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Suppressive Effect of IL-27 on Encephalitogenic Th17 Cells and the Effector Phase of Experimental Autoimmune Encephalomyelitis[1]

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IL-27 has been shown to play a suppressive role in experimental autoimmune encephalomyelitis (EAE) as demonstrated by more severe disease in IL-27R-deficient (WSX-1$^{-/-}$) mice. However, whether IL-27 influences the induction or effector phase of EAE is unknown. This is an important question as therapies for autoimmune diseases are generally started after autoreactive T cells have been primed. In this study, we demonstrate maximal gene expression of IL-27 subunits and its receptor in the CNS at the effector phases of relapsing-remitting EAE including disease peak and onset of relapse. We also show that activated astrocyte cultures secrete IL-27p28 protein which is augmented by the endogenous factor, IFN-γ. To investigate functional significance of a correlation between gene expression and disease activity, we examined the effect of IL-27 at the effector phase of disease using adoptive transfer EAE. Exogenous IL-27 potently suppressed the ability of encephalitogenic lymph node and spleen cells to transfer EAE. IL-27 significantly inhibited both nonpolarized and IL-23-driven IL-17 production by myelin-reactive T cells thereby suppressing their encephalitogenicity in adoptive transfer EAE. Furthermore, we demonstrate a strong suppressive effect of IL-27 on active EAE in vivo when delivered by s.c. osmotic pump. IL-27-treated mice had reduced CNS inflammatory infiltration and, notably, a lower proportion of Th17 cells. Together, these data demonstrate the suppressive effect of IL-27 on primed, autoreactive T cells, particularly, cells of the Th17 lineage. IL-27 can potently suppress the effector phase of EAE in vivo and, thus, may have therapeutic potential in autoimmune diseases such as multiple sclerosis. ***The Journal of Immunology,*** **2007, 179: 3268–3275.**

Multiple sclerosis (MS)[4] is a complex inflammatory, demyelinating disease of the CNS affecting over 2 million people worldwide. MS is second only to trauma as the leading cause of neurological disability in young adults (1). Experimental autoimmune encephalomyelitis (EAE) is a widely used animal model of CNS inflammatory demyelination and has directly led to the development of three currently approved treatments for MS (2). Although the pathogenesis is incompletely understood, MS is believed to be a T cell-mediated autoimmune disease which may have an as yet unidentified, infectious component (3). Th1 immune responses were thought to mediate inflammatory demyelination in MS and EAE; however, there is now growing evidence implicating the newly characterized IL-17-producing Th cell population (Th17) in the pathogenesis of these and other autoimmune diseases (4–6).

IL-27 is a novel heterodimeric cytokine consisting of an EBI-3 subunit and a p28 subunit which are structurally related to the IL-12/IL-23 subunits p40 and p35/p19, respectively (7). IL-27 signals through its heterodimeric receptor consisting of a WSX-1 (also known as TCCR) subunit and the signaling gp130 subunit which is shared by other cytokine receptor complexes (8). Receptor ligation induces intracellular signaling via heterogeneous Jak/STAT pathways, depending on the cell type (9–11).

Initial studies on IL-27 demonstrated a role in the early phase of Th1 induction (12, 13). IL-27 signaling was shown to activate the Th1-specific transcription factor, T-bet, resulting in up-regulated expression of IL-12Rβ2 on naive T cells. This renders these cells responsive to the Th1-inducing cytokine, IL-12 (14). However, more recent studies have demonstrated anti-inflammatory properties of IL-27 signaling. Studies with infectious and autoimmune inflammatory models have shown that mice lacking the WSX-1 subunit of the IL-27R complex develop excessive, pathological inflammation during both Th1 and Th2 responses suggesting broad anti-inflammatory functions of IL-27 (15, 16). Molecular mechanisms underlying such functions have begun to be elucidated. Hunter and colleagues (17) showed that IL-27 can limit IL-2 production during Th1 differentiation and Owaki et al. (18) showed that suppressor of cytokine signaling 3 (SOCS3) plays an important role in this inhibitory effect. It appears, therefore, consistent with many other cytokines, that IL-27 has pleiotropic roles in immune responses.

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[4] Abbreviations used in this paper: MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; RR, relapsing-remitting; MOG, myelin oligodendrocyte glycoprotein; DLN, draining lymph node; rm, recombinant murine; CBA, cytometric bead array; LFB, Luxol Fast Blue; SOCS, suppressor of cytokine signaling.

The recent identification of the Th17 lineage has resulted in a significant revision of the Th1-Th2 paradigm (5, 19). Th17 cells characteristically produce, among other cytokines, high levels of IL-17A and IL-17F. This effector T cell population has been shown to play an important role in immune responses to extracellular bacteria as well as a pathogenic role in autoimmunity (5, 20–22). Recently, two groups demonstrated that IL-27 suppresses Th17 immune responses. Stumhofer et al. (23) showed enhanced CNS inflammation associated with augmented Th17 responses in *Toxoplasma gondii*-infected WSX-1$^{-/-}$ mice. This group demonstrated that IL-27-mediated Th17 suppression was dependent on STAT1 but was independent of SOCS3 and was not attributable to competition with IL-6 for the shared gp130 receptor subunit. Batten et al. (24) showed that these IL-27R-deficient mice are hypersusceptible to EAE induced by active immunization with myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$ in CFA, and have elevated numbers of Th17 cells. However, the disease phase at which IL-27 exerted its suppressive effect was not established in the EAE model. This question is critical as optimal treatments for autoimmune conditions require efficacy following presentation of symptoms, which corresponds to the effector phase of disease. Thus, in the current study, we focused on the role of IL-27 in this phase of disease and, in particular, its effect on T cells that have already been primed with autoantigen using adoptive transfer EAE. We developed this investigation further by examining the effect of exogenous IL-27 on active EAE.

