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The Interdependent, Overlapping, and Differential Roles of Type I and II IFNs in the Pathogenesis of Experimental Autoimmune Encephalomyelitis

Rodrigo Naves,*1 Simer P. Singh,*2 Kevin S. Cashman,† Amber L. Rowse,† Robert C. Axtell,‡ Lawrence Steinman,‡ John D. Mountz,* Chad Steele,*† Patrizia De Sarno,§ and Chander Raman*§†

Type I IFNs (IFN-α and IFN-β) and type II IFN (IFN-γ) mediate both regulation and inflammation in multiple sclerosis, neuromyelitis optica, and in experimental autoimmune encephalomyelitis (EAE). However, the underlying mechanism for these Janus-like activities of type I and II IFNs in neuroinflammation remains unclear. Although endogenous type I IFN signaling provides a protective response in neuroinflammation, we find that when IFN-γ signaling is ablated, type I IFNs drive inflammation, resulting in exacerbated EAE. IFN-γ has a disease stage–specific opposing function in EAE. Treatment of mice with IFN-γ during the initiation phase of EAE leads to enhanced severity of disease. In contrast, IFN-γ treatment during the effector phase attenuated disease. This immunosuppressive activity of IFN-γ required functional type I IFN signaling. In IFN-α/β receptor–deficient mice, IFN-γ treatment during effector phase of EAE exacerbated disease. Using an adoptive transfer EAE model, we found that T cell–intrinsinc type I and II IFN signals are simultaneously required to establish chronic EAE by encephalitogenic Th1 cells. However, in Th17 cells loss of either IFN signals leads to the development of a severe chronic disease. The data imply that type I and II IFN signals have independent but nonredundant roles in restraining encephalitogenic Th17 cells in vivo. Collectively, our data show that type I and II IFNs function in an integrated manner to regulate pathogenesis in EAE. The Journal of Immunology, 2013, 191: 000–000.

The IFNs are a family of related cytokines exerting an essential role in inflammation and autoimmunity. They are classified into two subtypes according to receptor specificity and sequence homology (1). Type I IFNs consist of IFN-β and several other members, whereas type II IFN has only one single member, IFN-γ. Types I and II IFNs bind distinct cell surface receptor complexes, the IFN-α/β receptor (IFNAR) and the IFN-γ receptor (IFNGR), respectively. IFNAR and IFNGR are comprised of two transmembrane glycoproteins: IFNAR1 and IFNAR2, and IFNGR1 and IFNGR2 (2, 3). A common feature of both type I and II IFNs is the employment of the JAK-STAT signal transduction pathway to regulate nuclear gene expression (1).

Although types I and II IFNs bind distinctive receptors and differentially regulate the expression of a variety of other cytokines, they develop synergistic functions priming macrophages for tumor cell killing (4), enhancing CTL responses to melanoma cell vaccines (5), and inhibiting viral replication (6). Recruitment and modulation of STAT1 are central elements in the cross-talk between types I and II IFNs triggered in response to viral infection (7, 8). However, the cooperative action of types I and II IFNs and the pathophysiological significance of this interaction in autoimmunity have been less studied.

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the CNS and the leading cause of neurologic disability in young adults (9). There is compelling evidence supporting the individual role of types I and II IFNs in the pathogenesis of MS and its animal model, experimental autoimmune encephalomyelitis (EAE). The lack of either IFN-β or IFNAR results in a more severe and chronic EAE (10–12), and the systemic administration of IFN-β is, to date, the most commonly used therapy for MS providing longer periods of remission, reducing the severity of relapses, and decreasing the inflammatory lesion in the CNS (13–16). In contrast, controversial evidence has been reported in relationship to the role of IFN-γ in MS and EAE. A positive association between increased levels of IFN-γ and clinical manifestations attributed a pathological role of IFN-γ in MS (17–20). Subsequent studies have challenged the notion that IFN-γ is pathogenic, and they have even suggested a protective role for IFN-γ in EAE, and perhaps in some forms of MS (21–27). Furthermore, EAE can be ameliorated...
after IFN-γ-treatment (24, 28–30), whereas MS patients treated with IFN-γ exhibited exacerbations of disease (31). Therefore, the role of IFN-γ in MS and EAE is still unresolved.

Th cells producing either IFN-γ (Th1) or IL-17 (Th17) have been shown to play a critical role in the immunopathogenesis of MS and EAE (32–35). Recently, we have demonstrated that IFN-β is very effective in the treatment of EAE induced by Th1 cells but it is ineffective and induces exacerbations when disease is led by Th17 lymphocytes (36). Remarkably, IFN-β treatment significantly worsened EAE in IFNGR-deficient mice, suggesting that immunosuppression by IFN-β required functional activity of IFN-γ (36).

In this study we tested the hypothesis that IFN-β and IFN-γ act cooperatively to modulate autoimmune neuroinflammation. Our results revealed an intricate interaction between types I and II IFN signaling in the pathogenesis of EAE regulating the threshold of EAE susceptibility, the effector function of Th1 and Th17 cells, and the severity of disease. A functional and reciprocal interaction between types I and II IFN signaling was indispensable to promote a protective response.

