



The type I IFN induction pathway constrains Th17-mediated autoimmune inflammation in mice

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IFN- β , a type I IFN, is widely used for the treatment of MS. However, the mechanisms behind its therapeutic efficacy are not well understood. Using a murine model of MS, EAE, we demonstrate that the Th17-mediated development of autoimmune disease is constrained by Toll-IL-1 receptor domain-containing adaptor inducing IFN- β -dependent (TRIF-dependent) type I IFN production and its downstream signaling pathway. Mice with defects in TRIF or type I IFN receptor (IFNAR) developed more severe EAE. Notably, these mice exhibited marked CNS inflammation, as manifested by increased IL-17 production. In addition, IFNAR-dependent signaling events were essential for negatively regulating Th17 development. Finally, IFN- β -mediated IL-27 production by innate immune cells was critical for the immunoregulatory role of IFN- β in the CNS autoimmune disease. Together, our findings not only may provide a molecular mechanism for the clinical benefits of IFN- β in MS but also demonstrate a regulatory role for type I IFN induction and its downstream signaling pathways in limiting Th17 development and autoimmune inflammation.

Introduction

MS is a chronic autoimmune demyelinating disease characterized by the infiltration of inflammatory cells, including macrophages and T cells, into the CNS that results in the destruction of myelin sheath (1, 2). As is the case for most autoimmune diseases, the etiology of MS is not known. The interplay between inflammation and neuronal degeneration most likely contributes to the initiation and progression of CNS tissue damage. To date, there are no curative treatments for MS. However, type I IFN, specifically IFN- β , has proven to be beneficial for the treatment of MS, as demonstrated by decreased inflammatory lesion formation in the CNS, prolonged remission, and lower relapse rate (3–5). In contrast, IFN- γ , a type II IFN, appears to exacerbate MS (6). Although clinical data clearly demonstrate that IFN- β is effective for treating MS, the underlying mechanism responsible for its therapeutic effects remains elusive, and the physiological role of endogenous type I IFNs in inhibiting the development of autoimmune disease is also poorly understood.

MS and EAE, an animal model of MS, were previously thought to be mediated by Th1 cells. However, a number of recent studies provide strong evidence that IL-17-producing T cells play a dominant role in the pathogenesis of EAE (7–14). After activation by professional antigen-presenting cells, naive CD4⁺ T cells differentiate into distinct effector subsets characterized by the cytokines they produce. Traditionally, CD4⁺ effector T cells have been classified into 2 subsets: Th1 and Th2 lineages. Th1 cells are induced by IL-12 and produce large quantities of IFN- γ , whereas Th2 cells secrete IL-4, IL-5, and IL-13. While Th1 cells orchestrate cellular

immunity, Th2 cells regulate humoral immunity and allergic response. In addition, Tregs have been identified for their ability to control effector T cell responses. Recently, a new Th subset that produces IL-17 (Th17) was identified. Th17 cells are distinct from Th1 and Th2 cells in phenotype, function, and developmental pathways. Th17 cells have been shown to play a critical role in the pathogenesis of inflammatory diseases, such as adjuvant-induced arthritis and EAE (7, 10, 14–20).

The development of Th17 cells is regulated by a complex network of cytokines. A number of studies demonstrate that the differentiation of Th17 cells depends on TGF- β and IL-6, which induce naive T cells to secrete IL-21. IL-21 in turn functions in a positive autocrine loop to upregulate Th17 lineage-specific transcription factor ROR γ t and expression of IL-17, whereas IL-23 maintains and expands the Th17 cell populations (10, 12, 21–29). Interestingly, TGF- β is also essential for the generation of Foxp3⁺ Tregs. Reciprocal development pathways for the generation of pathogenic Th17 cells and Foxp3⁺ Tregs have therefore been proposed. While TGF- β alone promotes the generation of Tregs that are well known for their ability to suppress autoimmunity and inflammation, in the presence of IL-6 produced by the innate immune system during infection, TGF- β induces the differentiation of proinflammatory Th17 cells. In addition to IL-6 and TGF- β , other cytokines, including IL-1, IL-13, IL-18, IL-22, and TNF, have been shown to promote Th17 differentiation or expansion in combination with IL-6/TGF- β or IL-23 (16, 18, 30–32). Studies also demonstrate that certain cytokines can interfere with the development of Th17 cells. IL-4, IFN- γ , and IFN- α are found to inhibit IL-23-driven expansion of Th17 cells. IL-2, a cytokine important for T cell survival and generation of Foxp3⁺ Tregs, inhibits the differentiation and/or expansion of Th17 cells (33). Furthermore, IL-27, an IL-12/IL-23 family member, is a potent negative regulator of Th17 cell differentiation (34–36). IL-27 is a heterodimeric molecule composed of p28 and Epstein-Barr virus-induced gene 3 (*Ebi3*) subunits, which have homologies to IL-12p35 and p40 respectively. The IL-27 receptor complex consists

Nonstandard abbreviations used: BMDC, BM-derived DC; BMM, BM-derived macrophage; CM, conditional medium; IFNAR, type I IFN receptor; IRF, IFN regulatory factor; MOG, myelin oligodendrocyte glycoprotein; Th17, IL-17-producing Th subset; TRIF, Toll-IL-1 receptor domain-containing adaptor inducing IFN- β .

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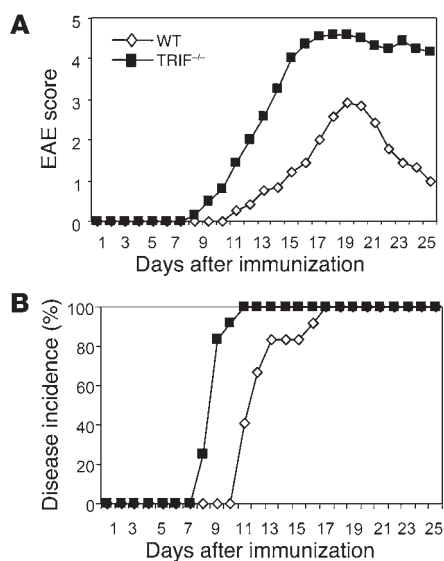


Figure 1

TRIF-deficient mice develop severe EAE. (A) Mean EAE score and (B) disease incidence in WT mice ($n = 12$) and $TRIF^{Lps2/Lps2}$ mice ($n = 12$) at each time point. WT and TRIF-deficient mice were immunized with MOG peptide (MOG_{35–55}) emulsified in CFA. Mice were assigned a disease score of 0 to 5 based on the severity of EAE.

of the unique subunit IL-27R and the gp130 chain of IL-6R. IL-27 is produced by innate immune cells and has various effects on T cell immunity. Recent data show that IL-27, via STAT1 signaling, can inhibit the differentiation of Th17 cells triggered by IL-6 plus TGF- β (34, 35, 37–39). Loss of IL-27 expression leads to increased Th17 differentiation, enhanced infiltration of IL-17-producing T cells in inflamed tissues, and exacerbated neuroinflammation.

Clinical studies demonstrate that MS is a T cell-mediated autoimmune disease of the CNS, which indicates that tolerance to the self antigen myelin is broken down, leading to the activation of autoreactive T cells. However, the existence of autoreactive T cells is not the only factor in the initiation and development of MS. Although myelin-reactive T cells are also present in healthy individuals, normal individuals have multiple layers of protective mechanisms to suppress the activation of autoreactive T cells. In EAE, the disease is induced peripherally by injection of self antigen in association with a strong adjuvant containing killed whole mycobacteria. Thus, TLRs play an important role by inducing inflammatory cytokine milieu, which may promote Th17 development and contribute to the pathogenesis of autoimmune disease. TLR signaling is mediated by a family of MyD88 adaptor proteins, primarily MyD88 and Toll-IL-1 receptor domain-containing adaptor inducing IFN- β (TRIF) (40, 41). We and others have identified 2 major types of TLR signaling pathways: the MyD88-dependent activation of NF- κ B, which results in the induction of inflammatory genes such as TNF, IL-6, and IL-1 β ; and TRIF-dependent pathways involving the induction of type I IFNs and secondary response genes activated by IFN- β in an autocrine/paracrine manner (40, 42, 43). The type I IFN family consists of IFN- β and multiple IFN- α subtypes. These cytokines bind to a common receptor, the type I IFN receptor (IFNAR), leading to the activation of the JAK/STAT signaling pathway. In addition to their well-known function as the first line of defense against viral infection, type I IFNs have a variety

of immunomodulatory effects on DCs, macrophages, T cells, and B cells. Recently, we found that the production of and signaling by type I IFN is required for LPS-induced IL-10 upregulation, suggesting that the type I IFN pathway may serve a novel antiinflammatory role in TLR-mediated signaling in macrophages (44).

