells cultured under Th17 cell differentiation conditions in the absence or presence of NPC supernatant or NPC supernatant preincubated with a LIF-neutralizing antibody or an isotype control.

(B) NPC supernatant or control medium was preincubated with a LIF-neutralizing antibody or an isotype control and subsequently intraperitoneally injected into EAE mice (n = 8 per groups) every other day, starting from the day of immunization. Clinical scores and intracellular staining of IL-17 and IFN-γ in CD4⁺ T cells derived from the spleen or CNS of these groups.

(C) Clinical scores of EAE mice treated intraperitoneally with LIF (10 μg/kg) (n = 6) or PBS (n = 6) daily, starting from the day of immunization.

(D) Intracellular staining of IL-17, IFN-γ, and Foxp3 in CD4⁺ T cells from the spleen or CNS of EAE mice as described above. The absolute counts of CD4⁺IL-17⁺ (Th17) cells in the spleen or CNS are presented in the right panel.

(E) Flow cytometric analysis of Th17, Th1, and Treg cell subsets under individual lineage-specific conditions in the presence or absence of LIF (50 ng/ml).

(F) Surface expression of LIFR on naive CD4⁺ T cells or gated Th17 cells in CD4⁺ T cells that were derived from EAE mice at the indicated stage (top) or under in vitro Th17 cell differentiation conditions at the indicated time point (bottom).

*p < 0.05 and **p < 0.01 (two-tailed Student's t test). Data are representative of three independent experiments (mean and SEM in B, C, and D).

Signaling Characteristics of LIF in Activated CD4⁺ T Cells

The data described above suggest that LIF inhibited Th17 cell differentiation by opposing STAT3 phosphorylation induced by IL-6. Because LIF was previously shown to act independently in upregulating STAT3 activity in some non-T cell types (Joly et al., 2008; Setati et al., 2010; Slaets et al., 2008; Yu et al., 2009), we examined the signaling characteristics of LIF in CD4⁺ T cells in the absence of IL-6. To this end, CD4⁺ T cells derived from *Il6*$^{-/-}$ mice were used to exclude any confounding effect resulting from endogenously produced IL-6. We demonstrated that unlike the constitutively expressed IL-6R, LIFR was expressed on *Il6*$^{-/-}$ CD4⁺ T cells only after TCR stimulation (Figure 6A). Consistently, LIF did not induce STAT3 phosphorylation in *Il6*$^{-/-}$ CD4⁺ T cells when LIFR was not expressed in the absence of TCR stimulation (data not shown). However, once the expression of LIFR was induced by TCR stimulation, LIF was capable of inducing a low and transient STAT3 phosphorylation in *Il6*$^{-/-}$ CD4⁺ T cells as compared to the strong and sustained STAT3 phosphorylation triggered by IL-6 (Figure 6B). As a result, exogenously added IL-6 stimulated Th17 cell differentiation of *Il6*$^{-/-}$ CD4⁺ T cells in the presence of TGF-β whereas

