Identification of an IL-27/osteopontin axis in dendritic cells and its modulation by IFN-γ limits IL-17–mediated autoimmune inflammation

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Dendritic cells (DCs) play a central role in determining the induction of T cell responses. IL-27 production by DCs favors induction of IL-10–producing regulatory T cells, whereas osteopontin (OPN) promotes pathogenic IL-17 T cell responses. The regulatory mechanisms in DCs that control these two cell types are not understood well. Here, we show that IFN-γ induces IL-27 while inhibiting OPN expression in DCs both in vitro and in vivo and that engagement of IFN-γR expressed by DCs leads to suppression of IL-17 production while inducing IL-10 from T cells. DCs modified by IFN-γ acquire IL-27–dependent regulatory function, promotes IL-10–mediated T cell tolerance, and suppress autoimmune inflammation. Thus, our results identify a previously unknown pathway by which IFN-γ limits IL-17–mediated autoimmune inflammation through differential regulation of OPN and IL-27 expression in DCs.

IL-27 is a potent antiinflammatory cytokine, which belongs to the IL-12 family and is comprised of an IL-12p40–related protein, encoded by the EBV-induced gene 3 (EBI3, also known as IL27), and a unique IL-12p35–like protein, IL-27p28v (1). Initial animal studies on the biology of IL-27 suggested a role for IL-27 in the initiation of the Th1 response (2, 3); however, subsequent animal studies on the biology of IL-27 indicated that a single DC subpopulation can elicit both T cell outcomes, whereas IL-27 levels were low in IFN-γR-deficient mice compared with WT mice. Engagement of IFN-γR expressed by DCs leads to suppression of IL-17 production and induction of IL-10 from T cells. Furthermore, IFN-γ-modified DCs ameliorate the disease severity of EAE through an IL-27–dependent mechanism. Taken together, our results identify a previously unknown pathway by which IFN-γ limits IL-17–mediated autoimmune inflammation through reciprocal DC modulation of OPN and IL-27.

Results

IFN-γ Reciprocally Regulates OPN and IL-27 Expression in DCs. To study the effect of IFN-γ on DCs, we first examined its effect on the production of both pro- and antiinflammatory cytokines from DCs. We found that IFN-γ stimulation of DCs markedly induced IL-27 levels while inhibiting OPN expression in DCs (Fig. 1 A and B). IFN-γ stimulation did not affect other DC-associated cytokines including IL-1β, IL-6, IL-12, 23, and TGF-β (Fig. S1). IFN-γ inhibited OPN and induced IL-27 at both mRNA and protein levels from WT-DCs in a dose-dependent manner. IFN-γ stimulation failed to inhibit OPN or induce IL-27 expression from IFN-γR−/− DCs. In addition we found that IFN-γR−/− DCs expressed high levels of OPN in comparison with WT-DCs, whereas IL-27 levels were low in IFN-γR−/− DCs compared with WT-DCs (Fig. 1 C and D). Taken together, these results demonstrate that IFN-γ reciprocally regulates OPN and IL-27 expression in DCs.

Altered OPN and IL-27 Expression in DCs Differentially Modulate T Cell Production of IL-17 and IL-10. To test whether IFN-γ reciprocally regulated DC expression of OPN and IL-27 in vivo, we induced EAE in WT and IFN-γR−/− mice. We found that OPN mRNA expression was markedly increased in DCs at the peak of disease in spleen, lymph node (LN), and CNS. Furthermore, OPN levels were higher in DCs from IFN-γR−/− mice compared with WT mice...
Indepent experiments, and the error bars represent the mean ± SD.

Fig. 1. IFN-γ reciprocally regulates OPN and IL-27 expression in DCs. (A and B) Real-time quantitative RT-PCR analysis of mRNA encoding IL-27p28 and OPN in DCs treated with or without mouse rIFN-γ (100 ng/ml). (C and D) Dose-dependent effect of IFN-γ on IL-27 and OPN expression in DCs from WT and IFN-γ−/− mice. Shown is real-time quantitative RT-PCR analysis of mRNA encoding IL-27p28 and OPN in DCs. Data are representative of five independent experiments, and the error bars represent the mean ± SD.