Building on our previous studies of gene expression analysis in chronic EAE (25), we sought to further dissect temporal and spatial characteristics of IL-27 expression, and that of its receptor, in relapsing-remitting (RR) EAE. In the spinal cord, we found highest expression of IL-27 and its receptor at the effector phases of RR-EAE, disease peak, and relapse. To investigate the influence of IL-27 signaling specifically on the effector phase of T cell-mediated autoimmune inflammation, we used murine adoptive transfer EAE models. In these models (26, 27), mice are immunized with myelin Ag in adjuvant but cells are harvested from the periphery before migration to the CNS. These cells are then restimulated in vitro which not only expands the effector, myelin-reactive population but also provides an opportunity for in vitro manipulation and characterization of these cells. In this study, we show that treatment of effector cells with IL-27 suppresses encephalitogenic Th17 responses in this population and reduces their capacity to transfer EAE to naive animals. Furthermore, we demonstrate that continual delivery of IL-27 by s.c. osmotic pump for 7 days significantly suppresses EAE, suggesting that IL-27 may have therapeutic potential in inflammatory autoimmune diseases such as MS.

Materials and Methods

Mice and EAE induction

Mice were purchased from The Jackson Laboratory. Experimental procedures were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University.

Chronic EAE. Female 8- to 12-wk-old C57BL/6 mice were immunized s.c. with 100 μg of $MOG_{35–55}$ in CFA containing 5 mg/ml *Mycobacterium tuberculosis* H37Ra (Difco), at two sites on the back. Mice were injected with 200 ng of pertussis toxin in PBS i.p. on days 0 and 2 and were scored daily using an EAE clinical scale as detailed below.

RR-EAE. Female 8- to 12-wk-old SJL mice were immunized s.c. with 100 μg of proteolipid peptide $(PLP)_{139–151}$ in CFA containing 5 mg/ml *M. tuberculosis* H37Ra (Difco), at two sites on the back. Mice were injected with 200 ng of pertussis toxin in PBS i.p. on days 0 and 2 and were scored daily using an EAE clinical scale as detailed below. Relapse was defined as an increase of at least one clinical grade after mice had previously improved at least a full clinical grade and stabilized (28).

Adoptive transfer EAE. Female 8- to 12-wk-old C57BL/6 mice were immunized in the footpads with 300 μg of $MOG_{35–55}$ in CFA containing 5 mg/ml *M. tuberculosis* H37Ra (Difco). Draining lymph node (DLN) or spleen cells were harvested 11 days postimmunization and cultured for 4 days in Click's medium (Irvine Scientific) with 15% FCS, penicillin/streptomycin, L-glutamine, nonessential amino acids, vitamins, and 2-ME (Invitrogen Life Technologies). All cells were cultured with $MOG_{35–55}$ (25 μg/ml) and either carrier-free recombinant murine (rm) IL-27 (20 ng/ml; R&D Systems) or PBS as a control. Cells were harvested, washed, and 1.5–2 $\times$ 10^7 cells were transferred to two groups (IL-27 or PBS) of naive, 8- to 12-wk-old, female C57BL/6 mice via the tail vein. Mice were given 250 ng of pertussis toxin i.p. on days 0 and 2 post transfer.

IL-23-driven adoptive transfer EAE. Female 8-to 10-wk-old female SJL mice were immunized in the footpads with 100 μg of $PLP_{139–151}$ in CFA containing 5 mg/ml *M. tuberculosis* H37Ra (Difco). Lymph node cells were harvested on day 9 postimmunization and cultured for 10 days in RPMI 1640 supplemented with 10% FCS, penicillin/streptomycin, l-glutamine, HEPES, sodium pyruvate, and 2-ME as previously described (4). All cells were cultured in the presence of $PLP_{139–151}$ (20 μg/ml), rmIL-23 (10 ng/ml), and either rmIL-27 (20 ng/ml) or PBS. Media were changed on days 5 and 8 and supplemented with rmIL-2 (2 ng/ml). On day 10, cells were harvested, washed, and transferred to naive female 8- to 10-wk-old SJL mice (5 $\times$ 10^6 cells/mouse) via the tail vein. Mice were given 250 ng of pertussis toxin i.p. on days 0 and 2 post transfer.

EAE was clinically assessed by daily scoring using a scale from 0 to 5 as follows: partial limp tail, 0.5; full limp tail, 1; limp tail and waddling gait, 1.5; paralysis of one hind limb, 2; paralysis of one hind limb and partial paralysis of other hind limb, 2.5; paralysis of both hind limbs, 3; ascending paralysis, 3.5; paralysis of trunk, 4; moribund, 4.5; death, 5.

Subcutaneous implantation of osmotic pumps

On day 11 postimmunization, mice were anesthetized and a shallow incision was made on the back, taking care to avoid the immunization sites. Osmotic minipumps (Alzet Osmotic Pumps; Durect) containing PBS or carrier-free rmIL-27 in PBS were inserted s.c. and the wound was closed.

Gene expression analysis by real-time PCR

Mice were sacrificed at the following phases of RR-EAE: onset (n = 6, all score 1), severe disease (n = 4, mean score 3.25 $\pm$ 0.14, range 3–3.5), remission (n = 5, all score 0), and relapse (n = 4, mean score 1.62 $\pm$ 0.12, range 1.5–2). Mice in the remission group had recovered by a score of at least 1.5 and mice in the relapse group had worsened by a score of at least 1 following a clear remission. In addition, six healthy mice were sacrificed to serve as healthy controls. Animals were extensively perfused transcardially with PBS. Spleens and spinal cords were removed and stored in RNAlater (Ambion). Tissues were individually homogenized using a TissueLyser and RNA was extracted using RNeasy Lipid Tissue Midi kits (Qiagen). cDNA was synthesized using Superscript II First strand synthesis kits (Invitrogen Life Technologies) and gene expression was analyzed by TaqMan real-time PCR (Applied Biosystems). *18S* gene expression was used as an endogenous control in all experiments and levels of gene expression were compared with healthy controls.

Astrocyte culture

Astrocyte cultures (>90% pure as assessed by glial fibrillary acidic protein staining) were established from neonatal C57BL/6 mice as previously described (29). Experiments were performed on cells from passages 1 to 3. On the day of treatment, medium were removed and cells were washed with PBS. LPS (1 μg/ml) $\pm$ IFN-γ (10 ng/ml) were added to selected wells. Supernatants and cell lysates were harvested at a range of time points from 6 to 72 h.