Since the identification of IL-17-producing cells, extensive studies have been performed to investigate the development and function of Th17 cells. There are also multiple studies that examine the association of these cells with autoimmune diseases, including EAE. However, the contribution of innate immunity to the development of the Th17 lineage has not been well characterized. Furthermore, the functional role of type I IFN induction pathways in modulating inflammatory response in EAE is not fully understood. In this study, we used EAE as a model to investigate the role of the TRIF-dependent IFN induction pathway of the innate immunity system in the regulation of autoimmune inflammation. Our results demonstrate that the TRIF pathway negatively regulates Th17-mediated autoimmune inflammation via type I IFN-induced IL-27 production in macrophages.

Results

TRIF-deficient mice develop severe EAE. To investigate the involvement of the TRIF pathway in CNS autoimmune diseases, we examined the development of EAE in TRIF mutant mice ($TRIF^{Lps2/Lps2}$), which carry a loss-of-function mutation in the TRIF gene. We hereafter refer to these mice as $TRIF^{-/-}$ mice. WT and $TRIF^{-/-}$ mice were immunized with myelin oligodendrocyte glycoprotein (MOG) peptide emulsified in CFA. The disease progression was monitored by physical examination and assigned a disease score of 0 to 5 based on the severity of EAE. Although both groups of mice eventually developed autoimmune disease, $TRIF^{-/-}$ mice exhibited significantly more severe EAE than WT control mice. We observed that $TRIF^{-/-}$ mice experienced earlier onset of EAE and exhibited higher disease incidence during the acute phase (Figure 1, A and B). For example, at day 10 after immunization, a significant proportion of $TRIF^{-/-}$ mice developed neurological symptoms, whereas WT mice did not exhibit any signs of EAE until later in the disease course. Moreover, after initial symptom onset, WT mice gradually recovered, while $TRIF^{-/-}$ mice developed progressive disease with higher EAE scores. To examine infiltration of inflammatory cells, histological analysis was performed on spinal cord sections from these 2 groups. As shown by H&E staining (Figure 2), $TRIF^{-/-}$ mice developed more severe inflammatory lesions associated with an increased number of inflammatory foci and cell infiltration compared with the immunized WT mice.

Infiltration of Th17 cells in the CNS of TRIF-deficient mice. Recent studies have demonstrated that Th17 cells are critical for the development of autoimmune diseases, including EAE (10, 16, 21, 23). To investigate whether increased inflammation was associated with infiltration of Th17 cells in CNS, we performed immunohistochemical analysis of spinal cord from WT and $TRIF^{-/-}$ mice. Anti-CD4 and anti-IL-17 staining revealed few CD4⁺ or IL-17-positive cells in the CNS from WT mice 10 days after immunization. In contrast, multiple inflammatory foci filled with CD4⁺ and IL-17-positive cells were observed in the spinal cord sections of $TRIF^{-/-}$ mice (Figure 2). To further confirm the infiltration of IL-17-producing CD4⁺ cells in CNS, we isolated mononuclear cells from CNS tissues of WT and $TRIF^{-/-}$ mice. Consistent with our immunohistochemical analysis, intracellular cytokine staining revealed a significant increase in the percentage of IL-17-positive

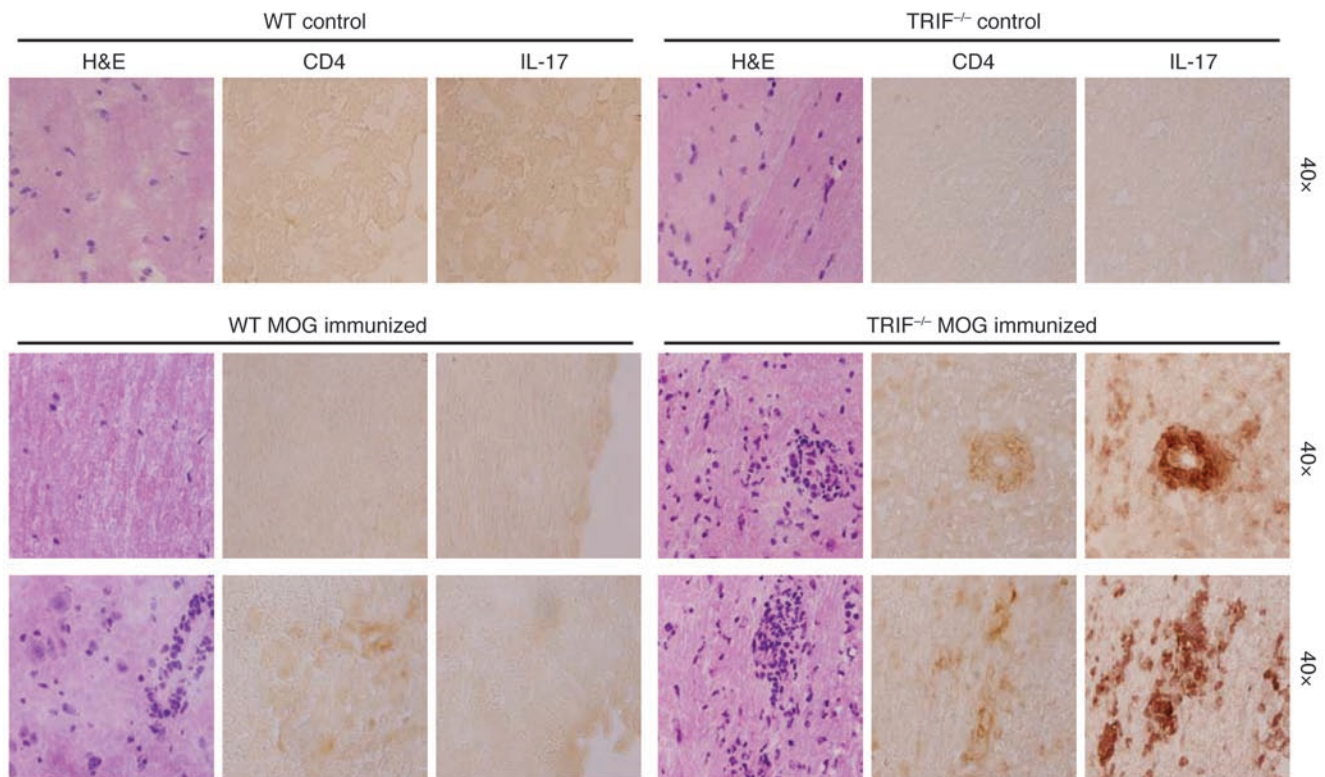


Figure 2 Increased CNS inflammation in TRIF-deficient mice during EAE. Representative sections of lumbar spinal cord from WT and TRIF-deficient mice at day 12 after MOG/CFA immunization were stained with H&E to determine inflammation or immunostained with anti-CD4 or anti-IL-17 for infiltration of CD4⁺ and Th17 cells. Data are representative of 3 experiments with similar results.

cells among the infiltrating CD4⁺ T cells from immunized *TRIF*^{-/-} mice as compared with WT mice (Figure 3A).

Enhanced generation of antigen-specific Th17 cells in TRIF-deficient mice. The increased inflammation and infiltration of Th17 cells in CNS suggest that the TRIF pathway in the innate immune system is involved in the regulation of Th17 development during EAE induction. To test this, total splenocytes were isolated from mice at day 7 after immunization and restimulated with MOG peptide *ex vivo*. As shown in Figure 3B, splenocytes from *TRIF*^{-/-} mice produced significant amounts of IL-17. In contrast, splenocytes from WT mice produced very low levels of IL-17 at this early stage of EAE induction. At day 21 after immunization, even though splenocytes from WT and *TRIF*^{-/-} mice produced significant amounts of IL-17 when restimulated with antigen, *TRIF*^{-/-} splenocytes still produced much higher levels of IL-17 (Figure 3C). We noticed that IFN- γ production was also relatively high in the TRIF-deficient cells (Figure 3D). In addition, increased serum levels of IL-17 were observed in TRIF-deficient mice after MOG/CFA immunization (data not shown).