In accordance with OPN mRNA expression, IFN-γ−/− DCs expressed higher OPN at the protein level as measured by Western blot (Fig. 2B). To investigate whether IL-27 induction is also involved in the inhibitory effects of IFN-γ on EAE development in vivo, we examined IL-27 expression in DCs from WT and IFN-γ−/− mice with EAE. We found that expression of IL-27 was up-regulated in WT-DCs of mice with EAE (Fig. 2C) and that DCs from IFN-γ−/− mice with EAE had much less IL-27 expression compared with WT mice (Fig. 2C). These results demonstrate that IFN-γ up-regulates the expression of IL-27 while inhibiting OPN in DCs to negatively regulate the progression of autoimmune disease. Consistent with this observation, we found that IFN-γ−/− mice had high serum levels of OPN with little or no increase in serum IL-27 levels compared with WT mice (Fig. S2). These results clearly indicate that IFN-γ differentially modulates OPN and IL-27 expression in DCs in vivo during the course of EAE and, thus, could impact on immune responses both in the periphery and CNS.

Polarization of T cells to Th1, Th2, or Th17 phenotypes is a critical feature of cell-mediated immunity and is influenced by production of cytokines by DCs. We and others have shown that DC-expressed OPN induces IL-17 while inhibiting IL-10 production from T cells (17, 20). In contrast, IL-27 has been shown to inhibit IL-17 while inducing IL-10 production from T cells both in humans and mice (6-13). Because we observed IFN-γ deficiency led to altered OPN and IL-27 expression in DCs during EAE, we investigated whether DC-derived OPN and IL-27 had a role in T cell differentiation, specifically T cell production of IL-17 and IL-10. To test this hypothesis, we induced EAE in WT and IFN-γ−/− mice with myelin oligodendrocyte glycoprotein (MOG) and, at day 15, postimmunization isolated splenic CD11c+ DCs. Naïve splenic DCs were used as controls. We cocultured naïve DCs and DCs derived from immunized mice from WT and IFN-γ−/− mice with MOG-specific TCR transgenic CD4+ T cells (2D2) and measured MOG Ag-specific IL-17 and IL-10 production. We found that T cells cultured with DCs from IFN-γ−/− mice showed increased IL-17 levels compared with T cells cultured with WT DCs (Fig. 3A). When IL-10 secretion was quantified, significant differences were observed between T cells cultured with DCs from IFN-γ−/− mice and WT controls. In accordance with low IL-27 expression, IFN-γ−/− DCs induced less IL-10 production by T cells whereas T cells cultured with DCs from WT mice show an increase in IL-10 levels (Fig. 3A). We then investigated whether the IFN-γ activation of DCs could alter the balance of IL-10 and IL-17 production from T cells through differential regulation of OPN and IL-27 in DCs. To test this hypothesis, the in vivo isolated DCs from EAE mice were pretreated with IFN-γ and cultured with 2D2 T cells in the presence of MOG. As shown in Fig. 3B, IFN-γ-treated DCs both from WT and IFN-γ−/− mice, which express IFNγR, inhibited IL-17 production while increasing IL-10 production from T cells. However, this effect was not observed when a neutralizing antibody to IL-27 was included in the culture. Taken together, our results demonstrate that IFN-γ signaling events in DCs play a major role in negative regulation of Th17 development and induction of IL-10 production from T cells.