Histopathology

Following an initial perfusion with PBS, animals were perfused transcardially with 4% paraformaldehyde and spinal cords were removed. Tissues were processed and blocked in paraffin wax. Ten-micrometer sections were cut and stained with H&E for assessment of inflammation and with Luxol Fast Blue (LFB) for demyelination. Sections were assessed as follows: inflammation: 0, none; 1, a few inflammatory cells; 2, organization of perivascular infiltrates; and 3, increasing severity of perivascular cuffing with extension into the adjacent tissue. For demyelination: 0, none; 1, rare foci; 2, a few areas of demyelination; 3, large (confluent) areas of demyelination.

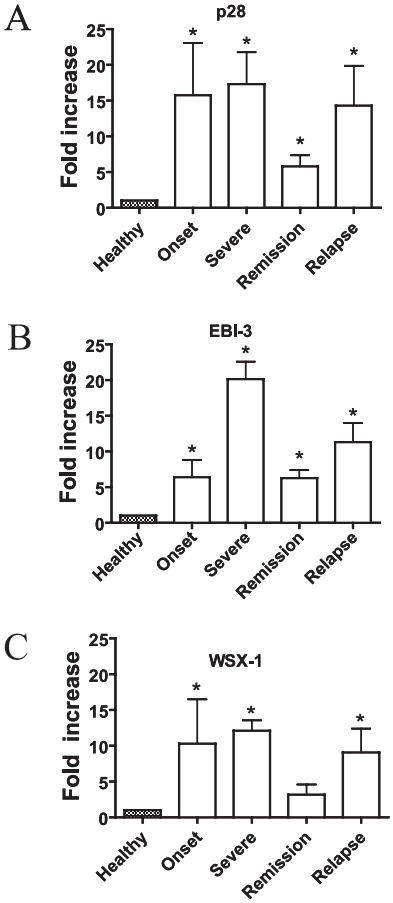

FIGURE 1. Temporal expression of IL-27 subunits and its receptor in the spinal cord of mice at various phases of RR-EAE. Groups of mice (n = 5/group) were sacrificed at disease onset, severe disease, remission, and relapse and gene expression was assessed by real-time PCR in comparison to healthy mice. Expression of IL-27 subunits *p28* (*A*) and *ebi3* (*B*) and the subunit of its receptor, *wsx-1* (*C*), correlated with the disease course in RR-EAE. *, $p < 0.05$.

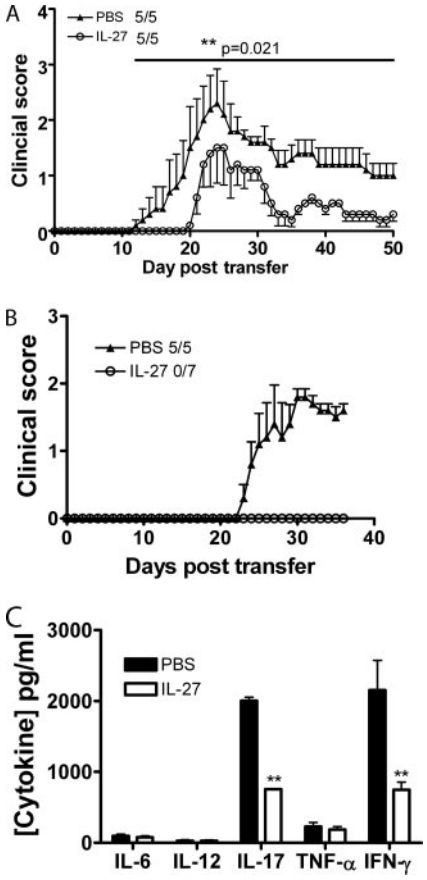

FIGURE 3. Exogenous IL-27 suppresses adoptive transfer EAE. DLN or spleen cells were harvested from C57BL/6 mice that had been immunized with $MOG_{35–55}$ in CFA. Cells were restimulated in vitro for 4 days with $MOG_{35–55}$ and IL-27 or PBS and transferred to naive recipient mice. *A*, Clinical scores of mice that received 2×10^7 encephalitogenic lymph node cells show that IL-27-treated cells induce less severe disease than PBS control cells. *B*, Clinical scores of mice that received 2×10^7 encephalitogenic spleen cells showing complete suppression of EAE transfer by IL-27. *C*, Levels of proinflammatory cytokine secretion by encephalitogenic DLN cells in culture were measured by CBA (IL-6, IL-12, TNF-α, IFN-γ) and ELISA (IL-17). One representative experiment of three is shown. *, $p < 0.05$; **, $p < 0.002$.

Cytometric bead array (CBA) and ELISA

Levels of the proinflammatory cytokines IL-6, IL-12, IFN-γ, and TNF-α in culture supernatants were assayed using a CBA kit as per manufacturer's instructions (BD Pharmingen) and data were acquired on a FACSAria cell sorter (BD Biosciences) Duoset ELISA reagents were used to quantify IL-17 and IL-27p28 was measured using a Quantikine ELISA kit (both R&D Systems).

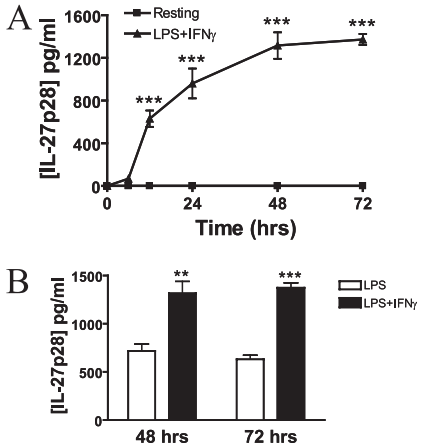

FIGURE 2. Secretion of IL-27p28 by activated astrocytes in vitro. Astrocyte cultures established from neonatal C57BL/6 mice were activated with LPS and IL-27p28 secretion into the supernatant was assayed by ELISA. *A*, Activated cultures secreted IL-27p28 protein in a time-dependent manner. *B*, IFN-γ potentiated the induction of IL-27p28 at 48 and 72 h. n = 4, one representative experiment of two is shown. **, $p < 0.02$; ***, $p < 0.0001$.