While TRIF is highly expressed in innate immune cells, including macrophages and DCs, there are no reports about its expression in T cells. To determine whether T cells express TRIF, highly purified CD4⁺ T cells were obtained through magnetic bead separation followed by FACS sorting. RT-PCR and Western blot analysis revealed that doubled-sorted CD4⁺ T cells expressed TRIF at both the mRNA and protein levels, albeit the expression level was lower than that in total splenocytes and BM-derived macrophages

(BMMs) (Supplemental Figure 1, A and B; supplemental material available online with this article; doi:10.1172/JCI33342DS1). To distinguish the role of TRIF in T cells and in APCs, we performed adoptive transfer experiments using encephalitogenic T cells from WT or TRIF-deficient mice. MOG-specific lymphocytes were isolated from WT and TRIF-deficient mice immunized with MOG peptide plus CFA. Encephalitogenic lymphocytes were restimulated *in vitro* with the antigen and transferred into WT or TRIF-deficient mice. As shown in Figure 4A, when encephalitogenic T cells from WT mice were used, TRIF-deficient recipient mice developed EAE with a more severe phenotype than that in WT recipient mice. On the other hand, when encephalitogenic *TRIF*^{-/-} T cells were used as donor cells, WT recipient mice had less severe EAE compared with *TRIF*^{-/-} recipient mice (Figure 4B). Accordingly, lymphocytes isolated from TRIF-deficient recipient mice produced more IL-17 proteins when restimulated with MOG peptide (data not shown). These results suggest that TRIF-mediated IFN pathways in non-T cell compartments, most likely macrophages and DCs, may play a significant role in inhibiting autoimmunity during the effector phase of EAE.

TRIF limits Th17 development through induction of antiinflammatory cytokine IL-27. These results indicate that activation of the TRIF pathway in innate immune cells may limit the development of proinflammatory Th17 cells. To test this hypothesis, naive T cells from WT mice were cocultured with BMMs or BM-derived DCs (BMDCs). As shown by intracellular staining in Figure 4C, while both LPS-stimulated WT and mutant macrophages could pro-

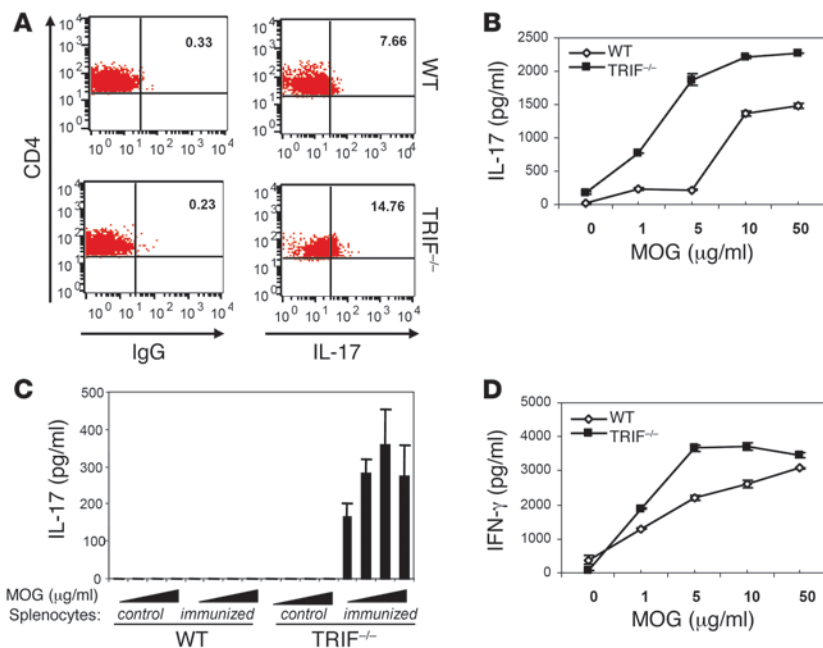


Figure 3

Th17 development in TRIF-deficient mice. (A) Flow cytometry analysis of CNS mononuclear cells from WT and TRIF-deficient mice at day 21 after immunization. CNS mononuclear cells isolated from WT and TRIF^{-/-} mice were stained for intracellular IL-17. Plots were gated on CD4⁺ T cells. Numbers indicate percentage of IL-17⁺CD4⁺ cells of total CD4⁺ cells. (B) T cells from TRIF-deficient mice immunized with antigen were hyperresponsive ex vivo. Total splenocytes were isolated from WT and TRIF-deficient mice 7 days after immunization and restimulated with MOG peptide ex vivo for 3 days. IL-17 production was measured by ELISA. (C and D) Ex vivo response of splenocytes from WT and TRIF-deficient mice 21 days after immunization. IL-17 or IFN-γ production was measured by ELISA. Results are reported as mean ± SD of duplicate samples from 1 representative experiment of 3 independent experiments.

mote Th17 differentiation, TRIF^{-/-} and IFNAR^{-/-} macrophages activated by LPS induced more IL-17-positive CD4⁺ T cells in coculture experiments. Furthermore, IL-17 production by T cells was significantly higher when cocultured with LPS-simulated TRIF^{-/-} macrophages. In addition to TRIF^{-/-} macrophages, LPS-stimulated IFNAR^{-/-} macrophages could significantly enhance IL-17 production (Figure 4D). In the parallel experiments, we found that LPS-stimulated DCs could promote Th17 development, and TRIF- and IFNAR-deficient DCs could induce slightly higher IL-17 production. However, the amount of IL-17 induced by WT and mutant DCs was much lower than that induced by macrophages (Supplemental Figure 2, A and B). Therefore, in our system, it seems that macrophages promoted robust IL-17 production from T cells. In this study, we also examined IL-17 production from purified TRIF-deficient T cells in the coculture system. We found that IL-17 production from TRIF^{-/-} cells was slightly higher, but the response pattern was similar. Like WT T cells, TRIF^{-/-} T cells produced more IL-17 protein when cocultured with TRIF^{-/-} or IFNAR^{-/-} macrophages stimulated with LPS (Figure 4E). Thus, our data indicate that the type I IFN pathway was still able to antagonize Th17 development in T cells lacking the TRIF molecule. Together, our results suggest that type I IFN induction and signaling events in macrophages are important for negatively regulating Th17 development.

Next, we investigated whether the TRIF signaling pathway in macrophages could limit Th17 development through production of negative regulators. To test this hypothesis, we analyzed a number of cytokines produced by WT versus TRIF-deficient macrophages in response to LPS stimulation (Figure 5A and Supplemental Figure 3). The defect in the TRIF molecule led to upregulation of proinflammatory cytokines in macrophages. However, the most striking observation was that TRIF-deficient macrophages produced much less IL-27 protein than WT cells. In addition, IL-27 production was also significantly reduced in TRIF-deficient DCs (Figure 5B). This result is consistent with a recent study showing that TLR4-induced IL-27 production in DCs critically depends on TRIF signals (45). IL-27,

a cytokine produced by innate cells, has previously been shown to inhibit the differentiation of Th17 cells and inflammatory autoimmune diseases (34, 35, 46). Consistent with previous reports, we found that IL-27 was a potent inhibitor of Th17 differentiation induced by TGF-β and IL-6 (Figure 5C). IL-27 also inhibited IL-17 production from antigen-specific T cells restimulated in vitro (Figure 5D). These data raise the possibility that TRIF signaling pathway may negatively regulate Th17 development through induction of antiinflammatory cytokines such as IL-27.

IFN-β can upregulate IL-27 expression in macrophages in an IFNAR-dependent manner. Since TRIF is vital for LPS-induced type I IFN production, we then addressed whether type I IFN was involved in LPS-induced IL-27 production. Remarkably, IL-27 production was significantly reduced in IFNAR-deficient macrophages after LPS stimulation (Figure 5E). We also found that IFNAR-deficient DCs produced decreased levels of IL-27 (Figure 5F). IL-27 induction by the TRIF pathway may occur via a feedback loop involving IFN-α/β. In addition to IL-27, IFN-α/β may affect expression of other cytokines involved in inflammation. For example, previous studies have shown that type I IFNs downregulate IL-12 (47). Therefore, we analyzed effects of type I IFN on the production of several cytokines, including IL-1β, IL-6, IL-10, IL-12, IL-23, and TNF-α, some of which have been shown to affect Th17 differentiation and expansion. Macrophages and DCs from WT or IFNAR mice were stimulated with LPS, and cytokines released into culture supernatants were measured by ELISA. Among the cytokines tested, as with our previous studies, defects in type I IFN signaling pathways resulted in reduced IL-10 production. Moreover, IFNAR-deficient cells had increased production of LPS-induced proinflammatory cytokines such as IL-1, IL-12, and TNF-α. On the other hand, IL-6 levels were almost not affected by IFN-α/β, and IL-23 production only increased slightly in IFNAR-deficient cells (Supplemental Figure 4). Since DCs are also major innate immune cells that provide cytokines and costimulatory molecules to T cells, we further analyzed cytokine production by DCs. Although the amount of each cytokine produced by these 2 different cell types varied, the