To directly demonstrate the effect of OPN and IL-27 on T cells, we stimulated CD4+ T cells with OPN in the presence or absence of rIL-27. We found that CD4+ T cells activated in the presence of OPN produced increased amounts of IL-17 and less IL-10 (Fig. 3C). However, the addition of IL-27 inhibited OPN-induced IL-17 while abrogating the inhibitory effect of OPN on IL-10 from T cells (Fig. 3C). Because we observed IFN-γ deficiency leads to increased OPN and low IL-27 levels in DCs during EAE, we next examined whether T cells from IFN-γ−/− mice.
mice exhibited changes in IL-17 and IL-10 expression. In accordance with increased OPN expression in DCs, T cells from IFN-γ−/− mice had high levels of IL-17 expression in spleen, LN, and CNS (Fig. 4A). When IL-10 levels were measured, we also found striking differences between T cells from WT and IFN-γ−/− mice. In accordance with increased IL-27 levels in DCs, T cells from mice that received neutralizing IL-27 antibody (Fig. 5A). Furthermore, the effect of IFN-γ-modified DCs on reducing the clinical severity of EAE was abrogated in the mice that received neutralizing IL-27 antibody (Fig. 5A). We then analyzed cytokine production in splenocytes of mice given IFN-γ-treated DCs or control DCs after ex vivo restimulation with MOG. We found large amounts of IL-17 and small amounts of IL-10 in splenocytes of mice given control DCs (Fig. 5B). In contrast, transfer of IFN-γ-treated DCs resulted in less secretion of IL-17 and increased synthesis of IL-10 in splenocytes of recipient mice (Fig. 5B). Coinjection of IFN-γ-treated DCs with anti-IL-27 antibody resulted in higher secretion of IL-17 and less IL-10 production by ex vivo-restimulated splenocytes than cells from mice that received IFN-γ-treated DCs. These results demonstrate a dominant tolerogenic effect of IFN-γ-treated DCs (Fig. 5B). To further examine the contribution of IL-27 to this tolerogenic effect, we injected IFN-γ-treated DCs or control DCs into WT and IL-27KO mice. We found that IFN-γ-modified DCs reduced clinical severity only in WT mice. Disruption of IL-27 pathway abrogated IL-10 deficiency differentially regulates IL-17 and IL-10 expression in T cells. (A) Real-time RT-PCR analysis of IL-17 and IL-10 in spleen, LN, and CNS-derived T cells were isolated from WT and IFN-γ−/− mice with or without EAE (n = 8 per group). (C) LN cells derived from WT and IFN-γ−/− mice were activated in vitro with MOG35-55 (20 μg/mL) for 72 h, and cell-free supernatants from cultures were assayed for IL-17 and IL-10.

Fig. 4. IFN-γ deficiency differentially regulates IL-17 and IL-10 expression in T cells. (A and B) Real-time RT-PCR analysis of IL-17 and IL-10 in spleen, LN, and CNS-derived T cells were isolated from WT and IFN-γ−/− mice with or without EAE (n = 8 per group). (C) LN cells derived from WT and IFN-γ−/− mice were activated in vitro with MOG35-55 (20 μg/mL) for 72 h, and cell-free supernatants from cultures were assayed for IL-17 and IL-10.

Fig. 3. Altered OPN and IL-27 expression in DCs differentially modulate T cell production of IL-17 and IL-10. (A) Total CD4+ T cells isolated from 2D2 mice were cocultured with CD11c+ DCs isolated from naïve and MOG35-55-immunized WT and IFN-γ−/− mice. Supernatants from cultures were harvested 72 h after initiation of cultures and assayed by ELISA for IL-17 and IL-10. (B) IFN-γ-treated DCs suppress IL-17 production while inducing IL-10 production from 2D2 T cells. WT and IFN-γ−/− DCs isolated from EAE mice were stimulated with IFN-γ for 12 h and were then cultured with 2D2 T cells in the presence of MOG peptide for 72 h. After 72 h, IL-17 and IL-10 production by CD4+ T cells was determined by ELISA. (C) OPN-induced IL-17 production or IL-10 inhibition from CD4+ T cells was abrogated by the addition of IL-27. CD4+ T cells were activated with anti-CD3 and anti-CD28 mAb (0.3 μg/mL) in the presence of 1 μg/mL mouse rOPN. In some culture conditions, 100 ng/mL of mouse rIL-27 was added together with 1 μg/mL mouse rOPN. Supernatants from cultures were analyzed for cytokines IL-17 and IL-10 by ELISA. Data are representative of three independent experiments, and the error bars represent the mean ± SD.

