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J Immunol published online 2 November 2012
http://www.jimmunol.org/content/early/2012/10/31/jimmunol.1200808

Supplementary Material
http://www.jimmunol.org/content/suppl/2012/11/01/jimmunol.1200808.8.DC1

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IFN-γ Limits Th9-Mediated Autoimmune Inflammation through Dendritic Cell Modulation of IL-27

Gopal Murugaiyan,1 Vanessa Beynon,1 Andre Pires Da Cunha, Nicole Joller, and Howard L. Weiner

IL-9–producing Th9 cells have been associated with autoimmune diseases, such as experimental autoimmune encephalitis. However, the factors that negatively regulate Th9 cells during autoimmune inflammation are unclear. In this article, we show that IFN-γ inhibits Th9 differentiation both in vitro and in vivo. This suppressive activity was dependent on the transcription factor STAT-1. In addition to its direct inhibitory effect on Th9 differentiation, IFN-γ suppressed Th9 cells through the induction of IL-27 from dendritic cells. In vitro, treatment of naive CD4+ T cells with IL-27 suppressed the development of Th9 cells, which was partially dependent on the transcription factors STAT-1 and T-bet. Moreover, IL-27 treatment completely abrogated the encephalitogenicity of Th9 cells in the experimental autoimmune encephalomyelitis model. Thus, our results identify a previously unknown mechanism by which IFN-γ limits Th9-mediated autoimmune inflammation through dendritic cell modulation of IL-27. The Journal of Immunology, 2012, 189: 000–000.

Experimental autoimmune encephalomyelitis (EAE), induced by immunization of mice with myelin oligodendrocyte glycoprotein 35–55 [MOG (35–55)] peptide in complete Freund’s adjuvant, is a well-established model for the human autoimmune disease multiple sclerosis. Evidence indicates the involvement of a specific type of Th cell, termed Th9 cells in the pathogenesis of EAE (1–4). Th9 cells are characterized by the expression of IL-9. Th9 cells have been established as a separate lineage of Th cells distinct from conventional Th1, Th2, and Th17 cells (5–7). It has been demonstrated that mice deficient for IL-9 or IL-9R are protected from EAE, and the suppression of EAE in IL-9−/− mice could be reversed when treated with rIL-9 (1–4). In addition, altered IL-9 or IL-9R expression has been linked to other autoimmune diseases such as asthma and arthritis (7–9). Although Th9 cells have been suggested to be mediators of the inflammation associated with autoimmune diseases, the factors that negatively regulate Th9 cells during autoimmune inflammation are not known. IL-27 is a potent anti-inflammatory cytokine, which belongs to the IL-12 family and is composed of an IL-12p40–related protein, encoded by the EBV-induced gene 3 (also known as IL-27), and a unique IL-12p35–like protein, IL-27p28 (10). Initial studies on the biology of IL-27 suggested a role for IL-27 in the initiation of the Th1 response (11, 12). However, recent studies have indicated that IL-27 has broad inhibitory effects on Th1 and Th2 subsets of T cells as well as on APC function (13, 14). In addition, we and others have shown that IL-27 is capable of inducing IL-10–producing regulatory type 1 (Tr1) cells while inhibiting IL-17–producing Th17 cells both from humans and mice and acts as a negative feedback mechanism against proinflammatory immune responses (15–19). Although IL-27 has been shown to negatively regulate other inflammatory T cell subsets, the role of IL-27 in the regulation of Th9 cells remains unknown.

In this article, we show that IFN-γ inhibits Th9 differentiation both in vitro and in vivo. In addition to its direct inhibitory effect on Th9 differentiation, IFN-γ suppressed Th9 cells through the induction of IL-27 from dendritic cells (DCs). IL-27 suppressed the development of Th9 cells, which was partially dependent on the transcription factors STAT-1 and T-bet. Furthermore, IL-27 treatment completely abrogated the encephalitogenicity of Th9 cells in the EAE model. Taken together, our results identify a previously unknown mechanism by which IFN-γ limits Th9-mediated autoimmune inflammation through DC modulation of IL-27.