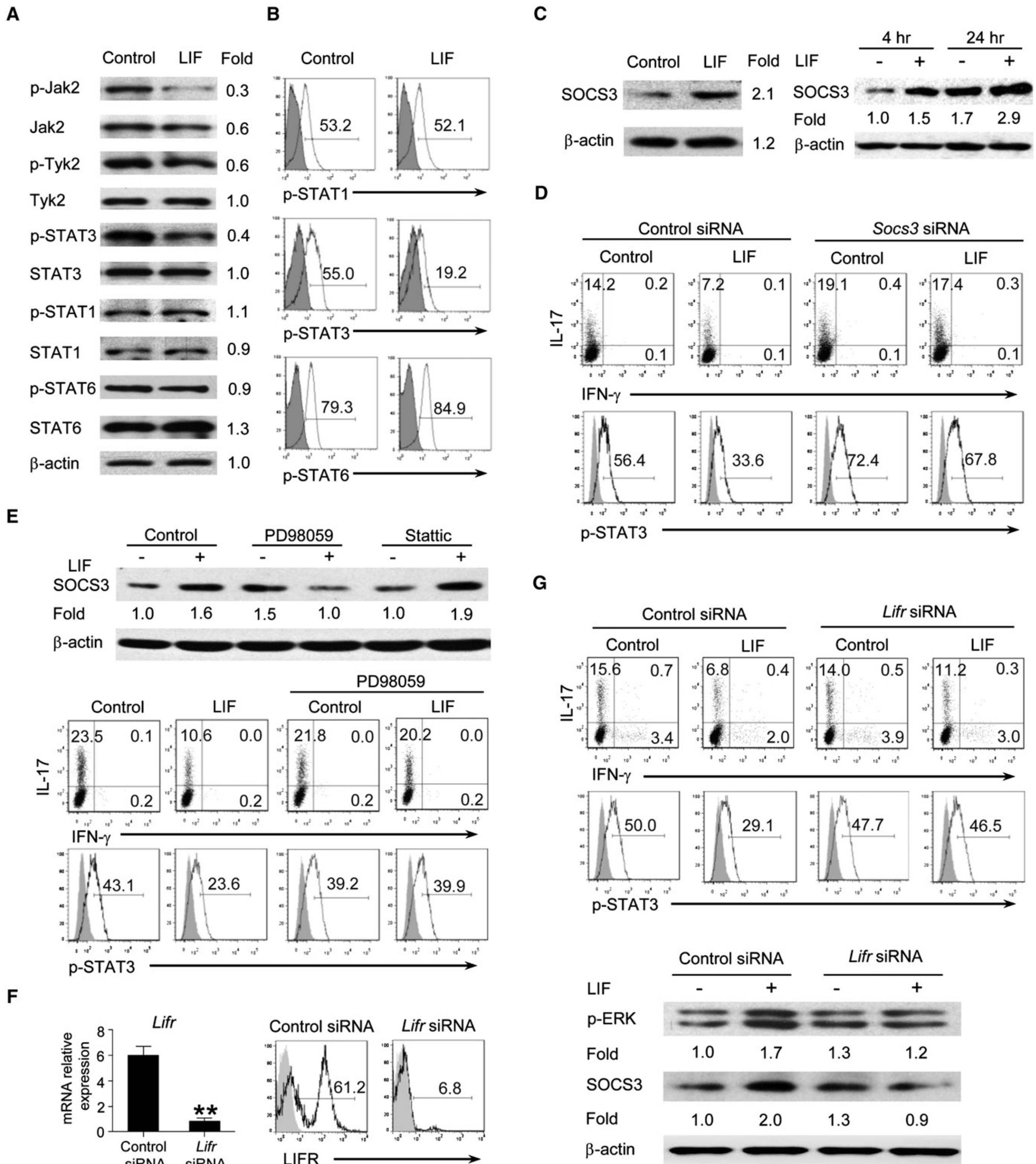


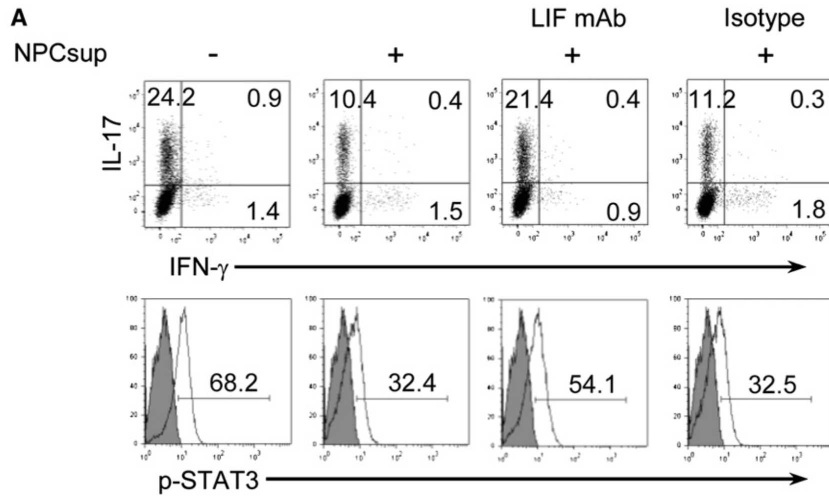
Figure 4. Mechanism Underlying the Inhibition of Th17 Cell Differentiation by LIF

(A) Immunoblot analysis of JAK-STATs in CD4^{+} T cells from lymph nodes of EAE mice treated with LIF or PBS.

(B) Flow cytometric analysis of the levels of phosphorylated STAT1, STAT3, and STAT6 in CD4^{+} T cells under in vitro Th1, Th17, or Th2 cell differentiation conditions in the presence of LIF (50 ng/ml).

(C) SOCS3 expression determined by immunoblotting in CD4^{+} T cells from lymph nodes of LIF-treated or control EAE mice (left) or under in vitro Th17 cell differentiation conditions in the presence of LIF (50 ng/ml) (right).

(D) Flow cytometric analysis of Th17 cell differentiation and STAT3 phosphorylation in CD4^{+} T cells transfected with *Socs3*-specific or control siRNAs under Th17 cell polarization conditions in the presence or absence of LIF (50 ng/ml).