Fig. 2. IFN-γ deficiency differentially regulates IL-17 and IL-10 expression in T cells. (A and B) Real-time RT-PCR analysis of IL-17 and IL-10 in spleen, LN, and CNS-derived T cells were isolated from WT and IFN-γ−/− mice with or without EAE (n = 8 per group). (C) LN cells derived from WT and IFN-γ−/− mice were activated in vitro with MOG35-55 (20 μg/mL) for 72 h, and cell-free supernatants from cultures were assayed for IL-17 and IL-10.

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IFN-γ-modified DCs Attenuate EAE in an IL-27-dependent Manner. To determine whether IFN-γ-modified DCs have enhanced regulatory function in vivo, we activated DCs with or without mouse recombinant IFN-γ, pulsed them with MOG, and transferred these cells into syngeneic naive mice. We then immunized the mice with MOG peptide and monitored disease progression. We found that pretreatment with IFN-γ-modified DCs markedly reduced clinical severity of EAE compared with mice treated with control DCs (Fig. 5A). Furthermore, the effect of IFN-γ-modified DCs on reducing the clinical severity of EAE was abrogated in the mice that received neutralizing IL-27 antibody (Fig. 5A). We then analyzed cytokine production in splenocytes of mice given IFN-γ-treated DCs or control DCs after ex vivo restimulation with MOG. We found large amounts of IL-17 and small amounts of IL-10 in splenocytes of mice given control DCs (Fig. 5B). In contrast, transfer of IFN-γ-treated DCs resulted in less secretion of IL-17 and increased synthesis of IL-10 in splenocytes of recipient mice (Fig. 5B). Coinjection of IFN-γ-treated DCs with anti-IL-27 antibody resulted in higher secretion of IL-17 and less IL-10 production by ex vivo-restimulated splenocytes than cells from mice that received IFN-γ-treated DCs. These results demonstrate a dominant tolerogenic effect of IFN-γ-treated DCs (Fig. 5B). To further examine the contribution of IL-27 to this tolerogenic effect, we injected IFN-γ-treated DCs or control DCs into WT and IL-27KO mice. We found that IFN-γ-modified DCs reduced clinical severity only in WT mice. Disruption of IL-27 abrogated the tolerogenic effect of IFN-γ-treated DCs, which were not able to limit disease severity (Fig. 5C). In accordance with the disease score, disruption of the IL-27 pathway abrogated the disease-modifying effect of DCs by modulating T cell cytokine secretion (Fig. 5D). In the clinical setting of MS, therapeutic intervention is often started after the onset of the symptoms. Therefore, it is important to investigate whether a treatment regimen, which is effective EAE prevention, can also reverse established disease. Thus, we tested the efficacy of IFN-γ-modi-
ified DCs on EAE after the onset of clinical symptoms. We found that treatment with IFN-γ-modified DCs after the onset of EAE (score of ≥1.5) resulted in rapid clinical recovery from EAE (Fig. 5E). These results indicate an essential role for IFN-γ in driving DCs that blunt Th17 responses and halt autoimmune inflammation through mechanisms involving IL-27 and IL-10.

**IL-27 Treatment Inhibits Clinical Severity of EAE in IFN-γR−Deficient Mice.** Our results thus far suggest that IFN-γ suppresses EAE development via induction of IL-27. To further investigate this observation, we asked whether IL-27 treatment in vivo could reverse the severe EAE phenotype observed in IFNγR−/− mice. To test this hypothesis, WT and IFN-γR−/− mice were administered recombinant mouse IL-27 after immunization with MOG. Consistent with recent studies (10), we found that IL-27 could inhibit EAE development in WT mice (Fig. 6A). Strikingly, injection of IL-27 suppressed the severe EAE phenotype observed in IFNγR−/− mice compared with PBS controls (Fig. 6A). In addition, LN cells from both IL-27-treated WT and IFN-γR−/− mice produced much less IL-17 and higher IL-10 when restimulated with MOG antigen (Fig. 6B). Furthermore, we found that IL-27 treatment inhibited OPN levels in DCs from both WT and IFN-γR−/− mice (Fig. 6C). Because we observed that IFN-γ-induced IL-27 expression was accompanied by reduced OPN levels in DCs, we examined whether IL-27 had any inhibitory effects on OPN expression in DCs. In accordance with the in vivo inhibition of OPN, we found that IL-27 significantly inhibited OPN expression in DCs both from WT and IFN-γR−/− mice (Fig. 6D). In addition we found that bone marrow-derived DCs from IL-27R−/− mice expressed higher levels of OPN than DCs from WT mice (Fig. 6E). Moreover, DCs isolated from spleen, LN, and CNS of IL-27R−/− mice had very high levels of OPN compared with WT mice (Fig. 6F), and serum OPN levels were significantly higher in IL-27R−/− mice in comparison with WT mice (Fig. 6G). These findings indicate that the reduced OPN expression observed in DCs from IL-27-treated mice is due to the inhibitory effect of IL-27 on OPN. Thus, IL-27-mediated resolution of inflammation is mediated both through induction of IL-10 and inhibition of IL-17 from CD4+ T cells and by inhibition of OPN expression in DCs. Taken together, these results demonstrate that IFN-γ serves a protective role in Th17-mediated CNS inflammation via IL-27 induction.