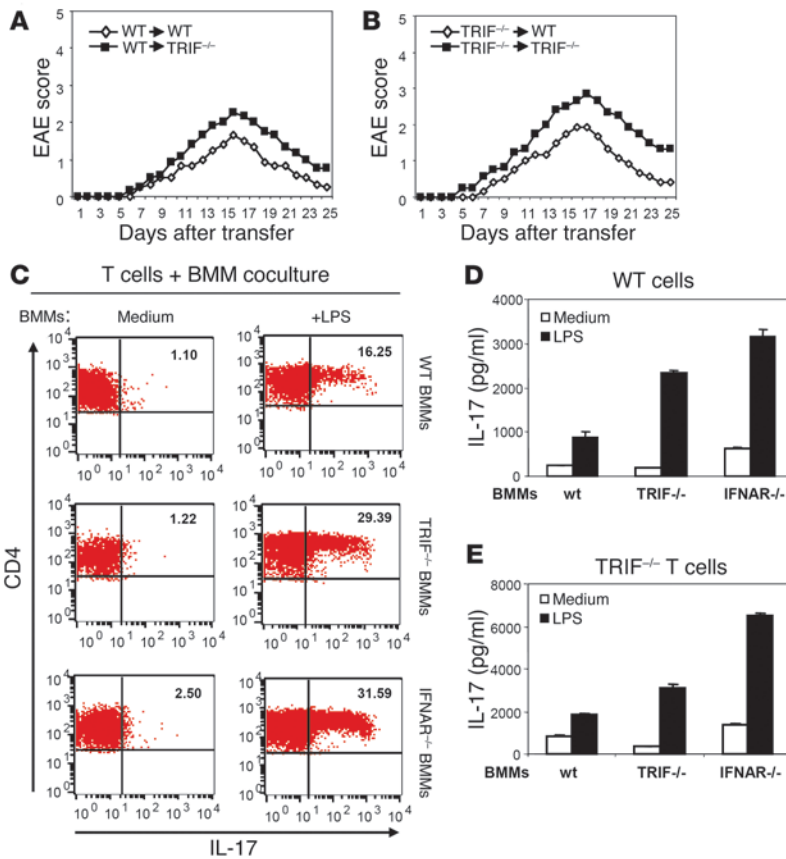


Figure 4

Type I IFN induction pathway in innate immune system constrains the development of Th17 development and CNS autoimmune disease. (A) Adoptive transfer experiments. Spleen cells and draining lymph node cells isolated from immunized WT mice were used as donor cells and restimulated with 20 $\mu\text{g/ml}$ of MOG peptides in vitro. 3×10^7 cells were transferred into WT or *TRIF*^{-/-} naive recipient mice via tail-vein injection (5 mice per group). The mice were monitored daily for clinical signs of disease. (B) Spleen cells and draining lymph node cells isolated from immunized *TRIF*^{-/-} mice were restimulated with MOG peptide in vitro for 72 hours. 3×10^7 cells were transferred into WT or *TRIF*-deficient naive recipient mice via tail-vein injection (5 mice per group). (C) Flow cytometry analysis of Th17 development in CD4⁺ T cells cocultured with BMMs. BMMs from WT mice, *TRIF*-deficient, or *IFNAR*-deficient mice were stimulated with LPS (100 ng/ml) for 24 hours, then were cultured with WT naive CD4⁺ T cells in the presence of anti-CD3 (1 $\mu\text{g/ml}$) for 72 hours. Cells were stained for surface CD4 and intracellular IL-17. Plots were gated on CD4⁺ T cells. Numbers indicate percentage of IL-17⁺CD4⁺ cells of total CD4⁺ cells. (D) IL-17 production in the coculture of WT CD4⁺ T cells and BMMs in experiments described in C. (E) IL-17 production from *TRIF*-deficient CD4⁺ T cells cocultured with BMMs.

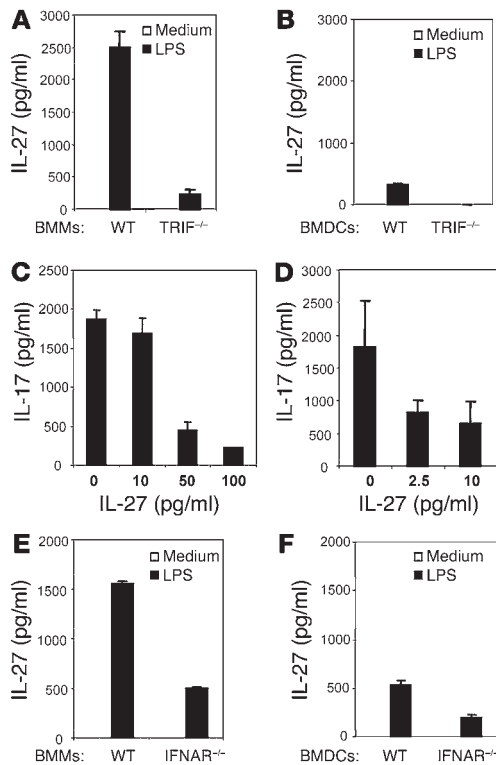
overall pattern was similar. The most significant difference is that macrophages usually produced a significantly higher level of IL-27 in response to LPS stimulation. Even though IL-27 plays a critical role in limiting Th17 development and autoimmune response, it does not exclude the possibility that other cytokines may participate in generating the phenotype of *IFNAR*-deficient mice. It is most likely that the inflammatory cytokine milieu with the reduced IL-27 level in *IFNAR*-deficient mice may lead to increased production of IL-17 and development of autoimmune disease.

Lack of IFNAR signaling in macrophages leads to increased Th17 development. Thus far, our data suggest that type I IFN-induced IL-27 production may represent an important mechanism for the immunoregulatory role of the *TRIF* pathway.

Next, we tried to determine whether supernatant, termed conditional medium (CM), from macrophages stimulated with IFN- β has an inhibitory effect. As shown in Figure 6A, the addition of CM from IFN- β -treated WT and *TRIF*-deficient macrophages, which express *IFNAR*, inhibits IL-17 production from Th17 culture. In contrast, CM from IFN- β -treated *IFNAR*-deficient macrophages failed to do so. To further confirm that type I IFNs directly mediate IL-27 expression, we determined whether IFN- β per se can stimulate IL-27 production in macrophages. We were surprised to find that WT macrophages treated with IFN- β produced IL-27 in a dose-dependent manner, whereas *IFNAR*^{-/-} macrophages failed to do so (Figure 6B). The defect in IL-27 induction in both *TRIF*- and *IFNAR*-deficient macrophages uncovers a potential link between the innate cytokine IL-27 and type I IFNs. The ability of exogenous type I IFN to inhibit IL-23-induced IL-17 production has been demonstrated previously (7). In our study, we also found that

both exogenous IFN- α and IFN- β can suppress IL-17 production from T cells stimulated with IL-6 and TGF- β (data not shown). Then, the question is whether *IFNAR*-mediated signaling events in macrophages are required for negatively regulating Th17 development. Interestingly, as shown in Figure 4, E and G, *IFNAR*^{-/-} macrophages induced more IL-17 production by T cells than WT macrophages, even though WT and *IFNAR*^{-/-} macrophages produced similar levels of type I IFNs. These findings indicate that type I IFN-induced signaling events in macrophages play a critical role in limiting Th17 development.

To determine whether IL-27 directly contributes to the inhibitory effect of the type I IFN pathway, we utilized an anti-IL-27 antibody to block IL-27 activity. Our results show that supernatants from IFN-treated macrophages inhibited the development of Th17 cells in vitro. When anti-IL-27 antibody was added to block the IL-27 activity in the CM from IFN-treated WT macrophages, the IFN- β -mediated inhibitory effect on Th17 cells was reversed, as demonstrated by IL-17 production and intracellular IL-17 staining from WT T cells (Figure 6C and Supplemental Figure 5A). We also got similar results with *TRIF*-deficient T cells (Supplemental Figure 5B). Finally, we tested to determine whether IL-27 also contributes to IFN-mediated inhibition of encephalitogenic T cells. Lymphocytes isolated from WT mice immunized with MOG/CFA were restimulated with MOG peptides in the presence of CM from IFN-treated macrophages plus anti-IL-27 antibody or control IgG. As shown in Figure 6D, CM from IFN-treated macrophages suppressed IL-17 production from antigen-specific T cells, and anti-IL-27 antibody could inhibit such suppression. Similar results were also obtained when *IFNAR*-deficient T cells were used (Figure 6E).