Materials and Methods

Mice

C57BL/6 wild-type (WT), IFN-γ−/−, IFN-γR−/−, IL-10−/−, T-bet−/−, and IL-21R−/− mice were purchased from The Jackson Laboratory (Bar Harbor, ME). 129S6/SvEv STAT-1−/− and 129S6/SvEv control mice were obtained from Taconic. MOG-specific TCR transgenic mice 2D2 and IL-27R−/− mice were obtained from Dr. V. Kuchroo (Harvard University, Cambridge, MA). STAT-3−/− mice were obtained from D.E. Levy (New York University Medical Center, New York, NY). All mice were used at 6–8 wk of age. Animals were maintained in a specific pathogen-free condition in the animal facility of Harvard Institutes of Medicine. All experiments were in accordance with guidelines from the committee on animals at Harvard Medical School.

Reagents

Cells were maintained with IMDM (Invitrogen). Neutralizing anti–IL-9 was obtained from BD Biosciences, and anti–IL-27 Ab was obtained from R&D Systems. FACS Abs to IL-9 and IFN-γ were obtained from BioLegend. Recombinant mouse TGF-β, IL-4, and IL-27 were obtained from R&D Systems. All RT-PCR primers and reagents were obtained from Applied Biosystems.
Induction and evaluation of EAE

Mice were injected s.c. in both flanks with 100 μg MOG (35–55) peptide (MEGVYSRSPFSRVHLYRNGK) dissolved in PBS emulsified in an equal volume of CFA-CFA (Difco) supplemented with 5 mg/ml Mycobacterium tuberculosis H37Ra and injected twice i.v. with 200 ng 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, hind-limb weakness or partial paralysis; 3, complete hind-limb paralysis; 4, forelimb and hind-limb paralysis; and 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.

Anti-IL-9 treatment

Mice (n = 8) received 50 μg IL-9 Ab (BD Biosciences) or IgG control i.p. every other day starting on day −1 postimmunization.

RNA isolation, cDNA synthesis, and real-time PCR

Total RNA was isolated from cell pellets using RNA easy 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 total RNA using Taqman reverse transcription reagents. cDNA was amplified using sequence-specific primers (the following were from Applied Biosystems): IL-27, Mm00461164_m1; IL-10, Mm00453655_m1; IFN-γ, Mm01168134_m1; IL-21, Mm00517640_m1; and IL-9, Mm00434305_m1 real-time PCR mix) 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.

Cytokine analysis

Spleens or draining lymph nodes (inguinal regions) were harvested and pooled from EAE mice, and single-cell suspensions were prepared. Cells were cultured at 5 × 10^5/well in 96-well U-bottom plates with 20 μg/ml MOG (35–55) peptide in RPMI 1640 medium supplemented with 10% FCS. For ELISA, supernatants were harvested at 72 h of culture. The concentrations of indicated cytokines were measured by quantitative capture ELISA according to the guidelines of the manufacturer (BD Biosciences).

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 twice and CD4^+ T cells were isolated from this suspension by magnetic separation using microbeads (Miltenyi Biotec).

Generation of DCs. DCs were derived from bone marrow progenitor cells. In brief, the femoral and tibial cells were harvested in DC culture medium (RPMI 1640 medium, 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 × 10^5 cells/ml/well. Culture medium was replaced with fresh medium every 3 d. At day 6, dislodged cells were used as bone marrow-derived DCs. Spleenic DCs were isolated using CD11c beads (Miltenyi Biotec).

IFN-γ treatment of DCs

DCs were stimulated with IFN-γ in the presence or absence of LPS for 48 h. Supernatants were collected as conditioned medium (CM) and stored at −70°C. The amount of IL-27 was measured using ELISA.

T Cell culture. Naive CD4^+ T (CD4^+CD44lo CD62L^+) cells were cultured in RPMI 1640 medium (Sigma-Aldrich). Medium was supplemented with 5% FCS, 1% penicillin/streptomycin, 1% L-glutamine and sodium pyruvate, and 50 μM 2-ME. Cells were stimulated with plate-bound anti-CD3 (2 μg/ml) and anti-CD28 (2 μg/ml). For Th9 cell differentiation, cells were stimulated in the presence of the following cytokines: 20 ng/ml IL-4 and 3 ng/ml TGF-β. In some culture condition, recombinant mouse IFN-γ (100 ng/ml) or IL-27 (100 ng/ml) was added. For adoptive transfer of EAE, 2D2 CD4^+ T cells were stimulated under Th9 differentiation condition in the presence of absence of IL-27. Restimulated cells were collected and extensively washed with PBS. A total of 5 × 10^5 cells were injected i.v. into Rag-1−/− mice. Recipient mice were injected i.p. with 200 ng pertussis toxin (List Biological Laboratories) on days 0 and 2 after T cell transfer.