Isolation and flow cytometric analysis of CNS cells

Following extensive transcardial perfusion with PBS, spinal cords were removed from mice and pooled for mononuclear cell isolation. Briefly, tissues were mechanically dissociated through a 100-μm strainer and washed with PBS. The resultant pellet was fractionated on a 60/30% Percoll gradient by centrifugation at 300 $\times$ g for 20 min. Microglia and infiltrating mononuclear cells were harvested from the interface, washed, counted, and cultured for 4 h in RPMI 1640 containing 10% FCS, penicillin/streptomycin, nonessential amino acids, L-glutamine, vitamins and 2-ME and stimulated with PMA (50 ng/ml), ionomycin (500 ng/ml), and GolgiPlug. Cells were harvested and stained as detailed below.

Flow cytometry

On the day of analysis, cells were restimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) (Sigma-Aldrich) and treated with GolgiPlug (1 μg/10^6 cells; BD Pharmingen) for 4 h. Cells were harvested, washed in staining buffer containing 1% FCS, 0.1% NaN_3 in PBS, and blocked with anti-CD16/CD32 Abs. Following another wash step, cells were stained with a fluorescently labeled Ab to CD4 for 20 min in the dark at 4°C. Cells were

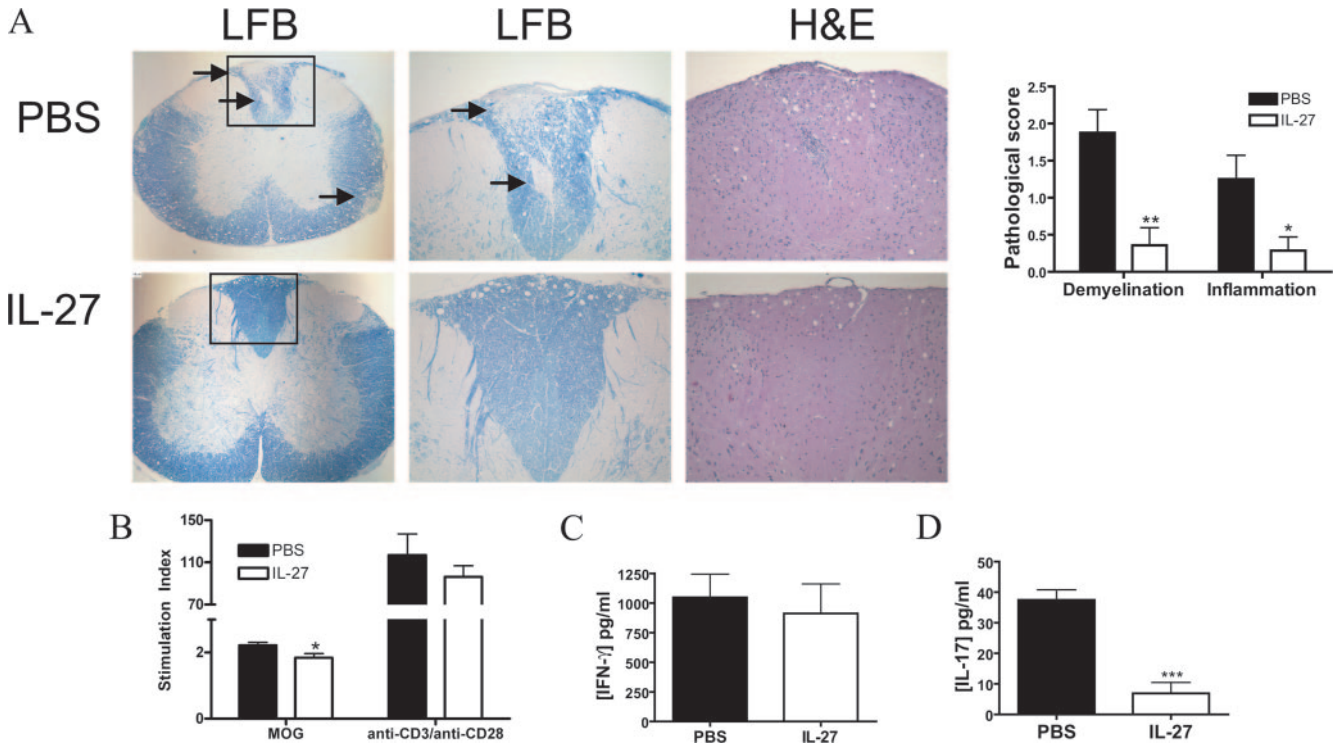

FIGURE 4. Immunological and histopathological analysis of recipient mice in adoptive transfer EAE. *A*, CNS histopathology of mice that received 2×10^7 encephalitogenic spleen cells (PBS $n = 5$, IL-27 $n = 7$). Mice were sacrificed at day 36 post transfer. After extensive perfusion, spinal cords were harvested and processed for histology. Ten-micrometer sections were stained with H&E (inflammation; magnification ×4) and LFB (demyelination; magnification ×4 and ×10). *B*, Proliferative responses of recipient mouse spleen cells stimulated with $MOG_{35–55}$ (25 μg/ml) or anti-CD3/anti-CD28 (1 μg/ml each) for 48 h. Proliferation was assessed by [^{3}H]thymidine incorporation in the final 12 h of culture and expressed as stimulation index relative to background. *C* and *D*, Cytokine production (IFN-γ and IL-17) by spleen cells of recipient mice in response to $MOG_{35–55}$ (25 μg/ml) at 48 h was assessed by ELISA. *, $p < 0.05$; **, $p < 0.002$; ***, $p < 0.0001$.

washed, fixed, and permeabilized using Fix and Perm cell permeabilization reagents (Caltag Laboratories. Cells were stained for intracellular cytokines with PE-conjugated rat anti-mouse IL-17 and IFN-γ Abs. All Abs were purchased from BD Pharmingen. Data were acquired on a FACSAria and analyzed using FlowJo Software.

Statistics

EAE experiments were analyzed by calculating the area under the curve for each mouse over the clinical period of the experiment and performing statistical analysis on each group using these values. All experiments were tested for statistical significance using unpaired, two-tailed, Student's *t* tests. Differences were considered significant if $p < 0.05$.