**Discussion**

Our results identify a previously unknown pathway by which IFN-γ limits IL-17–mediated autoimmune inflammation through DC modulation of OPN and IL-27. In EAE, it was initially thought that CD4+ T cells mediating autoimmunity had a Th1 phenotype characterized by the production of IFN-γ (26). However, this traditional view has been challenged by studies describing more EAE in IFN-γ–deficient animals (27). Evidence now indicates that T cells critical for EAE are characterized by the production of IL-17. In addition, IL-17 expression has been detected in the target tissue in human autoimmune diseases including MS, rheumatoid arthritis, and psoriasis (28). IL-17–deficient mice develop EAE with delayed onset and reduced severity (29), and anti-IL-17 antibody prevents chemokine expression in the brain and the subsequent development of EAE (30). Furthermore, T cell infiltration and inflammation in the brain in EAE occur only when Th17 cells outnumber Th1 cells (31).

IFN-γ plays an important role in Th17 cell biology. IFN-γ−/− mice immunized with collagen in CFA develop severe arthritis, and T cells from such mice secrete increased amounts of IL-17 after collagen restimulation in vitro (32). Along the same line, after mycobacterial infection, IFN-γ−/− mice had larger numbers of IL-17–producing T cells than WT mice (33). In addition, a disease-ameliorating effect of IFN-γ has been observed in lethal autoimmune myocarditis (34). Nonetheless, although en-
dogenous IFN-γ is protective in animal models of arthritis and in EAE, the mechanisms that orchestrate the anti-inflammatory effects of IFN-γ in controlling autoimmune inflammation are poorly understood. Here, we show that IFN-γ induces IL-27 while inhibiting OPN expression in DCs and that engagement of IFNγR by DCs leads to suppression of IL-17 production while inducing IL-10 from T cells. In accordance with these in vitro results, DCs from IFN-γR−/− mice expressed very high levels of OPN and low levels of IL-27 in comparison with DC from WT mice. T cells cultured with IFN-γR−/− DCs produced higher IL-17 and lower IL-10, whereas T cells cultured with DCs from WT mice produced lower IL-17 and higher IL-10. This difference in T cell production of IL-17 and IL-10 correlated with the pattern of OPN and IL-27 expression by DCs that are modulated by IFN-γ. We have reported that OPN-induced IL-10 production by CD4 T cells via β3-integrin receptor and inhibited IL-10 via CD44 receptor (16). We have also shown that OPN-induced IL-17 production is associated with an increase in the IL-17 lineage transcription factor RORγt expression. IL-27-mediated inhibition of IL-17 has been shown through the down-regulation of RORγt (7). It appears that IL-27 overrides the effect of OPN in activated T cells by down-regulating RORγt expression and indirectly affects IL-10 via the CD44 receptor. Thus, our results demonstrate that there is a reciprocal regulation in the induction of pathogenic cytokine IL-17 and protective cytokine IL-10 in the immune system depending on the cytokine secretion by the innate immune system.