Statistical analysis was performed using the unpaired t test. A p value < 0.05 was considered significant. Data are presented as mean SEM. For EAE, groups were compared using linear regression analysis.

Results

IFN-γ inhibits Th9 differentiation

We first examined the effect of IFN-γ on the production of IL-9 from Th9 cells. We found that IFN-γ stimulation significantly inhibited IL-9 production from Th9 cells (Fig. 1A). Activation of the intracellular signaling molecule STAT-1 is a common feature of IFN-γ signaling (20). We therefore tested whether the inhibition of IL-9 production by IFN-γ was dependent on STAT-1 using STAT-1−/− T cells. We found that IFN-γ did not suppress Th9 development in STAT-1−/− T cells (Supplemental Fig. 1A). These data indicate that IFN-γ-mediated suppression of Th9 development requires activation of STAT-1. To test whether IFN-γ regulates IL-9 expression in vivo, we induced EAE in WT and IFN-γ−/− mice. Consistent with previous studies, we found that IFN-γ−/− mice developed severe EAE (Fig. 1B). We observed altered IL-9 expression in T cells isolated from WT and IFN-γ−/− mice with EAE (Fig. 1C). In addition, we found that IFN-γ−/− mice had high serum levels of IL-9 compared with WT mice (Fig. 1D). To investigate whether IL-9 blockade in vivo could reverse the severe EAE phenotype observed in IFN-γ−/− mice, we induced EAE in IFN-γ−/− mice and administered neutralizing anti–IL-9 Ab. We

FIGURE 1. IFN-γ inhibits Th9 differentiation both in vitro and in vivo. (A) Real-time PCR and flow cytometry analysis of naive CD4^+ T cells stimulated under Th9 conditions in the presence or absence of IFN-γ (100 ng/ml). (B) Clinical scores of WT and IFN-γ−/− mice at various times after immunization with MOG (33–55) in CFA. (C) Real-time RT-PCR analysis of IL-9 in LN and CNS-derived CD4^+ T cells isolated from WT and IFN-γ−/− mice with or without EAE. (D) ELISA of serum IL-9 from WT and IFN-γ−/− mice with EAE. (E) Clinical scores of EAE in IFN-γ−/− mice (n = 8/group) treated with anti–IL-9 Ab or isotype control. Results are representative of three independent experiments.
found that anti–IL-9 Ab treatment delayed the onset of clinical disease and ameliorated the severity of EAE (Fig. 1E). These results suggest that IFN-γ plays an important role in the regulation of IL-9 production from T cells and that neutralization of IL-9 suppresses the production of encephalitogenic T cells.

**IFN-γ limits Th9 differentiation through dendritic cell modulation of IL-27**

We have previously shown that IFN-γ induces expression of IL-27 by DCs (21). To test whether IFN-γ regulates DC expression of IL-27 in vivo, we induced EAE in WT and IFN-γ−/− mice. We found that expression of IL-27 was upregulated at both the mRNA and protein levels in WT DCs of mice with EAE and that DCs from IFN-γ−/− mice with EAE had much less IL-27 expression compared with WT mice (Fig. 2A, 2B). Because we observed that IFN-γ deficiency led to reduced IL-27 expression in DCs and increased IL-9 in T cells during EAE, we investigated whether DC-derived IL-27 had a role in Th9 cell differentiation, specifically T cell production of IL-9. For this, we immunized WT and IFN-γ−/− mice with MOG and isolated splenocytes on day 10 postimmunization. Cells were then cultured with MOG in vitro in the presence or absence of anti–IL-27 Ab, and MOG Ag-specific IL-9 production was measured. We found that T cells from IFN-γ−/− mice showed increased IL-9 levels compared with T cells from WT mice (Fig. 2C). In addition, we found that the addition of neutralizing Ab to IL-27 significantly increased IL-9 production both from WT and IFN-γ−/− mice (Fig. 2C). Next, we tested whether CM from DCs stimulated with IFN-γ has an inhibitory effect on IL-9 production from T cells. We found that the addition of CM from IFN-γ−/−-treated DCs inhibited IL-9 production from Th9 culture (Fig. 2D). To determine whether IL-27 contributes to the inhibitory effect of the IFN-γ pathway, we used an anti–IL-27 Ab to block IL-27 activity. When anti–IL-27 Ab was added to block the IL-27 activity in the CM from IFN-γ−/−-treated DCs, the IFN-γ-mediated inhibitory effect on Th9 cells was reversed (Fig. 2D).