Results

Expression of p28, ebi3, and wsx-1 in the CNS correlate with RR-EAE disease activity

To identify the disease phases at which endogenous IL-27 signaling is significantly up-regulated during EAE, we performed temporospatial gene expression analysis. Previously, we showed that gene expression of IL-27 subunits *p28* and *ebi3* and the *wsx-1* subunit of the IL27R in lymph node and spinal cord, but not spleen, correlated with disease activity in chronic EAE (25). In this study, we examined additional disease phases, recovery and relapse, which are of particular clinical relevance in human RR-MS, the most common form of MS. We found that expression of *p28* (Fig. 1*A*), *ebi3* (Fig. 1*B*), and *wsx-1* (Fig. 1*C*) in the spinal cord directly correlated with clinical disease activity. Specifically, expression of all three genes increased at disease onset and was maximal during severe disease. Of particular interest, expression of these genes in the spinal cord was down-regulated during disease remission relative to severe disease and increased again during relapse. In the case of both *p28* and *ebi3*, gene expression during remission was still significantly higher than that of healthy controls. We found no significant difference in gene expression in the spleen at any disease phase examined (data not shown), which is in agreement with our chronic EAE studies (25). This suggests that during clinical EAE, regulation of IL-27 expression is pertinent in CNS-resident cells and/or infiltrating effector cells, as opposed to global alterations throughout the immune system. Astrocytes are important CNS-resident cells that demonstrate a range of immunoregulatory functions and so we proceeded to examine IL-27 expression in this population in vitro.

Activated astrocytes secrete IL-27p28 protein in vitro

Activated APCs produce IL-27 in the periphery (10). It is known that microglia express IL-27 in response to LPS plus IFN-γ (30) and our group showed that IL-27 is expressed by CNS $CD11b^+$ macrophages/microglia during EAE (25). In agreement with recent work by Stumhofer et al. (23), we found greatly increased expression of *p28* mRNA (>1000-fold) in neonatal astrocytes in response to LPS plus IFN-γ (data not shown). Stumhofer et al. (23) demonstrated relatively high constitutive expression of *ebi-3* in both normal mouse brain and resting astrocyte cultures and undetectable *p28*, which would suggest that expression of p28 is a limiting factor in the production of heterodimeric IL-27 in the CNS. Therefore, we examined secretion of IL-27p28 from astrocytes by ELISA and found a time-dependent increase in response to LPS plus IFN-γ (Fig. 2*A*). We did not detect any IL-27p28 in the supernatant of cultures that were treated with IFN-γ alone or in resting astrocytes (data not shown). To discern the contribution of

the endogenous factor IFN-γ, we also activated astrocytes with LPS alone. We observed a significant potentiation of IL-27p28 secretion from astrocytes by IFN-γ compared with LPS alone at 48 h, which was even more pronounced at 72 h (Fig. 2*B*). This suggests that endogenous factors such as cytokines can augment the production of IL-27 in the CNS during inflammation.

IL-27 suppresses adoptive transfer EAE

Given the correlation of IL-27 expression with the effector phases of EAE, we sought to directly examine the effect of IL-27 on pathogenic effector cells using adoptive transfer EAE. DLN or spleen cells harvested from immunized C57BL/6 mice were restimulated in vitro with $MOG_{35-55,}$ in the presence of IL-27 or PBS for 4 days, and transferred into naive mice. Animals that received IL-27-treated DLN cells showed significantly delayed onset of disease, reduced severity, and enhanced recovery compared with the control group (Fig. 3*A*). We repeated the experiment with spleen cells and 100% of mice in the control group (five of five) developed EAE while none (zero of seven) of the mice receiving IL-27-treated cells developed clinical disease (Fig. 3*B*). In comparison with the DLN cell transfer, animals receiving control (PBS) splenocytes showed slightly less severe clinical symptoms and a longer time to disease onset demonstrating the robustness of the effect of exogenous IL-27 during the culture period. We repeated the experiment a third time, in this case transferring a lower number of DLN cells (15×10^6 cells) to induce less severe disease and found a similar disease incidence and clinical profile as was observed in the splenocyte experiment (data not shown). We analyzed the cytokine profile of the supernatants of transferred cells by CBA and ELISA. There was no significant difference in the production of the proinflammatory cytokines IL-6, IL-12, or TNF-α, however, in both DLN and splenocyte cultures we found significantly lower levels of IL-17 in IL-27-treated cultures (Fig. 3*C*) suggesting that IL-27 may suppress adoptive transfer EAE by suppressing IL-17. We also observed suppression of IFN-γ in the supernatant of IL-27-treated cultures which is in apparent contrast to it role in early Th1 polarization (14). However, in a review of the literature, we find that Yoshimura and colleagues (34) clearly demonstrated that IL-27 synergizes with IL-12 to induce IFN-γ production from naive T cells but actually suppresses IFN-γ production from fully activated T cells.

Histopathological analysis of spinal cords showed considerable inflammation and demyelination in the control group but rare CNS pathology in the IL-27-treated group (Fig. 4*A*). We also examined the immune profile of recipient animals by harvesting spleens, culturing cells for 48 h with myelin Ag, and assessing proliferation and cytokine production. Proliferative responses were mildly impaired in mice receiving IL-27-treated cells which reached statistical significance in the cultures stimulated with myelin Ag (Fig. 4*B*, $p < 0.05$). We found no significant difference in secretion of IFN-γ between the two groups (Fig. 4*C*). However, release of IL-17 from Ag-specific cells was significantly suppressed in the IL-27-treated group (Fig. 4*D*).