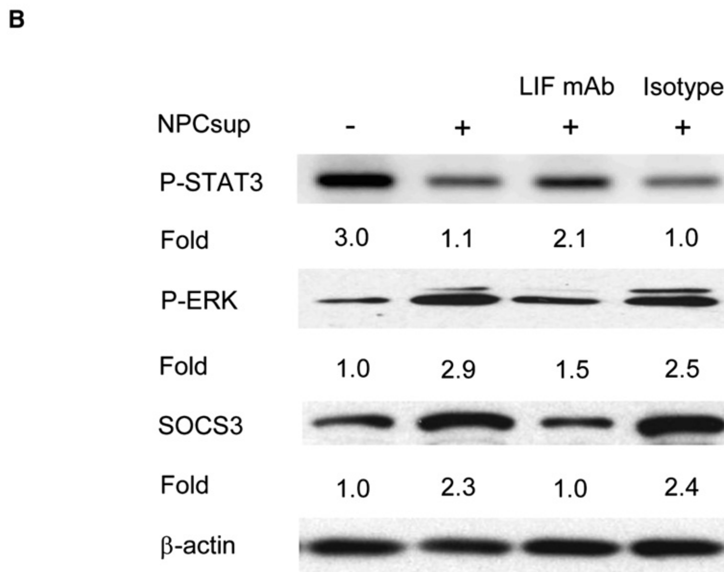


Figure 5. Role of LIF in the Signaling Cascade Triggered by NPC Supernatant in Th17 Cells

(A) $CD4^+CD25^-$ T cells were cultured under Th17 cell differentiation conditions in the presence of NPC supernatant preincubated with a LIF-neutralizing antibody or an isotype control. The percentage of Th17 cells and the level of phosphorylated STAT3 in $CD4^+$ T cells were investigated by flow cytometry.

(B) Immunoblot analysis of phosphorylated STAT3, phosphorylated ERK, and SOCS3 expression from the same cell preparations as in (A).

Data are representative of four independent experiments.

LIF did not (34.56% ± 3.42% versus 0.84% ± 0.17%, $p < 0.01$; Figure 6B), suggesting that this transient induction of STAT3 phosphorylation by LIF is not sufficient to induce Th17 cell differentiation.

We hypothesized that LIF-induced STAT3 phosphorylation could not sustain because the LIF signaling was subsequently dominated by increased ERK and SOCS3 activities that suppressed STAT3 phosphorylation. We investigated the transient nature of LIF-induced STAT3 phosphorylation by measuring the activities of ERK and SOCS3 in relation to STAT3 phosphorylation at both the early (30 min) and the later (4 to 24 hr) time points after LIF treatment. The initial STAT3 phosphorylation at the early time point corresponded to minimal SOCS3 activity, and its phosphorylation level decreased along with progressive increases in both ERK and SOCS activity at the later time points (Figure 6C). Correspondingly, when SOCS3 expression was silenced with specific siRNAs, LIF-induced STAT3 sustained throughout (Figure 6D). Taken together, as in non-T cells, LIF also induces STAT3 phosphorylation in $CD4^+$ T cells in the absence of IL-6. However, the failure of the initial STAT3 phosphorylation to persist is attributable to the activity of SOCS3 signaling initiated by LIF.

Inhibition of Human Th17 Cell Differentiation by NPC Supernatant and Recombinant LIF

A significant amount of LIF was secreted by human NPCs in the culture (0.72 ± 0.07 ng/ml at 72 hr, $p < 0.01$; Figure 7A). To determine the effect of LIF on human Th17 cell development, we first induced Th17 cell differentiation of purified $CD4^+CD25^-CD45RA^+$ T cells from healthy individuals in the presence of human NPC supernatant. Addition of the NPC supernatant inhibited Th17 cell differentiation and STAT3 phosphorylation in a dose-dependent manner, which was abolished by the addition of human LIF-neutralizing antibody (Figures 7B and 7C; Table S1). The direct role of LIF in Th17 cell differentiation and STAT3 phosphorylation was confirmed in parallel experiments with recombinant human LIF (Figure 7D;

(E) Purified $CD4^+$ T cells were preincubated with the indicated inhibitors and cultured in the presence or absence of LIF (50 ng/ml) under Th17 cell differentiation conditions. Immunoblotting analysis of SOCS3 expression (top) and flow cytometric analysis of the percentage of Th17 cells and the level of phosphorylated STAT3 (bottom) in these $CD4^+$ T cells.

(F) Purified $CD4^+$ T cells were transfected with *Lifr*-specific or control siRNAs and subsequently cultured under Th17 cell differentiation conditions in the presence of LIF (50 ng/ml). LIFR expression was analyzed by real-time PCR and flow cytometry.

(G) The percentage of Th17 cells and the level of phosphorylated STAT3 by intracellular staining (top) and the expression of phosphorylated ERK and SOCS3 by immunoblotting (bottom) were analyzed from the same cell preparations as in (F).

*$p < 0.05$ and **$p < 0.01$ (two-tailed Student's t test). Data are representative of four independent experiments (mean and SEM in F).