To determine whether IL-27 also contributes to IFN-γ-mediated inhibition of IL-9–producing encephalitogenic T cells, lymphocytes isolated from mice immunized with MOG/CFA were restimulated with MOG peptide in the presence of CM from IFN-γ−/−-treated DCs with or without IL-27 Ab. As shown in Fig. 2E, CM from IFN-γ−/−-treated DCs suppressed IL-9 production from Ag-specific T cells, and anti–IL-27 Ab inhibited this suppression. Moreover, the inhibition of Th9 was not due to the presence of IFN-γ in the culture supernatants as we used IFN-γR−/− T cells. Taken together, our results demonstrate that IFN-γ signaling events in DCs play a major role in negative regulation of Th9 development. These data suggested that IL-27 negatively regulates IL-9 production from T cells and prompted us to examine the function of IL-27 in the Th9 differentiation pathway in vitro. To directly investigate the effect of IL-27 on Th9 differentiation, we cultured naive CD4+ T cells under Th9-polarizing condition in the presence or absence of rIL-27. We found that addition of IL-27 significantly inhibited IL-9 from Th9 cells (Fig. 3A, 3B). IL-27 blocked Th9 cell differentiation even more than IFN-γ. It has been recently shown that IL-25 is involved in the amplification of Th9 cells, and expression of the IL-25R on Th9 cells has been described previously (22). Although addition of IL-25 to Th9 differentiation conditions increased IL-9 expression, even under these strong Th9 polarizing conditions IL-27 markedly inhibited IL-9 expression and IL-25R expression (Fig. 3C, 3D). Collectively, these data indicate that IL-27 inhibits the differentiation of Th9 cells. To confirm that IL-27 is indeed a negative regulator of IL-9 expression in vivo, we isolated lymphocytes from WT and IL-27R−/− mice with EAE and found that IL-27R−/− mice had elevated levels of IL-9 after MOG stimulation compared with cells from WT mice. In agreement with previous studies, we found that IL-27R−/− mice developed severe EAE (Supplemental Fig. 1B). Consistent with clinical scores, ex vivo CD4+ T cells isolated from the CNS of IL-27R−/− mice expressed higher levels of IL-9 in comparison with CD4+ T cells from WT mice (Fig. 3E). Moreover, we observed higher IL-9 expression by ex vivo-restimulated lymph node cells from IL-27R−/− mice than by cells from WT mice (Fig. 3F).

In addition to Th9 cells, Th17 cells can also produce IL-9. It has been demonstrated that IL-9 together with TGF-β can induce the differentiation of naive CD4+ T cells into Th17 cells in vitro and that IL-9 produced by Th17 cells themselves amplifies Th17 development in an autocrine manner (23). IFN-γ and IL-27 have been shown to inhibit Th17 differentiation both in humans and mice (16, 24). Moreover, T cells from IFN-γ and IL-27R−/− mice had elevated levels of IL-17 during the course of EAE (18, 21).