IL-27 suppresses IL-23-driven adoptive transfer EAE

To further examine the significance of IL-27-induced suppression of IL-17 in effector cells in EAE, we used a recently described IL-23-driven model of adoptive transfer EAE which results in a high proportion of pathogenic Th17 cells (4, 31). In this model, it was reported that as few as 10^5 Th17 cells induced CNS inflammation and transfer of 3×10^5 Th17 cells caused severe EAE (31). In our study, DLN cells were harvested from SJL mice that had been immunized with $PLP_{139-151}$ in CFA. These cells were stimulated with rmIL-23 and $PLP_{139-151}$ and treated with either IL-27

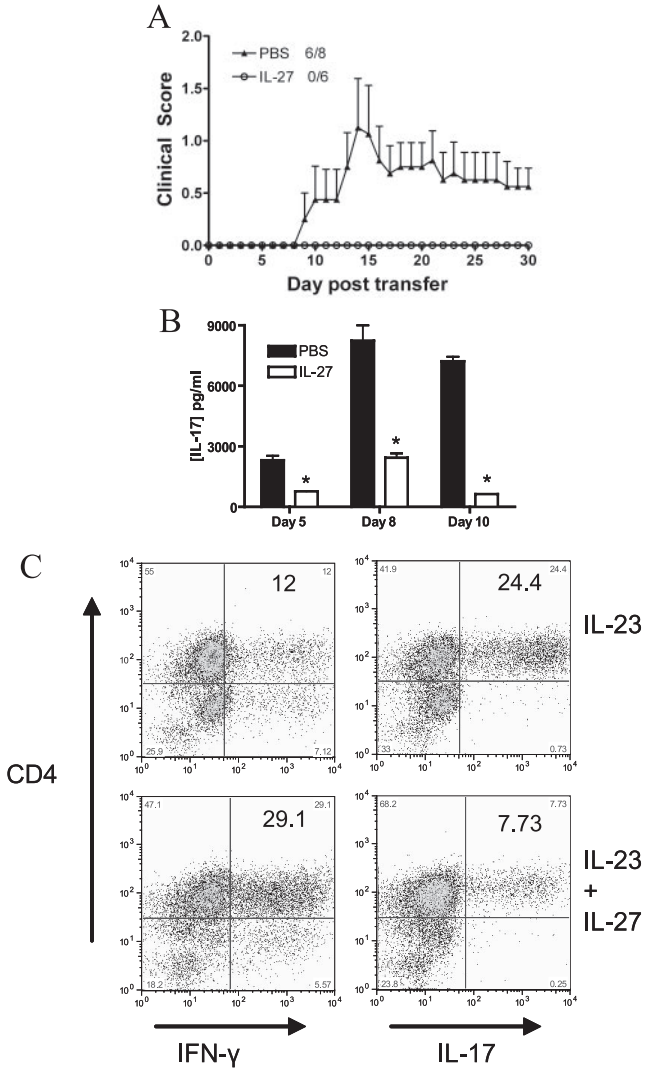

FIGURE 5. Exogenous IL-27 suppresses IL-23-driven adoptive transfer EAE. DLN cells were harvested from SJL mice that had been immunized with $PLP_{139-151}$ in CFA. Cells were restimulated in vitro for 10 days with $PLP_{139-151}$ in the presence of IL-23, IL-2 and either IL-27 or PBS and transferred to naive recipient mice (PBS $n = 8$, IL-27 $n = 6$). *A*, Clinical scores of mice that received 5×10^6 encephalitogenic lymph node cells from donor mice that had been immunized with $PLP_{139-151}$ in CFA. *B*, Levels of IL-17 secreted by $PLP_{139-151}$-reactive lymph node cells during the culture period as measured by ELISA. *C*, Intracellular cytokine staining of $CD4^+$ cells on day 9 of the culture period. Cells were restimulated with PMA/ionomycin and treated with GolgiPlug for 4 h before harvest. After staining for CD4, cells were fixed, permeabilized, stained for intracellular IFN-γ and IL-17 and analyzed by flow cytometry. Numbers represent percentages of cells. ∗, $p < 0.05$.

or PBS. A total of 5×10^6 total cells were transferred to two groups of naive mice. Although 75% of mice that received PBS-treated cells developed disease, albeit milder than previously reported (31), none of the mice that received IL-27-treated cells developed EAE (Fig. 5*A*). Again, we found lower levels of IL-17 in the supernatants of IL-27-treated cells throughout the culture phase (Fig. 5*B*).

We used flow cytometry to characterize the pathogenic cell populations that were being transferred. We found that IL-23- plus PBS-treated cultures had a higher proportion of Th17 ($CD4^+IL17^+$) than Th1 ($CD4^+$ IFN-γ^+) (24.4 vs 12%) cells. In contrast, cells cultured with IL-23 plus IL-27 had much fewer