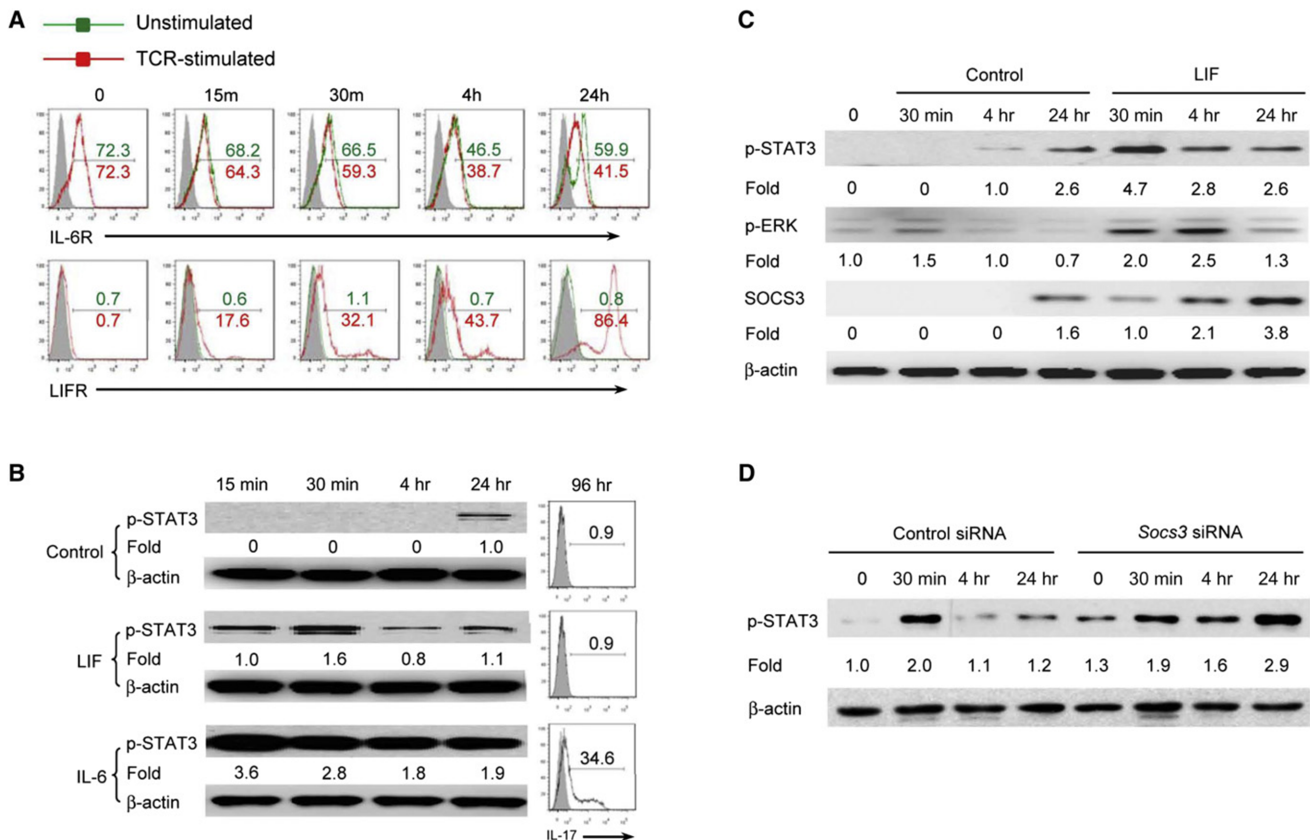


Figure 6. Signaling Cascades of LIF Independent of IL-6

(A) Expression of IL-6R and LIFR on purified *Il6*$^{-/-}$ CD4^{+} T cells stimulated with or without CD3- and CD28-specific antibodies (red or green, respectively) at the indicated time points.

(B) Purified *Il6*$^{-/-}$ CD4^{+} T cells were stimulated with CD3- and CD28-specific antibodies in the absence or presence of LIF (50 ng/ml) or IL-6 (20 ng/ml). Immunoblot analysis of the kinetics of STAT3 phosphorylation in these CD4^{+} T cells at the indicated time points (left) and the percentage of Th17 cells when these cells were cultured in the presence of TGF-β (right).

(C) Immunoblot analysis of the levels of phosphorylated STAT3, phosphorylated ERK, and SOCS3 expression in activated *Il6*$^{-/-}$ CD4^{+} T cells in the presence or absence of LIF (50 ng/ml).

(D) Purified *Il6*$^{-/-}$ CD4^{+} T cells were transfected with *Socs3*-specific or control siRNAs and then treated with or without LIF (50 ng/ml) in the presence of TCR stimulation. Levels of phosphorylated STAT3 were determined by immunoblotting at the indicated time points.

Data are representative of three independent experiments.