These results demonstrate that IFN-mediated IL-27 production by macrophages plays a major role in inhibiting Th17 development.

IFNAR^{-/-} mice develop severe EAE *in vivo*. Since defects in the IFNAR signaling pathway in macrophages lead to accelerated Th17 development, we hypothesized that mice with defects in this pathway should develop more severe Th17-mediated autoimmune disease. To test this hypothesis, we examined the development of

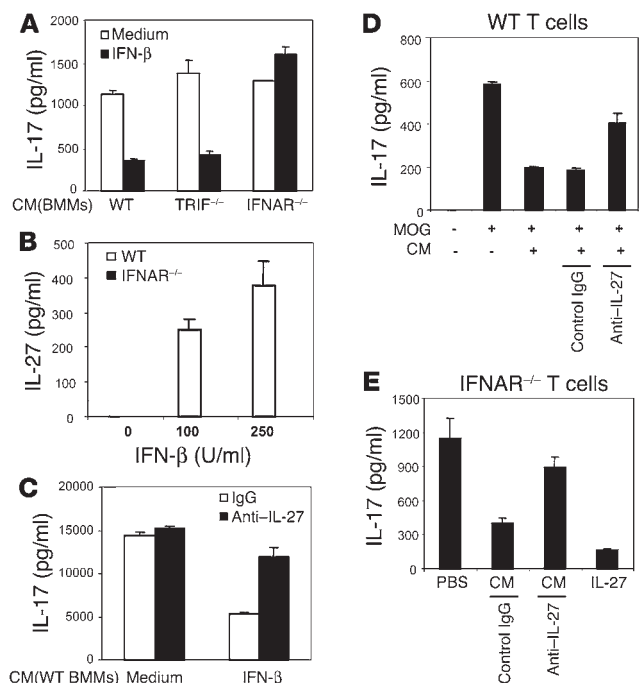
Figure 6

Type I IFN-mediated IL-27 production in macrophages contributes to inhibition of IL-17 production. (A) CM from IFN-β-treated macrophages suppresses Th17 development. WT, TRIF-deficient, and IFNAR-deficient BMMs were stimulated with IFN-β for 24 hours. Supernatants from IFN-β-stimulated macrophages were used as CM; they were added to Th17 culture and incubated for 72 hours. IL-17 production by CD4⁺ T cells was determined by ELISA. (B) WT and IFNAR-deficient BMMs were stimulated with IFN-β for 24 hours. The level of IL-27 protein was measured by ELISA. Data shown are representative of at least 3 experiments. (C) IFN-β-mediated inhibitory effects on Th17 development are reversed in the presence of anti-IL-27 antibody. CM from IFN-β-stimulated WT macrophages together with anti-IL-27 antibody or control IgG was added to Th17 cell culture. After 72 hours, IL-17 production by CD4⁺ T cells was determined by ELISA. (D) IL-27 contributes to IFN-β-mediated inhibition of encephalitogenic T cells. Lymphocytes isolated from immunized WT mice were restimulated with MOG peptide for 72 hours in the presence of CM from IFN-treated macrophages plus anti-IL-27 antibody or control IgG. IL-17 levels were measured by ELISA. (E) Lymphocytes isolated from immunized *IFNAR*^{-/-} mice were restimulated with MOG peptide in the presence of CM from IFN-treated macrophages plus anti-IL-27 antibody. IL-27 treatment was included as a positive control. IL-17 level was measured after 72 hours of culture.

Figure 5

The TRIF pathway limits Th17 development through induction of anti-inflammatory cytokine IL-27. (A) WT and TRIF-deficient macrophages were stimulated with 100 ng/ml LPS. The amount of IL-27 protein in culture supernatants was measured after 24 hours of stimulation. (B) WT and TRIF-deficient DCs were stimulated with 100 ng/ml of LPS. The amount of IL-27 protein was measured after 24 hours of stimulation. (C) IL-27 inhibits IL-17 production induced by IL-6 and TGF-β. Naive CD4⁺ T cells isolated from WT mice were activated for 72 hours in Th17 culture in the presence of IL-27 as indicated. IL-17 production by CD4⁺ T cells was determined by ELISA. (D) IL-27 inhibits antigen-induced Th17 response. Total splenocytes isolated from WT mice 21 days after MOG/CFA immunization were restimulated with MOG peptide *ex vivo* in the presence of IL-27 for 3 days. IL-17 production was measured by ELISA. Data shown are representative of at least 3 experiments. (E and F) WT and *IFNAR*^{-/-} BMMs or DCs were stimulated with 100 ng/ml LPS. The amount of IL-27 protein was measured after 24 hours of stimulation.

EAE in *IFNAR*^{-/-} mice. As expected, *IFNAR*^{-/-} mice were highly susceptible to EAE (Figure 7A). Notably, the lack of IFNAR mainly affected the progression of EAE, but not the onset or the incidence of disease. While most of the WT mice recovered after the disease activity peaked around days 17–20, *IFNAR*^{-/-} mice developed progressive and chronic EAE akin to what we observed in the *TRIF*^{-/-} mice. Accordingly, increased infiltration of mononuclear cells was observed in the CNS of *IFNAR*^{-/-} mice immunized with MOG/CFA (Figure 7B). These data are in agreement with prior studies showing that exogenous type I IFNs can be used to suppress EAE in mice and MS in humans. Consistent with our findings in TRIF- and IFNAR-deficient mice, *IRF3*^{-/-} (IRF, IFN regulatory factor) mice were also sensitive to EAE induction (data not shown). To determine whether Th17-mediated inflammation accounts for the severity of EAE in *IFNAR*^{-/-} mice, we examined Th17 development in *IFNAR*^{-/-} mice. Similar to *TRIF*^{-/-} mice, *ex vivo* restimulation of T cells revealed that IFNAR-deficient splenocytes secreted more



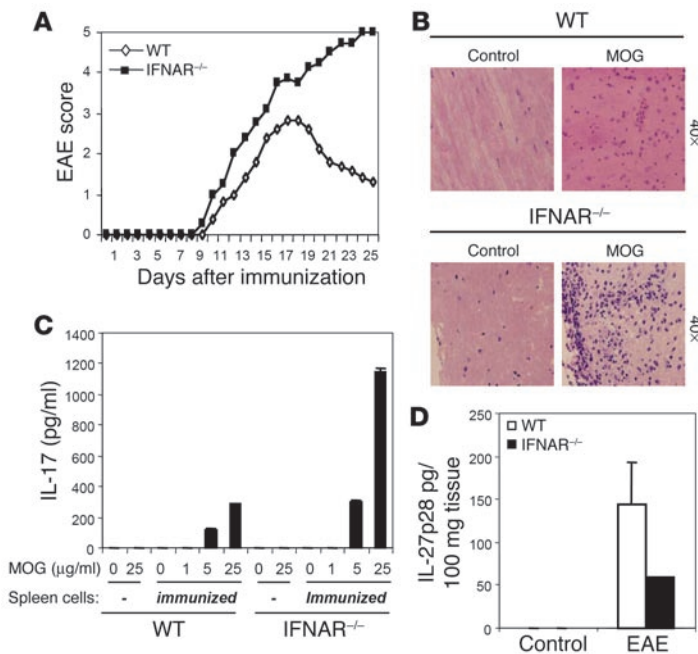


Figure 7
IFNAR^{-/-} mice are sensitive to EAE. (A) Mean disease score of WT mice and *IFNAR*^{-/-} mice during EAE induction. WT mice (n = 5) and *IFNAR*^{-/-} mice (n = 5) were immunized with MOG peptide (MOG₃₅₋₅₅) emulsified in CFA. Mice were assigned disease scores from 0 to 5 based on the severity of EAE. (B) Increased inflammation in *IFNAR*-deficient mice during EAE. Representative sections of spinal cord from WT and *IFNAR*-deficient mice at day 12 after MOG/CFA immunization were stained with H&E. (C) Total splenocytes isolated from WT and *IFNAR*^{-/-} mice 12 days after immunization were restimulated with MOG peptide ex vivo for 3 days. IL-17 production was measured by ELISA. (D) Spinal cord tissues isolated from WT (n = 3) and *IFNAR*^{-/-} (n = 3) mice 12 days after immunization were homogenized. The level of IL-27 protein in tissue samples was measured by ELISA. Data are representative of 3 experiments with similar results.