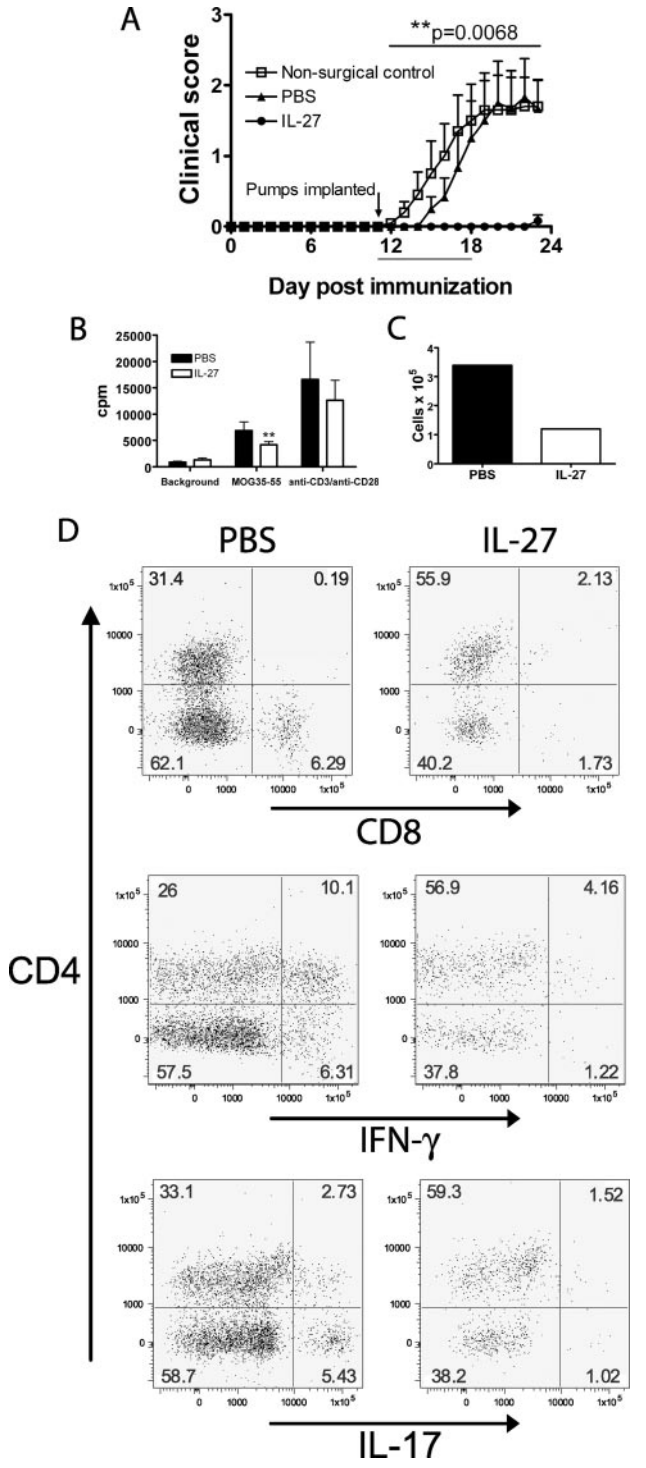

FIGURE 6. Exogenous IL-27 suppresses actively induced EAE. EAE was induced in C57BL/6 mice with $MOG_{35–55}$ and osmotic pumps (7-day delivery capacity) containing either rmIL-27 or PBS were implanted s.c. on day 11. Mice that did not undergo surgery were also assessed. *A*, Clinical scores of mice that received osmotic minipumps delivering PBS or IL-27 (400 ng/mouse/day) for 7 days and a control group that did not undergo surgery. *B*, Proliferative responses of spleen cells cultures without Ag, with the immunization Ag $MOG_{35–55}$ (25 μg/ml) or with anti-CD3/anti-CD28 (1 μg/ml) for 60 h were assessed following addition of [^{3}H]thymidine for the last 12 h of the culture period. *C*, Yield of mononuclear cells recovered from pooled spinal cords of mice receiving either PBS or IL-27 before short-term culture. *D*, Mononuclear cells recovered from pooled spinal cords were restimulated in vitro with PMA (50 ng/ml), ionomycin (500 ng/ml), and GolgiPlug for 4 h. Cells were harvested, stained for cell surface markers and intracellular cytokines and analyzed by flow cytometry. **, $p < 0.01$.

Th17 than Th1 cells (7.73 vs 29.1%) (Fig. 5*C*). Thus, the presence of IL-27 in the culture resulted in 3-fold less Th17 cells (7.73 vs 24.4%) demonstrating that IL-27 negatively regulates autoreactive effector Th17 cells even in the presence of IL-23. These results agree with our findings in the nonpolarized model above and support the view that inhibition of adoptive transfer EAE by IL-27 is mediated by Th17 suppression.

Exogenous IL-27 suppresses actively induced EAE

Given the suppressive effect of IL-27 on encephalitogenic cells in vitro, we sought to examine whether IL-27 suppresses actively induced EAE in vivo. Initially, we tested i.p. injection of recombinant carrier-free IL-27, however, these experiments did not provide reproducible findings. To achieve continual, consistent delivery of cytokine throughout the treatment period, we implanted osmotic minipumps, containing either PBS or IL-27, s.c. on day 11 postimmunization. This resulted in the delivery of either PBS or 400 ng of IL-27 per day for 7 days. IL-27 significantly suppressed the development of clinical EAE compared with the control PBS group (Fig. 6*A*). A nonsurgical control group was included to account for the effect of the surgical procedure on the development of EAE and while a short delay in disease onset was observed, PBS pump mice developed comparably severe disease as nonsurgical controls. We repeated the experiment and sacrificed mice on day 18, the last day of IL-27 administration from the pump. Proliferative responses of spleen cells were assessed in response to the immunization Ag, $MOG_{35–55}$, or to nonspecific T cell stimulation, anti-CD3/anti-CD28 Abs. Splenocytes of IL-27-treated mice showed significantly lower proliferation in response to $MOG_{35–55}$ suggesting that IL-27 inhibited peripheral inflammatory responses to myelin Ag (Fig. 6*B*). We isolated mononuclear cells from pooled spinal cords of these mice and observed much fewer inflammatory cells in IL-27-treated mice (Fig. 6*C*). We immunophenotyped the cells recovered from spinal cords by flow cytometry and observed $CD4^+$ and $CD8^+$ cells in PBS spinal cords but virtually no $CD8^+$ cells in IL-27-treated mice. Furthermore, while $IL\text{-}17^+$ and $IFN\text{-}\gamma^+$ cells were readily detectable in the CNS of control PBS mice, few $IFN\text{-}\gamma^+$ and almost no $IL\text{-}17^+$ cells were observed in IL-27-treated mice (Fig. 6*C*). These data strongly support the view that IL-27 suppresses the effector phase of EAE.

Discussion

We previously showed that expression of IL-27 and its receptor subunit, WSX-1, correlated with disease course in a chronic model of EAE (25). RR-MS is the most common form of MS affecting up to 80% of patients. In this study, we extended our previous gene expression studies to examine the expression of IL-27 and its receptor in the remission and relapse phases of EAE using a RR-EAE model. We showed that expression of the IL-27 subunits *p28* and *ebi3* and the IL-27R subunit, *wsx-1*, correlated with clinical disease activity in the spinal cord. Expression of all three genes was maximal during severe disease, declined during remission, and increased again when relapses began. In the case of both *p28* and *ebi3*, gene expression during remission was still significantly higher than in healthy controls. This may reflect residual immunological signaling from the first attack or subclinical activity of RR-EAE which would be interesting to address in a model of monophasic EAE with complete recovery. These data demonstrate that IL-27 subunits and its receptor are differentially expressed in the CNS at various phases of disease and up-regulation of expression correlate with the effector phases of RR-EAE.

Although infiltrating cells of the peripheral immune system most likely contribute to expression of IL-27 in the spinal cord

during EAE, resident cells are also probable sources of IL-27 during CNS inflammation. Activated microglia have been shown to express IL-27 (30) and we found, in agreement with recent studies by Stumhofer et al. (23), that astrocytes greatly up-regulate expression (>1000-fold) of *p28* mRNA in response to activation with LPS plus IFN-γ. We went on to demonstrate secretion of IL-27p28 protein by astrocytes in response to LPS and this effect was augmented by IFN-γ. These data demonstrate that endogenous factors can influence the secretion of IL-27p28 from astrocytes which likely plays an immunomodulatory role during CNS inflammation.