Table S1). As shown in EAE mice, LIF did not affect the differentiation of Th1 or Treg cells from the same CD4^{+} T cell preparations (Figure S4). Furthermore, in experiments with purified CD4^{+} T cells from a panel of 18 subjects with MS, LIF was found to suppress the production of IL-17 under Th17 cell differentiation conditions (Figure 7E). Therefore, human NPC supernatant and recombinant human LIF had a similar role in the inhibition of Th17 cell differentiation in healthy individuals or MS subjects as it did in EAE.

DISCUSSION

It is generally thought that the therapeutic efficacy of NPCs in EAE is primarily mediated through a direct neural repair mechanism. Based on this notion, NPC-based cell therapy has been investigated as a potential treatment modality for MS. Although there is experimental evidence supporting the repair mechanism of NPCs in EAE after direct injection into the CNS, the current hypothesis does not address the possible contribution of a non-neural peripheral mechanism potentially involving immunoregulatory mechanism. Here, we provide compelling evidence showing that when peripherally administered, NPCs have potent immunoregulatory properties that can account for their treatment efficacy in EAE, independent of the myelin repair mechanism. Our experimental systems employed here allow the separation of anti-inflammatory action from the central myelin repair mechanism. This conclusion is supported by the lack of NPCs in the CNS tissue of treated EAE mice and by the lack of evidence for myelin repair. The definitive evidence for the peripheral regulatory mechanism comes from the observation that the EAE efficacy can be achieved with cell-free supernatant derived from NPCs or minimally irradiated NPCs that are unable to differentiate but are capable of secreting cytokines and neurotrophins.

One of the most challenging aspects of this study was to identify the critical factor(s) secreted by NPCs responsible for their treatment efficacy in EAE as well as the corresponding molecular

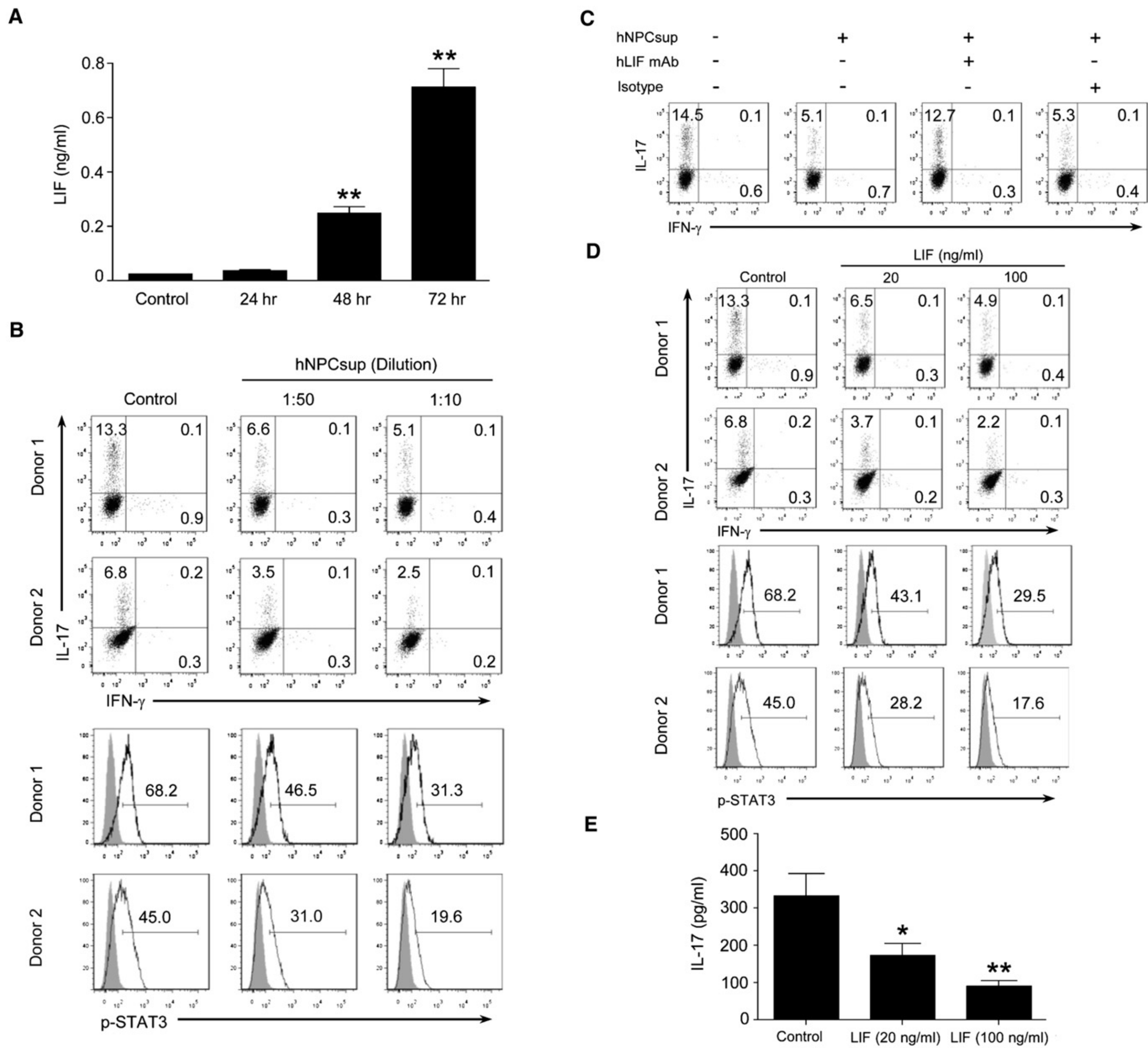


Figure 7. Effect of Human NPC Supernatant and Recombinant LIF on Human Th17 Cells