IL-17 than did WT cells from mice 12 days after MOG/CFA immunization (Figure 7C). These data suggest that the defect in IFN signaling pathway in *IFNAR*-deficient mice contributes to Th17 development and EAE progression in vivo. Altogether, the results from this study demonstrate that TRIF-dependent type I IFN induction pathway and *IFNAR* signaling may serve a protective role in limiting Th17-mediated CNS autoimmune inflammation.

To investigate whether IL-27 induction is involved in the inhibitory effects of IFN- α/β on EAE development in vivo, we examined IL-27 production in the CNS from *IFNAR*^{-/-} mice with EAE. After onset of clinical disease, the CNS tissues were isolated from WT and *IFNAR*-deficient mice and homogenized; then the IL-27 protein levels were measured by IL-27p28-specific ELISA. As shown in Figure 7D, expression of IL-27 was upregulated in CNS when mice developed EAE. Notably, *IFNAR*^{-/-} mice with EAE had much less IL-27 protein in the CNS compared with WT mice. These results suggest that type I IFN may upregulate the expression of IL-27 in CNS to negatively regulate the progression of autoimmune disease. The increased production of IL-27 may represent an attempt by the immune system to dampen the inflammatory response. These data also suggest that IFN-mediated IL-27 production may be one of the mechanisms by which IFN- β exerts its therapeutic effects in the treatment of EAE and MS.

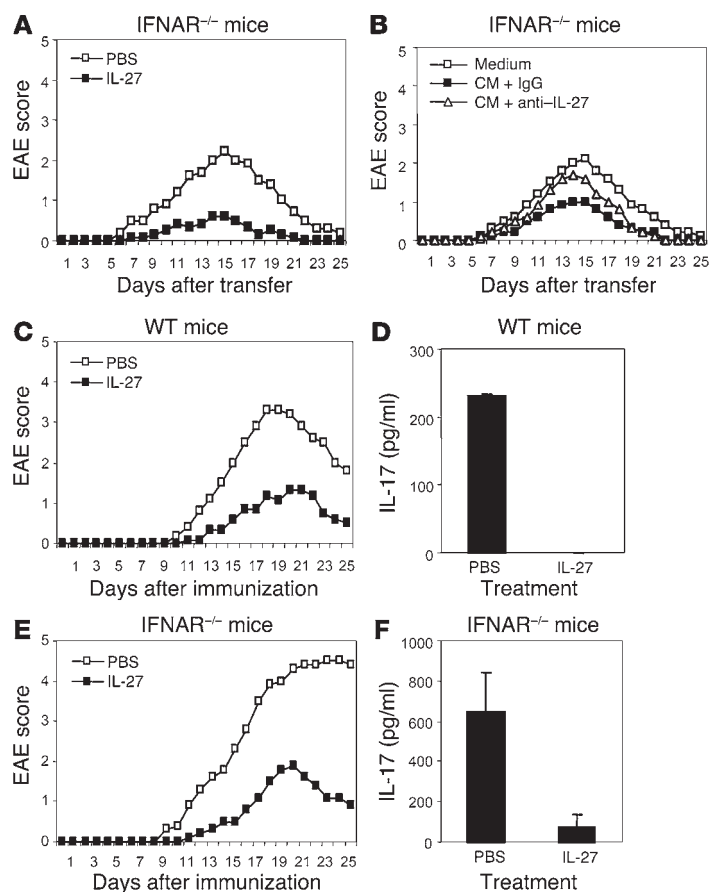
To determine whether this TLR/IFN/IL-27 regulatory loop is relevant in vivo, we utilized adoptive transfer experiments combined with anti-IL-27 blockade. Spleen and lymph node cells isolated from immunized *IFNAR*^{-/-} mice were restimulated in vitro with MOG peptide in the presence of supernatants from IFN-treated macrophages with or without anti-IL-27 antibody. As a positive control, we also added recombinant IL-27 to the ex vivo culture. Exogenous IL-27 inhibited the development of EAE in *IFNAR*^{-/-} recipient mice induced by adoptive transfer of antigen-specific *IFNAR*^{-/-} T cells (Figure 8A). Interestingly, CM from IFN- β -treated macrophages could ameliorate the adoptive transfer of EAE. Furthermore, the inhibitory effect of CM was significantly reduced when IL-27 activity was blocked. As shown in Figure 8B, MOG-

specific T cells cultured with CM in the presence of anti-IL-27 antibody induced more severe EAE compared with T cells cultured with CM plus control IgG. We also noticed that IL-27 blocking was not completed; this might result from the amount or the affinity of antibody used. Alternatively, other pathways may also contribute to the inhibitory effects of type I IFNs. Further studies using IL-27 or IL-27R knockout mice may clarify this issue.

As our results suggest that type I IFN may suppress EAE development via induction of IL-27, we determined whether IL-27 treatment in vivo could reverse the severe EAE phenotype in *IFNAR*-deficient mice. To test this, mice were administered purified murine IL-27 right after immunization. Consistent with recent publications (34, 48), our results showed that IL-27 could significantly inhibit EAE development in WT mice (Figure 8C). Strikingly, injection of IL-27 also suppressed the severe EAE phenotype in *IFNAR*-deficient mice compared with PBS controls. *IFNAR*-deficient mice treated with IL-27 showed much milder symptoms. In addition, splenocytes from IL-27-treated WT and *IFNAR*-deficient mice produced much less IL-17 when restimulated with antigen (Figure 8, D and F). Together, these results indicate that the type I IFN induction and signaling pathways may serve a protective role in Th17-mediated CNS inflammation via IL-27 induction.

Discussion

Recently, Th17 was identified as an inflammatory Th lineage that plays a critical role in a number of animal models of autoimmunity, including EAE (7, 10, 14–20, 22). Numerous studies have shown that Th17 development is regulated by an extensive cytokine network. Using EAE as a model, we demonstrate that TRIF-dependent type I IFN induction pathway constrains the differentiation of Th17 cells both in vitro and in vivo. Defects of key signaling molecules, including TRIF, IRF3, and *IFNAR*, in this pathway lead to increased CNS inflammation mediated by Th17 cells. Furthermore, we found that type I IFNs inhibit Th17 development in an autocrine/paracrine manner to upregulate IL-27 in macrophages. These results reveal that the endogenous type I IFN production

**Figure 8**

IL-27 reverses severe EAE phenotype in *IFNAR*^{-/-} mice in vivo. (A) IL-27 inhibits adoptive transfer of EAE in *IFNAR*^{-/-} mice. Spleen and lymph node cells isolated from immunized *IFNAR*^{-/-} mice were restimulated in vitro with MOG peptide in the presence of IL-27 or PBS for 72 hours. 3×10^7 cells were transferred into *IFNAR*^{-/-} naive recipient mice via tail-vein injection (5 mice per group). (B) IFN- β -mediated IL-27 production inhibits adoptive transfer EAE in *IFNAR*^{-/-} mice. Spleen and lymph node cells isolated from immunized *IFNAR*^{-/-} mice were restimulated in vitro with MOG peptide in the presence of CM from IFN-treated macrophages with or without anti-IL-27 antibody. After 72 hours, 3×10^7 cells were transferred into naive *IFNAR*^{-/-} recipient mice via tail-vein injection (5 mice per group). (C) IL-27 inhibits EAE development in WT mice. WT mice ($n = 5$) were immunized with MOG peptide emulsified in CFA. Recombinant carrier-free mouse IL-27 (0.25 μ g in 100 μ l PBS) was administered by s.c. injection to immunized WT mice every other day from day 2 until day 20. (D) Splenocytes from IL-27-treated WT mice represented in C were restimulated in vitro with MOG peptides for 72 hours, and IL-17 production was measured. (E) IL-27 treatment reverses the phenotype of EAE in *IFNAR*^{-/-} mice. Recombinant mouse IL-27 was administered by s.c. injection to immunized *IFNAR*^{-/-} mice ($n = 5$) every other day from day 2 until day 20. (F) Splenocytes from IL-27-treated *IFNAR*^{-/-} mice represented in E were restimulated in vitro with MOG peptides for 72 hours, and IL-17 production was measured.

and signaling in macrophages play an important role in the regulation of CNS inflammation and autoimmunity.