Previous studies have shown that the differentiation of Th17 cells is suppressed by IFN-γ. In vitro, treatment with IFN-γ-neutralizing antibody leads to increased frequency of Th17 cells, whereas exogenous IFN-γ reduces Th17 populations (35). Our results identify a previously unknown pathway by which IFN-γ limits IL-17-mediated autoimmune inflammation through DC modulation of OPN and IL-27. The negative impact of IFN-γ on IL-17 production from T cells was mediated through inhibition of OPN and induction of IL-27 from DCs that, in turn, leads to suppression of IL-17 from T cells. In addition, Th17 generation was also suppressed by expression of the IFN-γR receptor on DCs and macrophages, thus demonstrating the potential similarities between IFN-γ and IFN-α/β-dependent suppression of the Th17 response (17, 36).

The nature of specialized DCs that selectively dampen effector T cell-driven immunity is not well understood. Our results suggest a dominant function for IFN-γ-modified DCs to dampen autoimmunity through the induction of IL-10 and/or inhibition of IL-17 production from T cells in an IL-27-dependent manner. It has been shown that 27-ILR−/− mice develop exacerbated EAE, owing to high IL-17 or low IL-10 production from T cells (8–10). Consistent with these findings, the tolerogenic effect of IFN-γ-modified DCs we observed was completely abrogated in 27-ILR−/− mice. The increased expression of IL-27 in WT vs. IFN-γ-deficient mice correlates with increased IL-10 expression in T cells during EAE, which suggests that IFN-γ contributes to dampening inflammatory responses and driving the resolution of autoimmune pathology.

An additional mechanism to limit immune responses is the negative feedback of inflammatory process by inflammatory cytokines. DC expressed OPN promotes induction of IFN-γ in addition to IL-17 from T cells both in humans and mice (19). Our results demonstrate that OPN induces a negative feedback loop whereby IFN-γ limits IL-17 production by T cells through the induction of IL-27 and inhibition of OPN from DCs, and that this feedback loop is perturbed by the absence of IFN-γ (Fig. S3). The role of IFN-γ in antagonizing the function of IL-17, a critical pathogenic cytokine in autoimmune conditions, might be paradoxical for IFN-γ, a proinflammatory cytokine. However, it is now recognized that IFN-γ has regulatory function in limiting inflammation. At the height of inflammation during the course of an autoimmune pa-

Fig. 6. IL-27 treatment inhibits clinical severity of EAE in IFN-γR−/− mice. (A) IL-27 inhibits clinical severity of EAE in WT and IFN-γR−/− mice. WT and IFN-γR−/− mice (n = 6) were immunized with MOG peptide emulsified in complete Freund’s adjuvant. Recombinant IL-27 (0.25 μg per mouse) was administered s.c. to immunized WT and IFN-γR−/− mice every other day from day 2 until day 18. (B) LN cells from IL-27-treated and control mice were activated in vitro with MOG35–55 (20 μg/mL) for 72 h, and cell-free culture supernatants were assayed for IL-17 and IL-10 by ELISA. (C) Real-time RT-PCR analysis of OPN in DCs isolated from LN and CNS of WT and IFN-γR−/− mice (n = 5–6 per group) treated with or without IL-27. (D) Real-time RT-PCR analysis of OPN in DCs isolated from spleen of naive WT and IFN-γR−/− mice treated with or without rIL-27 (100 ng/mL). (E) Real-time RT-PCR analysis of OPN in DCs isolated from spleen of naive WT and IL-27R−/− mice. (F) Real-time RT-PCR analysis of OPN in DCs isolated from spleen, LN, and CNS of naive and EAE bearing WT and IL-27R−/− mice (n = 6 per group). (G) ELISA of serum OPN from WT and IL-27R−/− mice with or without EAE.
thology, the immune system is mobilized to restrict excess inflammation, and it appears that IFN-γ acts as a master upstream regulator of both inflammatory and regulatory pathways (37, 38). Our study identifies an important IFN-γ–dependent self-regulatory process that serves to control immune system homeostasis through an OPN/IL-27 axis in dendritic cells.