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** IFN-γ limits IL-9 production through induction of IL-27 from DCs. (A) Real-time RT-PCR analysis of IL-27p28 in DCs isolated from spleen of EAE bearing WT and IFN-γ−/− mice (n = 5/group). (B) DCs from EAE bearing WT and IFN-γ−/− mice (n = 5/group) were stimulated with LPS (100 ng/ml). IL-27 production was measured by ELISA. Results are representative of three independent experiments. (C) Splenocytes isolated from immunized WT and IFN-γ−/− mice on day 10 were restimulated with MOG peptide in the presence or absence of anti–IL-27 Ab. IL-9 levels were determined by real-time PCR and ELISA. (D) WT DCs were stimulated with IFN-γ for 24–48 h. Supernatants from IFN-γ-stimulated DCs were used as CM: they were added to Th9 culture in the presence or absence of anti–IL-27 Ab and cells were incubated for 72 h. IL-9 production by CD4+ T cells was measured by ELISA. (E) IL-27 contributes to IFN-γ–mediated inhibition of encephalitogenic T cells. Splenocytes isolated from immunized IFN-γ−/− mice were restimulated with MOG peptide for 72 h in the presence of CM from IFN-γ–treated DCs plus anti–IL-27 Ab or an isotype control. IL-9 levels were measured by ELISA. Data shown are representative of three independent experiments.
IL-9 expression at RNA and protein levels in both IFN-γ and IL-27. We found that the addition of IL-27 to Th9 cells blocked IL-9 production (Fig. 5). Following this, we tested whether the inhibition of IL-9 by IL-27 was dependent on IL-21. We found that both IFN-γ and IL-27 inhibited IL-9 expression from Th17 cells (Fig. 4A, 4C). Thus, IFN-γ and IL-10 alone were not responsible for the suppression of IL-9 expression by IL-27. IL-27 drives the expansion of Tr1 cells by inducing the expression of IL-21, a member of the IL-2 family of cytokines, which acts as an autocrine growth factor for Tr1 cells (25). Thus, we asked whether the inhibition of IL-9 production by IL-27 was dependent on IL-21. We found that IL-27-mediated Th9 cell suppression was not secondary to the induction of IL-21, because addition of IL-27 to IL-21R−/− Th9 cells completely inhibited IL-9 expression both at RNA and protein levels (Supplemental Fig. 3). Collectively, these data indicate that IL-27 inhibits Th9 differentiation while inducing Tr1 cytokines and that the inhibition of Th9 cells is not mediated through any of the induced cytokines. Thus, our results demonstrate that there is a reciprocity in the regulation of the pathogenic cytokine IL-9 and the protective cytokine IL-10 in the immune system depending on IL-27 secretion by the innate immune system.

IL-27 inhibits IL-9 production through STAT-1 and T-bet dependent pathway

IL-27 induces the phosphorylation of STAT-1 and STAT-3 proteins both of which play an important role in T cell function (26, 27). To analyze whether the differentiation of Th9 cells in the presence of IL-27 alters their phosphorylation profile, we stimulated naive CD4+ T cells activated with anti-CD3 and anti-CD28 under Th9-inducing conditions in the presence or absence of IL-27. Results are representative of three independent experiments. (Supplemental Fig. 2A) and IL-10−/− cells (Supplemental Fig. 2B). Thus, IFN-γ and IL-10 alone were not responsible for the suppression of IL-9 expression by IL-27. IL-27 drives the expansion of Tr1 cells by inducing the expression of IL-21, a member of the IL-2 family of cytokines, which acts as an autocrine growth factor for Tr1 cells (25). Thus, we asked whether the inhibition of IL-9 production by IL-27 was dependent on IL-21. We found that IL-27-mediated Th9 cell suppression was not secondary to the induction of IL-21, because addition of IL-27 to IL-21R−/− Th9 cells completely inhibited IL-9 expression both at RNA and protein levels (Supplemental Fig. 3). Collectively, these data indicate that IL-27 inhibits Th9 differentiation while inducing Tr1 cytokines and that the inhibition of Th9 cells is not mediated through any of the induced cytokines. Thus, our results demonstrate that there is a reciprocity in the regulation of the pathogenic cytokine IL-9 and the protective cytokine IL-10 in the immune system depending on IL-27 secretion by the innate immune system.

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IL-27 induces the phosphorylation of STAT-1 and STAT-3 proteins both of which play an important role in T cell function (26, 27). To analyze whether the differentiation of Th9 cells in the presence of IL-27 alters their phosphorylation profile, we stimulated naive CD4+ T cells for 24 h in the presence of Th9 differentation conditions. After 24 h, IL-27 was added, and phosphorylation of STAT-1 and STAT-3 were measured by flow cytometry. Results are representative of three independent experiments.

**FIGURE 5.** IL-27 inhibits Th9 differentiation while inducing Tr1 cells. Real-time PCR and flow cytometry analysis of IL-9, IFN-γ, IL-10, and IL-21 in naive CD4+ T cells activated with anti-CD3 and anti-CD28 under Th9-inducing conditions in the presence or absence of IL-27. Results are representative of three independent experiments.

**FIGURE 4.** IFN-γ and IL-27 inhibits Th17-produced IL-9. (A) Flow cytometry analysis of naive CD4+ T cells stimulated under Th17 condition in the presence or absence of IFN-γ (100 ng/ml) and IL-27 (100 ng/ml). (B) Real-time PCR analysis of RORγt in Th17 cells in the presence or absence of IFN-γ and IL-27. (C) Flow cytometry analysis of naive CD4+ T cells stimulated under Th9 condition in the presence or absence of IFN-γ (100 ng/ml) and IL-27 (100 ng/ml). (D) Real-time PCR analysis of RORγt in Th17 and Th9 cells.