We were surprised to find that TRIF-deficient mice had exacerbated symptoms of EAE. This phenotype is in stark contrast to that of *MyD88*^{-/-} mice, which are protected from the development of CNS autoimmunity (49). The severe EAE phenotype in *TRIF*^{-/-} mice is associated with increased production of proinflammatory cytokines and enhanced development of Th17 cells both in vitro and in vivo. Our data suggest that different TLR adaptors in macrophages and DCs may have different effects on the development of EAE. These results also imply that the TRIF pathway may serve a regulatory role to suppress or modulate Th17 development and inflammatory response under certain disease conditions such as EAE. Based on our model, TLR agonists that activate MyD88-dependent pathways would function as potent adjuvants for EAE induction. On the other hand, TRIF-dependent TLR agonists would be less effective, or even protective, in EAE induction. This conclusion is supported by previous studies that showed that only certain TLR agonists have adjuvant activities in the induction of CNS autoimmune inflammation (50, 51). It may also explain why mycobacteria are effective in EAE induction experiments. Mycobacteria stimulate primarily TLR2-induced MyD88 pathways and, to a lesser extent, TLR4-dependent MyD88 and TRIF pathways. Therefore, mycobacteria elicit strong inflammatory responses that may promote Th17 development in MOG/CFA-immunized mice. We speculate that the antagonism between MyD88 and TRIF might have evolved as a counterbalance during inflammatory response. It would be interesting to determine whether such antagonism also

exists in other autoimmune or inflammation conditions. At present, it is unclear how TLRs or other intracellular detection pathways are activated during the initiation and progression of MS. It is possible that some yet unidentified endogenous TLR ligands released during tissue damage may contribute to the development of this CNS autoimmune disease.

In this study, we also found that the type I IFN induction pathway in macrophages plays an important role in modulating Th17 response. Various cytokines produced by macrophages in response to LPS, including IL-6, IL-23, IL-1, IL-13, and TNF, have been shown to promote Th17 differentiation or expansion. While inflammatory cytokines are essential for host innate and adaptive immune response, they are thought to also contribute to disease pathogenesis of infectious agents or endogenous ligands by inducing inflammation and immune injury to the host. Our results indicate that the TRIF pathway may function as a brake to reduce the inflammatory response induced by Th17 cells. We found that macrophages from mice deficient in the TRIF signaling pathway were able to induce high levels of IL-17 production when cocultured with T cells. These findings underscore the important role of macrophages in modulating inflammatory response through a type I IFN autocrine/paracrine loop. It has been reported that microglia, the resident macrophage-like cells in the CNS, can express TLRs (1, 52, 53). Therefore, it would be interesting to determine whether microglia cells have the ability to modulate Th17 development during CNS inflammation.

Systemic administration of IFN- β has been shown to reduce the relapse rate, slow disease progression, and decrease the num-



ber of CNS lesions in MS, although the mechanisms responsible for its therapeutic effects remain unclear (3–5). Nevertheless, these results implicate the potential involvement of endogenous IFN- α/β in regulating inflammatory response in the CNS. This conclusion is supported by our results showing that IFNAR-deficient mice developed more severe EAE compared with WT mice. This is in agreement with a previous study that showed that lack of IFN- β renders mice more susceptible to EAE (54). However, in that study, the deficiency of IFN- β did not alter T cell function and cytokine production when T cells were restimulated with antigen. This is in contrast with our findings that T cells from *TRIF*^{-/-} and *IFNAR*^{-/-} mice exhibited enhanced IL-17 as well as IFN- γ production in ex vivo recall response. More importantly, our study demonstrates that endogenous type I IFN induction pathways are imperative in the regulation of CNS inflammation and autoimmunity. Consistent with a recent study that revealed that type I IFN can inhibit IL-23-dependent Th17 cell expansion (7), we also demonstrated that IFN- α and IFN- β can directly inhibit TGF- β /IL-6-mediated Th17 development (data not shown). An intriguing question that needs to be addressed is why there is such a profound difference between type I IFNs and type II IFN (IFN- γ) in the treatment of MS patients. Even though both type I and type II IFNs can inhibit Th17 development and EAE disease progression in mice, only type I IFNs have been shown to be effective in the treatment of MS patients. In fact, clinical trials have shown that the administration of type II IFN in MS patients actually exacerbated the disease. Therefore, the involvement of Th17 cells and the interplay between different T cell subsets in the pathogenesis of MS require further evaluation.

The means by which IFN- β is able to effectively modulate innate and adaptive immune responses in MS are undoubtedly complex and most likely cannot be explained by a single mechanism of action. Nevertheless, our results here suggest that type I IFN-induced IL-27 and perhaps other antiinflammatory cytokines in macrophages may contribute to the therapeutic efficacy of IFN- β . In this study, we found that the TRIF pathway controls IL-27 production in response to LPS stimulation. IL-27 has been shown to directly inhibit the differentiation of Th17 cells and Th17-mediated inflammatory response (34, 35, 46). IL-27 is produced by macrophages under inflammatory conditions through TLRs. Since its promoter region contains NF- κ B- and IRF-binding sites, several studies have confirmed that its expression depends on NF- κ B and IRF transcription factors (36, 45, 55). We also found that IL-27 production was significantly reduced in TRIF-deficient cells. However, IL-27 production was not totally blocked, which suggests that MyD88-dependent pathways also participate in the regulation of IL-27 production. Strikingly, we found that IL-27 production is also affected by type I IFNs and that TRIF-dependent IL-27 production in macrophages is mainly orchestrated through the type I IFN-mediated autocrine/paracrine loop. Furthermore, IFN- α/β per se is able to induce IL-27 production in macrophages. These results provide a potential mechanism for the clinical benefits of type I IFNs in MS. Results from other studies and this study indicate that IL-27 is upregulated in the CNS of mice with EAE (38, 56). We also found that IL-27 production in CNS tissues from *IFNAR*^{-/-} mice with EAE was significantly decreased. Furthermore, the severe phenotype in *IFNAR*^{-/-} mice can be rescued by administration of purified IL-27 protein. These results imply that IFN- β -mediated IL-27 production by inflammatory infiltrates or microglia cells may protect the CNS from immune injury. Together, our data suggest that the beneficial effects of IFN- α/β in the treat-

ment of CNS inflammatory disease may be, at least in part, contributed by the induction of IL-27.

Even though type I IFN-mediated IL-27 production plays a critical role in limiting Th17 development and autoimmune response, this finding does not exclude the possibility that other cytokines, such as IL-1, IL-10, IL-12, and TNF- α , may also be involved in modulating the phenotype of IFNAR-deficient mice, either independently or in compliment with IL-27 induction. IL-10 has long been known as an antiinflammatory cytokine, as IL-10-deficient mice have increased susceptibility to autoimmune disorders such as inflammatory bowel disease and autoimmune encephalomyelitis. While reduced IL-10 production may provide an alternative explanation for the phenotype of IFNR-deficient mice, IFN-induced IL-10 production may complement the induction of IL-27. Recent studies demonstrated that IL-27 is able to induce IL-10 production from T cells and generation of Tregs that suppress the development of Th17 cells (57–59). On the other hand, defects in type I IFN signaling are associated with increased production of proinflammatory cytokines. While increased IL-12 production may promote Th1 development and inflammatory response, IL-1 and TNF- α have been reported to enhance Th17 development in combination with IL-6 and TGF- β . It is noteworthy that IL-1 is one of the major cytokines that drives human Th17 differentiation from naive T cells. Therefore, the function of type I IFNs is possibly linked to modulation of the cytokine environment by upregulation of antiinflammatory cytokines such as IL-10 and IL-27 and downregulation of proinflammatory cytokines such as IL-1 and TNF, eventually leading to reduced autoimmune inflammation mediated by Th17 cells. Consequently, the deficiency in IFN production or signaling pathways in the innate immune system may result in generation of inflammatory milieu that promote tissue inflammation and injury. Therefore, it is possible that some MS patients might not have proper type I IFN induction or signaling during CNS inflammation. This mechanism may also be responsible for the inhibitory effects of IFN- β in human MS, although further clinical studies are needed.

In summary, the present study demonstrates that TRIF-dependent type I IFN induction and signaling pathways constrain IL-17 production and CNS inflammatory response through IL-27 production. Information learned from this study may help us to further develop immunotherapeutic strategies for the treatment of MS in the future. For example, using TLR ligands or chemical drugs that selectively activate TRIF or type I IFN induction pathways may significantly improve the clinical outcomes of MS with fewer side effects. It would also be interesting to determine whether TRIF-mediated type I IFN induction and signaling pathways also play important roles in the regulation of Th17 cells in other autoimmune diseases or infectious conditions.