**Materials and Methods**

DCs and CD4+ T cells were purified from spleens and LNs by positive selection using CD11C+ and CD4+ microbeads, respectively (Miltenyi Biotec). Bone marrow–derived DCs were generated by using a described method (39). For in vitro assays, DCs were activated with recombinant mouse IFN-γ for 12–60 h. Splenocytes or LN cells were activated with MOG 35–55 peptide (20 μg/mL) for 60–72 h. For DC-T cell cocultures, total CD4+ T cells isolated from MOG-specific TCR-transgenic mice (Z22) were cocultured with CD11C+ DC isolated from naive and MOG35-55-immunized WT and IFN-γ−/− mice. Cocultures were performed at a 1:3 DC/T cell ratio in U-bottom 96-well plates. Cytokines were measured by using real-time quantitative RT-PCR, and specific ELISA and Western blotting were performed with primers and antibodies as listed in *SI Materials and Methods*. For EAE induction, mice were injected s.c. with 100 μg of MOG35-55 peptide emulsified in CFA (Difco), supplemented with 5 mg/mL *Mycobacterium tuberculosis* and injected twice i.v. with 200 μg of pertussis toxin. Clinical assessment of EAE was performed as described in *SI Materials and Methods*. See *SI Materials and Methods* for full methods.

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Supporting Information

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SI Materials and Methods

Mice. C57BL/6 WT, IFN-γ−/−, and IFN-γR−/− mice were purchased from the Jackson Laboratory. MOG-specific TCR transgenic mice—2D2 and IL-27R−/− mice—were obtained from Vijay Kuchroo (Harvard University, Boston). Animals were maintained in a specific pathogen-free condition in the animal facility of Harvard Institutes of Medicine. All mice were 6–8 wk old at the beginning of experiments. All experiments were in accordance with guidelines from the committee on animals at Harvard Medical School.

Induction and Evaluation of EAE. Mice were injected s.c. in both flanks with 100 μg of MOG35–55 peptide (MEVGWYRSPFSR- VHLYRNGK) dissolved in PBS emulsified in an equal volume complete Freund’s adjuvant, CFA (Difco), supplemented with 5 mg/mL Mycobacterium tuberculosis H37Ra and injected twice i.v. with 200 ng of pertussis toxin (List Biological Laboratories) administered on the day of immunization and 48 h later. Clinical assessment of EAE was performed daily after disease induction according to the following criteria: 0, no disease; 1, tail paralysis; 2, hindlimb weakness or partial paralysis; 3, complete hindlimb paralysis; 4, forelimb and hindlimb paralysis; 5, moribund state. Mean clinical scores on separate days were calculated by adding scores of individual mice and dividing total number of mice in each group, including mice that did not develop signs of EAE.

IL-27 Treatment. For in vivo IL-27 treatment, carrier-free recombinant mouse IL-27 (0.25 μg/mL suspended in 100 μL of PBS) was administered by s.c. injection to MOG-immunized mice every day from day 2 until day 18.

Generation and Isolation of DCs. DCs were derived from bone marrow progenitor cells. In brief, the femoral and tibial cells were harvested in DC culture medium (RPMI medium 1640, 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, 20 ng/mL GM-CSF, and 10 ng/mL IL-4) and seeded in 24-well plates at a density of 1 x 106 cells/mL per well. Culture medium was replaced with fresh medium every 3 d. At day 6, dislodged cells were used as bone marrow-derived DCs. Splenic DCs were isolated from using CD11c beads (Miltenyi Biotec). DCs were stimulated with or without recombinant IFN-γ and then pulsed with MOG peptide. The MOG-pulsed DCs were harvested, washed extensively with sterile PBS, and used for in vivo priming. Mice were injected s.c. three times at 5-d intervals with 2 x 106 DC before MOG immunization.

RNA Isolation, cDNA Synthesis and Real-Time PCR. Total RNA was isolated from cell pellets by using RNAeasy Micro Kit (QIAGEN). RNA was stored at −80 °C. First-strand cDNA synthesis was performed for each RNA sample from 0.5 to 1 μg of total RNA by using Taqman reverse transcription reagents. cDNA was amplified by using sequence specific primers (probes used were identified by Applied Biosystems assay identification number: IL-27, Mm00461164_m1; OPN, Mm00436767_m1; IL-17, IL-10, Mm99999062_m1; IL-23, Mm01160011_m1; IL-12, Mm99999066_m1; IL-6, Mm99999064_m1; IL-1p, Mm01356189_m1; TGF-β, Mm-03024053_s1) (Applied Biosystems) and real-time PCR mix (Applied Biosystems) on ABI7500 cycler. GAPDH gene was used as an endogenous control to normalize for differences in the amount of total RNA in each sample. All values were expressed as fold increase or decrease relative to the expression of GAPDH.