**FIGURE 3.** IL-27 inhibits Th9 differentiation both in vitro and in vivo. Real-time PCR (A) and flow cytometry analysis (B) of naive CD4+ T cells activated with anti-CD3 and anti-CD28 under Th9-inducing conditions in the presence or absence of IL-27. Results are representative of five independent experiments. (C) Real-time PCR and ELISA of IL-9 in naive CD4+ T cells cultured under Th9 conditions together with IL-25 in the presence or absence of IL-27. Results are representative of three independent experiments. (D) Real-time PCR analysis of IL-25R on Th9 cells stimulated with or without IL-27. (E) Real-time PCR for IL-9 mRNA in CD4+ T cells isolated from the CNS of WT and IL-27R−/− mice with EAE. (F) Spleocytes from WT and IL-27R−/− mice were harvested 10 d after immunization and were stimulated ex vivo with MOG (35–55) peptide. IL-9 levels were measured by real-time PCR. Results are representative of three independent experiments.

Thus, we tested whether IFN-γ and IL-27 had suppressive function on IL-9 production by Th17 cells. We found that both IFN-γ and IL-27 inhibited IL-9 expression from Th17 cells (Fig. 4A). Consistent with previous reports, we also found that IFN-γ and IL-27 inhibited IL-17 and RORγt from these cells (Fig. 4A, 4C). However, in vitro-polarized Th9 cells produced no IL-17 and these cells did not express RORγt (Fig. 4B, 4D). Thus, our data indicate that the decrease in IL-9 production that we observed during EAE in WT mice can be attributed to the suppressive effect of IFN-γ/IL-27 on development of both Th9 and Th17 cells.

**IL-27 reciprocally regulates Th9 and Tr1 cells**

We and others have shown that IL-27 induces IFN-γ+IL-10+ Tr1 cells both from humans and mice (15, 16, 21). Thus, we asked whether activation of Th9 cells in the presence of IL-27–induced Tr1 cells. We found that addition of IL-27 inhibited Th9 differentiation while inducing Tr1 cytokines IL-10, IFN-γ, and IL-21 (Fig. 5). Following this, we tested whether the inhibition of IL-9 production by IL-27 was dependent on any of these induced cytokines. We found that the addition of IL-27 to Th9 cells blocked IL-9 expression at RNA and protein levels in both IFN-γ−/−

**FIGURE 2.** IL-27 inhibits Th9 differentiation through DC induction of IL-27.

IL-27 inhibits Th9 differentiation while inducing Tr1 cells. Real-time PCR and flow cytometry analysis of IL-9, IFN-γ, IL-10, and IL-21 in naive CD4+ T cells activated with anti-CD3 and anti-CD28 under Th9-inducing conditions in the presence or absence of IL-27. Results are representative of three independent experiments.
STAT-1 and STAT-3 was analyzed. We found that both STAT proteins were phosphorylated at the same level in the presence of Th9 conditions (Fig. 6A). Thus, IL-27 induces efficient phosphorylation of STAT-1 and STAT-3 after differentiation of CD4+ T cells under Th9 conditions, suggesting that both proteins could be involved in IL-27–mediated suppression of Th9 development. Thus, to determine whether the ability of IL-27 to inhibit IL-9 production involved these transcription factors, we stimulated naive CD4+ T cells from STAT-1<sup>−/−</sup> or STAT-3<sup>−/−</sup> mice and stimulated the cells under Th9-inducing conditions in the presence or absence of IL-27. The addition of IL-27 resulted in substantial inhibition of IL-9 production by WT and STAT-3<sup>−/−</sup> CD4+ T cells, but the effect was partially compromised in the absence of STAT-1 (Fig. 6B, 6C). Thus, STAT-3 was not responsible for the suppression of IL-9 expression by IL-27. Previous studies have shown that signaling through IL-27R leads to the activation of the transcription factor T-bet (12, 27, 28). We therefore tested whether the differentiation of Th9 cells in the presence of IL-27 leads to T-bet induction. We found that the addition of IL-27 induced T-bet expression in Th9 cells (Fig. 6D). To determine whether the ability of IL-27 to inhibit IL-9 production is dependent on T-bet, we cultured naive CD4+ T cells from WT and T-bet<sup>−/−</sup> mice under Th9-inducing conditions in the presence of IL-27. The addition of IL-27 resulted in complete inhibition of IL-9 production by WT CD4+ T cells, but the effect was partially compromised in the absence of T-bet (Fig. 6E). Taken together, our results suggest that IL-27–mediated inhibition of Th9 cells is mediated partially through the transcription factors T-bet and STAT-1.