(A) Concentrations of LIF in the supernatant of human NPC culture measured by ELISA at the indicated time points.

(B) $CD4^{+}CD25^{-}CD45RA^{hi}$ naive T cells derived from healthy individuals (n = 15) were cultured under Th17 cell polarization conditions in the presence of human NPC supernatant at the indicated dilutions and analyzed for the percentage of Th17 cells (top) and the level of phosphorylated STAT3 (bottom).

(C) The percentage of Th17 cells analyzed in the same culture as described above in the presence of a LIF-neutralizing antibody or an isotype control.

(D) The same cell preparations as in (B) were cultured under Th17 cell polarization conditions in the presence of recombinant human LIF at the indicated concentrations. The percentage of Th17 cells and the level of STAT3 phosphorylation were determined by flow cytometry.

(E) Purified $CD4^{+}$ T cells from MS subjects (n = 18) were treated with recombinant LIF under the Th17 cell differentiation conditions. Supernatants were measured for IL-17 production by ELISA.

$^{*}p < 0.05$ and $^{**}p < 0.01$ (two-tailed Student's t test). Data are representative of and reproducible from at least ten individual specimens (mean and SEM in A and E).

mechanisms of action. We provide compelling evidence supporting a critical role of LIF in inhibiting Th17 cell differentiation and in mediating the efficacy of NPCs in EAE. Mouse and human NPCs used here produce large amounts of endogenous LIF. Neutralization of LIF in NPC supernatant or administration of LIF antibody in NPC-treated mice abolished the effects on Th17 cell differentiation and the efficacy against EAE. Moreover, direct administration of recombinant LIF was efficacious in EAE and LIF directly inhibited Th17 cell differentiation in both mouse $CD4^{+}$ T cells and human $CD4^{+}$ T cells. This study sheds light on the critical role of LIF in Th17 cell biology in an autoimmune disease setting.

It is remarkable that the anti-inflammatory properties of NPCs and the effects of LIF treatment in EAE mice are mediated not

through generalized immune suppression but through a highly selective inhibition of Th17 cell differentiation, sparing Th1, Treg, and Th2 cells (data not shown). Our study provides a detailed account of the underlying molecular mechanism and the key signaling events responsible for the inhibition of Th17 cell differentiation by LIF. In this regard, our in vitro and ex vivo studies revealed the SOCS3-STAT3 pathway to be the key axis in the signaling cascade that mediates the inhibition of Th17 cell differentiation in response to LIF treatment. The role of SOCS3 described here is consistent with previous reports indicating that SOCS3 deficiency in T cells results in higher numbers of Th17 cells both in vitro and in vivo (Chen et al., 2006). Furthermore, the critical involvement of SOCS3 in Th17 cell differentiation offers an explanation for the selective effect of LIF on Th17 cells, sparing other $CD4^+$ T cell subsets, because SOCS3 deficiency has no significant effect on Th1 and Th2 cell differentiation and Treg cells do not express SOCS3 (Chen et al., 2006; Kinjyo et al., 2006).

How does LIF regulate signaling pathways in $CD4^+$ T cells in relation to Th17 cell differentiation in the context of the dynamic expression of LIF receptors? A LIF receptor is composed of a signaling subunit (gp130) and a specific receptor binding subunit for LIF (LIFR) (Kristensen et al., 2005). IL-6 receptors share gp130 with LIF receptors in addition to IL-6R as a specific subunit (Yu et al., 2007). We provide a compelling account of the LIF signaling mechanism in relation to that of IL-6 in Th17 cell differentiation. On one hand, IL-6 signals through gp130 and induces STAT3 phosphorylation by recruiting JAK1, JAK2, and tyrosine kinase 2 (Tyk2) (Heinrich et al., 2003), leading to the Th17 cell differentiation. On the other hand, through the inducible and progressive expression of LIFR during Th17 cell differentiation, LIF triggers an inhibitory signaling cascade by upregulating ERK and SOCS3 activities. The interaction of LIF signaling with IL-6-induced events leads to the inhibition of STAT3 phosphorylation and Th17 cell differentiation. It is conceivable that LIF may act through the recruitment of SH2 domain-containing protein-tyrosine phosphatase (SHP2) to activate the ERK pathway because SHP2 functions as an adaptor by linking growth factor receptor-bound protein 2 (Grb-2) in the process (Clahsen et al., 2005).