Proliferative Responses of T Cell and Cytokine Analysis. Spleens—2D2 and IL-27R−/− mice were cultured with plate-coated anti-CD3 and anti-CD28 mAb (0.3 μg/mL) in the presence or absence of 1 μg/mL mouse rOPN. In some culture conditions, 100 ng/mL of mouse rIL-27 was added together with 1 μg/mL of mouse rOPN. For DC- T cell cocultures, total CD4+ T cells isolated from MOG-specific TCR transgenic mice (2D2) were cocultured with CD11c+ DC isolated from naïve and MOG35-55 immunized WT and IFN-γ−/− mice. Cocultures were performed at a 1:3 DC/T cell ratio in U-bottom 96-well plates. Supernatants from cultures were harvested 72 h after initiation of cultures. IL-10 and IL-17 in the supernatants were assayed by ELISA kits (BD Biosciences).

Cytokine Assays. Mouse IL-10 and IL-17 were assayed by ELISA kits (BD Biosciences). OPN and IL-27p28 ELISA kits were from R&D Systems.

Western Blot Analysis. Whole-cell extracts (WCE) were prepared by using Roche complete lysis buffer and protease inhibitor tablets. Twenty micrograms of WCE was resolved on 10% acrylamide gels (Invitrogen) and transferred onto nitrocellulose membrane. Membranes were blocked at 5% nonfat milk powder at room temperature. For Opn expression, membranes were then incubated with 0.2 μg/mL anti-Opn antibody (R & D Systems) for 1 h at room temperature. Expression was visualized by using secondary HRP antibody and SuperSignal substrate kit from Pierce. For α-tubulin expression, nitrocellulose membranes were stripped and reprobed with antibody to α-tubulin.

Preparation and Evaluation of CNS Cells. Animals were perfused with cold PBS. Brains and spinal cords were dissected and incubated in 2.5 mg/mL collagenase D for 30 min at 37 °C. Single-cell suspensions were prepared by passing through a 70-μm strainer. Cells were washed in RPMI medium 1640, and mononuclear cells were isolated by using a discontinuous Percoll gradient (Pharmacia). Cells were washed twice and CD11c+ cells were isolated from this suspension by magnetic separation using microbeads (Miltenyi Biotec).

Statistical Analysis. Statistical analysis was performed by using the unpaired Student t test. A value of P < 0.05 was considered significant. Data are presented as mean ± SEM. For EAE, groups were compared by using linear regression analysis.
Fig. S1. IFN-γ inhibits OPN while inducing IL-27 without affecting other DC-associated cytokines. Real-time quantitative RT-PCR analysis of mRNA encoding OPN, IL-27, IL-1β, IL-6, IL-23, TNF-α, and TGF-β in DCs treated with or without mouse rIFN-γ (100 ng/mL).

Fig. S2. IFN-γ deficiency leads to altered serum OPN and IL-27 levels. (Left) IFN-γ-deficient mice develop severe EAE. WT and IFN-γ−/− mice (n = 6) were immunized with MOG peptide emulsified in CFA and monitored for EAE. ELISA of serum OPN (Center) and IL-27 (Right) from WT and IFN-γ−/− mice with or without EAE.

Fig. S3. OPN/IL-27 axis in dendritic cells is modulated by IFN-γ to limit IL-17-mediated autoimmune inflammation. DC-expressed OPN induces production of IFN-γ and IL-17 while inhibiting IL-10 from T cells (green arrows). At the height of inflammation, IFN-γ limits OPN-induced IL-17 production by T cells through the induction of IL-27 and inhibition of OPN from DCs (red arrows), and this feedback loop is perturbed by the absence of IFN-γ.