**IL-27 inhibits encephalitogenity of Th9 cells**

Because in vitro treatment of T cells with IL-27 inhibited the differentiation of Th9 cells, we next investigated whether IL-27 treatment inhibited encephalitogenity of Th9 cells in vivo. For this we stimulated MOG-specific 2D2 T cells under Th9-polarizing conditions in the presence or absence of IL-27 (Fig. 7A) and transferred the resulting T cells into Rag-1<sup>−/−</sup> mice. We found all Th9 cell recipients developed EAE after transfer. However, IL-27 treatment completely suppressed EAE induced by adoptive transfer of Th9 cells (Fig. 7B). Histopathological analysis of the spinal cords revealed that Th9 cells reconstituted Rag-1<sup>−/−</sup> EAE mice had increased amounts of infiltrating inflammatory cells and increased demyelination. In contrast, IL-27–treated Th9 cells failed to induce CNS inflammation, which correlated with the absence of clinical EAE (Fig. 7C, 7D). These data demonstrate that IL-27 controls the adoptive induction of EAE by IL-9–producing cells and that the disease inhibitory effect of IL-27 correlates with the inhibition of IL-9. Taken together, our findings identify IL-27 as an antagonist of Th9 development and indicate a possible therapeutic target for treating inflammatory diseases associated with Th9 cells.
IFN-γ mechanisms that orchestrate the anti-inflammatory effects of IFN-γ than WT mice (30, 31). In addition, IFN-γ mice deficient in IL-12p35 or IFN-γ autoimmune myocarditis (33), suggesting that IFN-γ disease ameliorating effect of IFN-γ with collagen in CFA develop severe arthritis (32). Moreover, a describing more severe EAE in IFN-γ dependent experiments. (Figure 7)

**Discussion**

It was initially thought that CD4+ T cells mediating autoimmunity in EAE had a Th1 phenotype characterized by the production of IFN-γ (29). However, this view has been challenged by studies describing more severe EAE in IFN-γ-deficient animals. In fact, mice deficient in IL-12p35 or IFN-γR develop more severe EAE than WT mice (30, 31). In addition, IFN-γ−/− mice immunized with collagen in CFA develop severe arthritis (32). More over, a disease ameliorating effect of IFN-γ has been observed in lethal autoimmune myocarditis (33), suggesting that IFN-γ may actually have a protective function. Nonetheless, although endogenous 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. In this study, we show that IFN-γ inhibits Th9 differentiation both in vitro and in vivo and that anti–IL-9 Ab treatment delays the onset of clinical disease and ameliorate the severity of EAE in IFN-γ−/− mice. Our finding that IFN-γ mediated inhibition of Th9 development provides a mechanistic basis for the severe EAE phenotype observed in IFN-γ−/− mice. Thus, through its antagonism of Th9 development, IFN-γ prevents the development of the pathogenic CD4+ T cell that is necessary for disease.

In addition to its direct inhibitory effect on Th9 differentiation, IFN-γ could also suppress Th9 cells through the induction of IL-27. Indeed, we found that IFN-γ induces IL-27 expression in DCs, which in turn leads to suppression of IL-9 from T cells. T cells cultured with supernatants from IFN-γ-treated DCs produced lower IL-9, whereas addition of anti–IL-27 to these supernatants restored IL-9 production. In accordance with these in vitro results, DCs from IFN-γ−/− mice expressed low levels of IL-27 in comparison with DCs from WT mice. The increased expression of IL-27 in WT versus IFN-γ-deficient mice correlates with decreased IL-9 expression in T cells during EAE, which suggests that in this setting IL-27 contributes to dampening inflammatory responses and driving the resolution of autoimmune pathology. In addition to Th9 cells, Th17 cells can also produce IL-9, which in turn amplifies and expands differentiated Th17 cells. Previous studies have suggested a pathogenic role of IL-9 as a Th17-derived cytokine that can contribute to inflammatory disease (2). In a recent study, it has been shown that IL-9 induces CCL20 production by astrocytes in vitro, thereby establishing a link between IL-9 and the infiltration of Th17 cells into the CNS (34). IFN-γ and IL-27 have been shown to inhibit Th17 differentiation in vitro. In addition, T cells from IFN-γ- and IL-27R−/− deficient mice had elevated levels of IL-17 during the course of EAE (18, 21). We found that IFN-γ and IL-27 inhibited IL-9 expression not only from Th9 cells but also from Th17 cells. Thus, our data indicate that the decrease in IL-9 production that we observed during EAE in WT mice can be attributed to the suppressive effect of IFN-γ/IL-27 on development of both Th9 and Th17 cells.