How do our findings reconcile with the previous observations that LIF independently induces STAT3 phosphorylation in some other cell types? In short, our study has confirmed that, in the absence of IL-6, LIF induces STAT3 phosphorylation in $CD4^+$ T cells immediately after TCR stimulation, at which time the opposing signaling mediated through ERK and SOCS3 is not fully initiated. However, the initial STAT3 phosphorylation induced by LIF is rapidly suppressed by the opposing signaling dominated by SOCS3 at the later time points. Therefore, our findings explain why the transient STAT3 phosphorylation is neither sustainable nor sufficient to induce the Th17 cell phenotype. This phenomenon of the transient induction of STAT3 phosphorylation has also been seen in mouse ESCs (Boyle et al., 2009; Forrai et al., 2006).

The discovery described here supports LIF as a potential therapeutic target for MS. The potential therapeutic advantage of LIF stems from its dual action involving an immunoregulatory mechanism observed in both EAE mice and MS-derived T cells (as described here) and a neural repair mechanism previously reported by Kilpatrick and colleagues (Slaets et al., 2010). In their studies with both EAE and a chemically induced demyelination model, a significant fraction of the peripherally administered LIF was shown to reach the CNS compartment, corresponding to the reduction in the EAE severity and the extent of demyelination by enhancing oligodendrocyte survival (Butzkueven et al., 2002; Marriott et al., 2008). It is conceivable that both mechanisms induced by LIF can work synergistically to achieve desirable therapeutic effects in MS in which the CNS pathology involves both inflammation and demyelination. This therapeutic advantage of LIF is rare among the current MS therapies and is urgently needed for the development of a more effective disease-modifying treatment for MS.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 mice were purchased from Shanghai Laboratory Animal Center. *Ifng*$^{-/-}$ and *Il6*$^{-/-}$ mice were from the Jackson Laboratory. *Stat1*$^{-/-}$ mice were purchased from Taconic. All experiments were performed with mice 6–10 weeks old with protocols approved by the Institutional Animal Care and Use Committee.

Neural Progenitor Cell Culture and Supernatant Preparation

Colonies of undifferentiated mouse ESCs (C57BL/6 mouse line) were generously provided by Y. Jin (Chinese Academy of Sciences) and cultured in LIF-supplemented mouse ESC medium (Ogawa et al., 2007). Differentiation and culture of ES-derived NPCs were described in detail in Supplemental Experimental Procedures. For irradiated NPCs, NPCs were irradiated (18 Gy) to render them incapable of differentiation. Human NPCs derived from hESCs (H9 cell line) were purchased from Invitrogen (N7800-100) and cultured according to the manufacturer's instructions. Supernatant of both mouse and human NPCs was harvested at 24 hr, 48 hr, and 72 hr from NPC cultures at a cell density of 1×10^6 cells/ml.

Induction and Treatment of EAE

EAE was induced by complete Freund's adjuvant-$MOG_{35\text{-}55}$ peptide immunization and scored daily (Wang et al., 2007). For a prevention protocol, NPCs or irradiated NPCs were intravenously injected into mice on the day of immunization (2×10^6 cells per mouse). NPC supernatant or control medium (200 μl per mouse) preincubated with LIF-neutralizing antibody (10 μg per mouse, R&D systems) or an isotype control antibody (10 μg per mouse, Jackson ImmunoResearch) was intraperitoneally injected into mice every other day starting from the day of immunization. LIF-neutralizing antibody or isotype control antibody was administrated intraperitoneally into NPC-treated or control EAE mice every other day, starting from the day of immunization. For a therapeutic protocol, NPCs (2×10^6 cells per mouse) were intravenously injected into EAE mice on day 10 postimmunization. Recombinant mouse LIF (Millipore) was injected intraperitoneally into mice every day, starting from the day of immunization (per prevention protocol) or from day 10 postimmunization (per therapeutic protocol).

Histology and Immunofluorescence

Spinal cords from treated or control EAE mice were fixed in 4% paraformaldehyde and paraffin embedded. Sections of 5 μm were stained with Luxol fast blue or H&E. Tissue sections of brains, spinal cords, and peripheral lymphoid organs (spleen and lymph nodes) were obtained from EAE mice treated with Q-dot-labeled NPCs at days 2, 7, 14, 21, and 30 after injection. To enumerate Q-dot-positive cells, a total of 80 spleen sections, 20 lymph node sections (4 lymph nodes per mouse), 120 brain sections, and 50 spinal cord sections were obtained from each mouse at 25 μm intervals and analyzed by fluorescence microscopy. Undifferentiated NPCs were stained for Nestin and Sox2 (Chemicon), and after 7 days of differentiation, NPCs were stained for Tuj-1, CNPase, and GFAP (Chemicon).