Methods

Reagents and mice. Murine IL-17, IL-23, IL-27, IL-6, TGF- β , and anti-IL-27p28 antibodies were purchased from R&D Systems. Murine IFN- α and IFN- β proteins were from PBL Biomedical Laboratories. Anti-CD3, anti-CD28, anti-IFN- γ , and anti-IL-4 were from BD Biosciences. Anti-IL-17 PE, anti-CD4 FITC, and anti-IFN- γ APCs were purchased from eBioscience. MOG_{35–55} peptides (MEVGWYRSPFSRVVHLYRNGK) were purchased from AnaSpec. LPS was purchased from Sigma-Aldrich.

All mice used were on a C57BL/6 genetic background. IFN Alpha Ro/o 129/Sv (*IFNAR*^{-/-}) mice were from B&K Universal Ltd. and were backcrossed with C57BL/6J mice (The Jackson Laboratory) for 6 generations.



TRIF^{Lps2/Lps2} mutant mice were kind gifts from Bruce Beutler (The Scripps Research Institute). *IRF3^{-/-}* and littermate WT mice were kind gifts from Tadatsugu Taniguchi (University of Tokyo, Tokyo, Japan). All mice were maintained and bred at the UCLA Department of Laboratory Animal Medicine mouse facility under specific pathogen-free conditions. All animal experiments were approved by the Animal Research Committee (ARC) at UCLA and were conducted in accordance with federal regulations as well as UCLA guidelines and regulations on animal studies.

EAE induction. EAE was induced in WT and different mutant mice 8 to 12 weeks of age by immunization of mice s.c. with 200 μ l of an emulsion containing 200 μ g MOG₃₅₋₅₅ peptides in 100 μ l PBS and 100 μ l CFA. CFA was prepared by mixing of incomplete Freund's adjuvant (DIFCO Laboratories) with 8 mg/ml of *Mycobacterium tuberculosis* H37RA (desiccated; DIFCO Laboratories). At the time of immunization and 2 days later, mice were injected i.v. with 200 ng of pertussis toxin (List Biological Laboratories) in 100 μ l PBS. Mice were monitored daily for clinical signs of disease and assigned disease scores from 0 to 5 based on the severity of EAE as follows: 0, no disease; 1, limp tail; 2, hind limb weakness; 3, hind limb paralysis; 4, hind limb and forelimb paralysis; 5, moribundity and death.

For in vivo IL-27 treatment, carrier-free recombinant mouse IL-27 (0.25 μ g suspended in 100 μ l of PBS) was administered by s.c. injection to MOG-immunized mice every other day from day 2 until day 20. The mice were monitored daily for clinical signs of disease.

Adoptive transfer experiments. For adoptive transfer experiments, donor mice were immunized with MOG in CFA as described above. Spleen cells and draining lymph node cells were isolated from mice 12 days after immunization and were restimulated with 20 μ g/ml of MOG peptides in vitro for 72 hours. Suspension cells were collected and washed extensively, and 3×10^7 cells were transferred into each naive recipient mouse via tail-vein injection (5 mice per group). On the same day and 2 days later, each mouse received 200 ng of pertussis toxin via i.v. injection. The development of EAE in these recipient mice was monitored daily. In some experiments, IL-27 or CM from IFN- β -stimulated macrophages was added during ex vivo culture.

Histology and immunohistochemistry. Brains and spinal cords obtained from mice at different times after induction of EAE were quickly frozen and cut into sections 5 μ m in thickness. For each mouse, 10 sections of the brain and 40 sections representing the entire spinal cord (10 each from the cervical, thoracic, and lumbar regions) were obtained. Sections were stained with H&E for routine histological analysis, and sections of the spinal cord were immunostained with anti-CD4, anti-IL-17, and anti-F4/80 (all from BD Biosciences) for infiltration of immune cells. Positive immunostaining was detected with a streptavidin-biotin immunoperoxidase system (Vector Laboratories) according to manufacturer's protocols.

CNS mononuclear cell isolation. Brains or spinal cords were removed and washed with cold PBS. Tissues were then passed multiple times through an 18-gauge needle, and the suspension was then passed through a 70- μ m cell strainer, resuspended in 40 ml of complete RPMI 1640, and pelleted at 2,000 *g* for 10 minutes at 4°C. Cells were resuspended in 60% isotonic Percoll (Amersham) solution and overlaid with 30% Percoll. The Percoll gradient was centrifuged at 1,000 *g* for 25 minutes at 25°C without brakes, and the top myelin layer was removed before harvesting BMNCs at the 60% and 30% interphase layer. Cells were washed with complete RPMI 1640 and were processed for ex vivo surface staining and intracellular staining as described.

Ex vivo restimulation with MOG₃₅₋₅₅. Spleens and lymph nodes were collected from the various strains of mice, and single-cell suspensions were prepared by mechanical disruption in complete RPMI 1640 medium. Viable cells were cultured for 72 hours in complete medium with or without MOG peptide at various concentrations. Culture supernatants were collected for ELISA of cytokine production.

Tb17 cell culture. Single-cell suspensions were prepared from spleens from WT or different mutant mice. Naive CD4⁺ T cells were enriched from spleen mononuclear cells by magnetic cell sorting with a mouse CD4⁺ T cell isolation kit (Miltenyi Biotec). CD4⁺ T cells were cultured in RPMI 1640 (Gibco BRL; Invitrogen) supplemented with 10% FBS (HyClone), penicillin and streptomycin, and 0.5 μ M 2-mercaptoethanol. CD4⁺ T cells in 96-well plates were stimulated with anti-CD3 (1 μ g/ml) and anti-CD28 (1 μ g/ml). Where indicated, cultures were supplemented with anti-mouse IFN- γ (5 μ g/ml), anti-mouse IL-4 (5 μ g/ml), IL-6 (5 ng/ml), TGF- β (1 ng/ml), IL-27 (1–25 ng/ml), IFN- α (0–250 U/ml), or IFN- β (0–250 U/ml). Seventy-two hours after stimulation, supernatants were collected and determined by ELISA.

ELISA. IL-1 β , IL-6, IL-10, IL-12, IL-17, IL-21, TNF- α , and IFN- γ were detected in culture supernatants with ELISA sets or antibody pairs from BD Biosciences. IL-27 and TGF- β were detected with ELISA kits (R&D Systems). IL-17 and IL-23 were detected with ELISA kits (eBioscience) per the manufacturer's instructions.

Flow cytometry. For intracellular cytokine staining, splenocytes or CD4⁺ T cells were first restimulated for 4 hours with 50 ng/ml of phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml of ionomycin in the presence of 5 μ g/ml of brefeldin A (Sigma-Aldrich). Cells were surface stained with fluorescein isothiocyanate-conjugated anti-CD4. Cells were then permeabilized with the Cytotfix/Cytoperm Plus Kit (BD Biosciences – Pharmingen) according to the manufacturer's protocol. Cells were stained intracellularly with PE-conjugated anti-mouse IL-17 and allophycocyanin-conjugated anti-mouse IFN- γ . Samples were acquired on a FACSCalibur, and data were analyzed with CellQuest Pro software (BD Biosciences).

Coculture of CD4⁺ T cells with BMMs or BMDCs. Naive CD4⁺ T cells were enriched from spleen mononuclear cells by magnetic cell sorting with a mouse CD4⁺ T cell isolation kit (Miltenyi Biotec) as described above. CD4⁺ T cells were further purified by sorting via FACS machine. BMMs and BMDCs were treated with TLR ligands 24 hours before coculture. T cells were cultured with BMMs or BMDCs at a ratio of 5:1, and cells were stimulated with 1 μ g/ml of anti-CD3 in complete medium. Where indicated, cultures were supplemented with recombinant cytokines. Supernatants or cells were analyzed on day 3 of culture.

IFN- β treatment of macrophages. WT and *IFNAR^{-/-}* BMMs were stimulated with IFN- β as indicated for 24 hours. Supernatants were collected as CM and stored at -20°C. The amount of IL-27 protein was measured using ELISA.

Preparation of BMMs and BMDCs. Murine BMMs were generated by flushing BM cells from femurs and tibiae of mice. These cells were cultured for 7 days in DMEM (Gibco BRL; Invitrogen) containing 10% FBS, penicillin, streptomycin, and 10% conditioned media from L929 cells overexpressing M-CSF.

For BMDCs, 3×10^6 BM cells were cultured in petri dishes in 10 ml culture medium containing 10 ng/ml of murine GM-CSF (R&D Systems). On day 3 of culture, this was replaced with fresh GM-CSF medium. Loosely adherent cells were transferred to a fresh dish and cultured for an additional 4 days.

Statistics. Statistical analysis was performed with a 2-tailed Student's *t* test for differences among groups. *P* < 0.05 was considered statistically significant. Quantitative data are expressed as means \pm SD unless otherwise stated.

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