IL-27 was first characterized as a proinflammatory cytokine associated with Th1 differentiation (12). However, subsequent studies with mice deficient in IL-27R showed exacerbated inflammatory responses to a variety of challenges, suggesting that IL-27 has important immunoregulatory functions in vivo. It has been shown that IL-27R−/− mice develop exacerbated EAE (18). Consistent with these findings, we observed increased IL-9 expression in T cells isolated from IL-27R−/− mice in comparison with WT mice. IL-27 has been shown to have suppressive effects on Th1, Th2, and Th17 cell differentiation. In this study, we found that IL-27 inhibits Th9 cell differentiation while inducing IL-10–producing Tr1 cells. In our experiments, IL-27 was more efficient than IFN-γ in suppressing IL-9 production. IL-27 induces both IL-10 and IFN-γ in T cells. Although IL-27 induced IFN-γ and IL-10 in Th9 cells, IL-27-mediated suppression of Th9 development occurred even in IFN-γ−/− and IL-10−/− T cells. IL-27 has been shown to induce the phosphorylation of STAT-1 and STAT-3 proteins and transcription factor T-bet. Our data demonstrate that IL-27–induced suppression of IL-9 production by Th9 cells is mediated by STAT-1 and T-bet. Although the activation of STAT-1 and T-bet by IFN-γ or IL-27 has been associated primarily with the development of Th1 responses, our results underscore that this signaling pathway also mediates anti-inflammatory activities.

The role of IFN-γ in antagonizing the function of IL-9, a critical pathogenic cytokine in autoimmune conditions may be viewed as paradoxical because IFN-γ is considered a proinflammatory cytokine (20, 35). However, it is now recognized that IFN-γ has regulatory function in limiting inflammation. At the height of inflammation during the course of autoimmune pathology, 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. In summary, as shown in Supplemental Fig. 4, our study identifies an important IFN-γ–dependent regulatory process that serves to control IL-9–mediated autoimmune inflammation through its direct effect on T cells or by inducing IL-27 from DCs.

Disclosures
The authors have no financial conflicts of interest.

References
Supplementary Fig. 1A. Naïve CD4+ T cells isolated from WT and STAT-1-/- mice stimulated under Th9 conditions in the presence or absence of IFN-γ. IL-9 levels were measured by real-time PCR and flow cytometry. Results are representative of three independent experiments.

Supplementary Fig. 1B. Clinical scores of WT and IL-27R-/- mice (n=6/group) at various times after immunization with MOG(33–55) in CFA. Results are representative of three independent experiments.
Supplementary Fig. 2A. Naïve CD4+ T cells isolated from WT and IFN-γ−/− mice stimulated under Th9 conditions in the presence or absence of IL-27 (100ng/ml). IL-9 levels were measured by real-time PCR and flow cytometry. Results are representative of three independent experiments.

Supplementary Fig. 2B. Naïve CD4+ T cells isolated from WT and IL-10−/− mice stimulated under Th9 conditions in the presence or absence of IL-27 (100ng/ml). IL-9 levels were measured by real-time PCR and flow cytometry. Results are representative of three independent experiments.
Supplementary Fig. 3. Naïve CD4+ T cells isolated from WT and IL-21R−/− mice stimulated under Th9 conditions in the presence or absence of IL-27 (100ng/ml). IL-9 levels were measured by real-time PCR and flow cytometry. Results are representative of two independent experiments.
Supplementary Fig.4. IFN-γ dependent regulatory process that serves to control IL-9 mediated autoimmune inflammation through its direct effect on T cells or by inducing IL-27 from DCs.