Our gene expression studies provide insight into the regulation of IL-27 expression during various phases of CNS inflammation. Taking into account the increased severity of EAE in IL-27R-deficient mice (24), it is reasonable to hypothesize that increased expression of IL-27 at effector phases of disease, suppresses encephalitogenic cells during EAE. Indeed expression of SOCS3, which has been shown to negatively regulate autoimmune inflammation (32), in the CNS, has also been shown to correlate with disease activity in RR-EAE (33). In chronic EAE, Kleinschek et al. (31) recently showed that IL-25 is up-regulated in microglia during EAE and exerts protective, anti-inflammatory effects in the CNS. Taking these studies into account, it is reasonable to speculate that up-regulation of IL-27 at the effector phase of disease may be a suppressive response of the host to autoimmune attack within the CNS. Such control is essential to regulation of inflammatory responses in general and in particular, in comparatively immunoprivileged sites such as the CNS where edema can have very severe consequences to the host. Astrocyte-derived IL-27 may be a key immunomodulator of this glial population and may play an important role in controlling inflammation within the CNS.

During EAE, peripheral myelin-reactive T cells proliferate and migrate to the CNS where they are reactivated by endogenous myelin Ag. Given the increased expression of IL-27 during the effector phases of EAE, we sought to address the role of IL-27 on the pathogenic effector cell population using adoptive transfer EAE. We showed that treatment of donor DLN or spleen cells with IL-27, while being reactivated in culture with myelin Ag, diminishes the capacity of these cells to transfer disease to naive animals. Animals receiving IL-27-treated DLN cells showed delayed onset, reduced severity of disease, and enhanced recovery and when the experiment was repeated with donor spleen cells, IL-27 completely suppressed the transfer of disease. Histopathological analysis of spinal cords showed considerable inflammation and demyelination in the control (PBS) group. We observed a mild degree of inflammation and demyelination in two of seven animals in the IL-27 groups despite the lack of clinical symptoms. These results demonstrate that although IL-27 suppressed the capacity of these cells to transfer clinical disease, it did not completely suppress their potential pathogenicity. At the time of transfer, levels of IL-17 were significantly reduced in the supernatants of IL-27-treated cultures. We also observed suppression of IFN-γ by IL-27 after 96 h in culture. This is in contrast to the profile of IL-27 as an early supportive factor for Th1 commitment in naive, unprimed T cells (14) but consistent with the previously reported suppression of IFN-γ production from fully activated T cells (34). It is also possible that reduced IFN-γ in the supernatants may be a result of a suppressive effect of IL-27 on non-T cell sources of IFN-γ in our cultures. This observation requires further study and is currently under investigation. Regardless of the source, suppression of IFN-γ in our IL-27-treated culture system suggests that IL-27 inhibits Th17 development directly rather than via IFN-γ and this has been shown by others in vitro (34). We also observed an inhibition of T cell proliferation and IL-17, but not IFN-γ production (Fig. 4*C*), in response to activation with myelin Ag, in spleens of mice receiving IL-27-treated cells, demonstrating a sustained inhibition of encephalitogenicity in this group (Fig. 4*D*). These data indicate that IL-27 plays a suppressive role in the effector phase of EAE. Suppression of IL-17 in donor cell cultures and sustained inhibition of IL-17 production from myelin-reactive spleen cells of recipient mice led us to examine the effect of IL-27 in a Th17-biased adoptive transfer EAE model.

Using the IL-23-driven model of adoptive transfer EAE described by Langrish et al. (4), we found that IL-27 potently suppressed the expansion of encephalitogenic Th17 cells in culture. Although we did not observe disease as severe as previously reported (4) in our control group (IL-23 only), we did find that IL-23- plus IL-27-treated cells did not transfer EAE to naive animals. Again, we found that IL-27 suppressed the production of IL-17 in these cultures. Flow cytometric profiling of the cell populations confirmed the pro-Th17 effect of IL-23 in this model of adoptive transfer EAE. Importantly, IL-27 negated this effect as demonstrated by a low proportion of Th17 cells and a high proportion of Th1 cells in IL-27- plus IL-23-treated cultures. These findings provide further evidence that suppression of adoptive transfer EAE by IL-27 is mediated by Th17 suppression.

To investigate the therapeutic potential of IL-27 in CNS autoimmune inflammation, we administered carrier-free rmIL-27 to mice that were actively immunized to develop EAE during the onset phase of disease. Strikingly, exogenous IL-27 significantly suppressed the development of EAE. Disease inhibition was associated with diminished peripheral immune responses to myelin Ag and less inflammatory infiltration of the CNS. In particular, we found very few IL-17-producing cells in the spinal cords of IL-27-treated mice, suggesting that disease suppression by IL-27 is mediated by suppression of encephalitogenic IL-17-producing cells. These findings suggest that IL-27 may be a useful therapeutic agent for suppressing autoimmune inflammation.

In summary, we have shown that autoreactive cells are a target cell population of IL-27 in EAE and, specifically, Th17 effector cells are particularly sensitive to suppression by IL-27. We have demonstrated in two mouse models of EAE that IL-27 can potently suppress the effector phase of CNS autoimmune inflammation. The s.c. delivery of exogenous IL-27 to inhibit disease presents an attractive avenue of translational research. The minimum requirement for a diagnosis of MS is at least one clinical attack and evidence of further, temporospatially distinct, disease activity by magnetic resonance imaging or clinical presentation. Thus, by the time that long-term MS therapies can be started, the autoreactive effector T cell population has already been primed by CNS Ag. For this reason, a therapeutic agent that demonstrates efficacy in the effector phase of disease is particularly desirable. Suppressive efficacy of IL-27 on established, autoreactive cell populations, particularly when delivered s.c. in vivo as we have demonstrated here, has significant translational relevance to the development of new therapies for human autoimmune diseases such as MS.

Disclosures

The authors have no financial conflict of interest.

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