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Proliferation Analysis and ELISAs

For recall experiments, splenocytes were stimulated with MOG_{35-55} (20 μg/ml) or ConA (2 μg/ml) 3 days prior to 18 hr pulse with [^{3}H]-thymidine. For Treg cell suppression assays, freshly isolated splenic $CD4^+CD25^-$ T cells from EAE mice were used as responder (4×10^4 cells/well) and cultured with 2 μg/ml ConA and 8×10^4 irradiated (18 Gy) syngeneic splenic APCs from EAE mice in the absence or presence of $CD4^+CD25^+$ Treg cells at a density of 4×10^4/well. During the last 18 hr, cells were pulsed with [^{3}H]-thymidine before harvest. Supernatants from MOG-restimulated or NPC cultures were harvested and levels of cytokines or LIF production were measured by ELISA Kits (R&D Systems).

T Cell Purification

$CD4^+$ T cells were purified by a CD4 Negative Isolation Kit (Miltenyi Biotec), and $CD4^+CD25^-$ T cells were further prepared by FACS sorting from MACS-sorted $CD4^+$ T cells. CNS-infiltrating mononuclear cells from EAE mice were prepared by Percoll gradient separation (Wang et al., 2006). Peripheral blood mononuclear cells were obtained from patients (n = 18) with clinically defined MS or healthy volunteers (n = 15) after informed consent and the protocol was approved by the Institutional Review Board at Baylor College of Medicine and Institute of Health Sciences. $CD4^+CD45RA^{hi}CD25^-$ naive T cells were sorted by FACS.

In Vitro T Helper Cell Differentiation

Purified $CD4^+CD25^-$ T cells were stimulated with antibodies to CD3 (5 μg/ml) and CD28 (2 μg/ml) under Th17 cell differentiation conditions (rmIL-6, 20 ng/ml; R&D Systems; rhTGF-β1, 3 ng/ml; anti-IL-4, 10 μg/ml; and anti-IFN-γ, 10 μg/ml; BD Biosciences), Th1 cell differentiation conditions (rmIL-12, 10 ng/ml and anti-IL-4, 10 μg/ml; BD Biosciences), Th2 cell differentiation conditions (rmIL-4, 20 ng/ml; rmIL-2, 10 ng/ml; R&D Systems and anti-IFN-γ, 10 μg/ml; BD Biosciences), or Treg cell differentiation conditions (rhTGF-β1, 5 ng/ml and rmIL-2, 40 ng/ml; R&D Systems) in the presence or absence of NPC supernatant or recombinant mouse LIF (50 ng/ml). In some experiments, T cell preparations were preincubated with specific inhibitors (PD98059 or Stattic, Calbiochem) for 1 hr.

$CD4^+CD45RA^{hi}CD25^-$ naive T cells prepared from healthy individuals were differentiated into Th17 cells as previously described (Yang et al., 2008a). $CD4^+$ T cells prepared from MS subjects were stimulated with a cocktail containing rhIL-6 (50 ng/ml), rhIL-1β (10 ng/ml), and rhIL-23 (20 ng/ml) plus antibodies to IFN-γ and IL-4 (10 μg/ml). Human NPC supernatant or recombinant human LIF expressed by HEK293 cells (293-6E) were added when indicated. In some experiments, mouse NPC supernatant or human NPC supernatant was preincubated with LIF-neutralizing antibodies (10 μg/ml) or isotype control for 1 hr prior to adding into culture.

Immunoblotting

$CD4^+$ T cells from lymph nodes of EAE mice or in vitro differentiated Th17 cells were lysed in ice-cold buffer containing a protease inhibitor cocktail (Roche). The lysates were fractionated by SDS-PAGE and analyzed by immunoblotting with specific antibodies to phospho-Jak2, Jak2, phospho-Tyk2, Tyk2, phosphor-STAT1, STAT1, phospho-STAT3, STAT3, phospho-STAT6, STAT6, phosphor-ERK, ERK, SOCS3 (Cell Signaling Technology), and β-actin (Sigma).

siRNA Transfection

Purified $CD4^+$ T cells were transfected with *Socs3*, *Lifr*, or nontargeting control siRNAs with a Mouse T Cell Nucleofector Kit (Amaxa, Germany) according to the manufacturer's instructions. Mouse *Lifr*, *Socs3*, and nontargeting control siRNAs were from Dharmacon. Gene expression analysis was assessed by real-time PCR with SYBR Green Master Mix under standard thermocycler conditions (Applied Biosystems). Gene expression was normalized to the *Actb* reference gene.

Statistical Analysis

Statistical significance was determined by performing a two-tailed Student's t test. p values < 0.05 were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at doi:10.1016/j.immuni.2011.06.011.

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