Materials and Methods

Mice

C57BL/6 (45.1 and 45.2) mice were purchased from Frederick Cancer Research. B6.129S7-Ifnagr1tmAgt/J (Ifngr1−/−) mice were purchased from The Jackson Laboratory and backcrossed onto C57BL/6 (B6) background for 10–12 generations. The B6.

Ifnar1−/− mouse (37) was obtained from Dr. Jocelyn Demengeot (Instituto Gulbenkian de Ciência, Oeiras, Portugal). Ifng1−/− mice were developed by crossing Ifnar1−/− and Ifngr1−/− mice. The Stat1−/− mice were provided to us by Dr. R. Lorenz (University of Alabama at Birmingham). All mice were maintained and bred at the University of Alabama at Birmingham under facility and treated in accordance with National Institutes of Health and the University of Alabama Animal Care and Use Committee guidelines.

Induction of EAE, scoring, and treatment

Active EAE was induced by immunizing wild-type (WT) and knockout mice 8–12 wk of age with a s.c. injection of MOG35–55 peptide (MOGp) (CPC Research). B6.129S7-Ifnagr1tmAgt/J (Ifngr1−/−) mice were purchased from Frederick Cancer Research. B6.129S7-Ifnagr1tmAgt/J (Ifngr1−/−) mice were purchased from The Jackson Laboratory and backcrossed onto C57BL/6 (B6) background for 10–12 generations. The B6.

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beginning on the day of immunization developed exacerbated EAE with greater mortality than did those treated with PBS (Fig. 2A, Table III). rIFN-γ treatment did not alter the mean day of onset (Table III). A lower daily dose of rIFN-γ (400 ng) had no effect on disease severity or mortality. Most remarkably, initiation of treatment with either 1 µg or 400 ng rIFN-γ after the first sign of clinical symptoms (days 10–19) suppressed disease progression for the course of the treatment (Fig. 2B). Following cessation of treatment, EAE severity bounced to levels equivalent to those of PBS-treated mice. Reinitiation of rIFN-γ therapy moderately induced recovery (data not shown). The mean day of onset was also significantly delayed by both doses of rIFN-γ treatment (Table III).

Our results from active EAE (Fig. 1A) suggested that IFN-α/β signaling work in cooperation with IFN-γ signaling to modulate EAE severity. Therefore, we interrogated whether type I IFN signals participates in the disease amelioration induced by IFN-γ treatment. To test this hypothesis we induced EAE in Ifnar1−/− mice and on day 10 we initiated treatment with IFN-γ or PBS. Remarkably, EAE in Ifnar1−/− mice treated with IFN-γ was slight but significantly more severe than in PBS-treated mice (Fig. 2C, Table IV). This leads us to infer that type I IFN signals cooperate with IFN-γ signals to attenuate disease. Not surprisingly, IFN-γ treatment has no effect on EAE in mice lacking STAT1, the major STAT activated in response to engagement of both IFN-γ and IFN-α/IFN-β receptors (Fig. 2D, Table IV) (1).

Type I and II IFN signals are required to sustain Th1 EAE but not Th17 EAE

Both Th1 and Th17 cells contribute to the pathogenesis of EAE, albeit through different mechanisms (32, 43). To determine the T cell-intrinsic role of type I and II IFN signals in Th1 versus Th17 EAE, we generated nonpolarized, Th1-polarized, or Th17-polarized encephalitogenic T cells from WT mice and the differential IFNR-deficient mice and transferred them into WT naive recipients. The donor cell populations from all groups of mice contained equal proportions of CD4+, CD8+, B220+, and regulatory T cells (CD4+CD25+Foxp3+), and no CD11b+ or CD11c+ cells at the time of transfer (Supplemental Fig. 1 and data not shown). Irrespective of the genotype of donor mice, restimulation cultures under nonpolarizing conditions contained Th1 cells, Th17 cells, and a small proportion of CD4+ T cells that coexpressed activity of IFN-γ in EAE

In addition to classical EAE, mice with loss of IFNGR signals also develop an atypical form of disease (41, 42). Ifngr1−/− mice developed atypical EAE at an incidence >85%, and additional loss of IFNAR signaling, unlike classical EAE, had no effect in this form of disease (Fig. 1B, Table II).

Opposing activity of IFN-γ in EAE

To test the prediction that IFN-γ has disease stage–specific opposing activity in EAE, we treated mice daily with IFN-γ beginning at the time of immunization (days 1–9) or after onset of clinical symptoms (days 10–19). Mice treated with 1 µg rIFN-γ

Table I. Classical EAE severity in type I and II IFN-deficient mice is not sensitive to changes in inducing Ag dose

<table>
<thead>
<tr>
<th>Group of Mice</th>
<th>Day of Onset (Mean ± SD)</th>
<th>Maximum Score (Mean ± SD)</th>
<th>Time to Peak (d) (Mean ± SD)</th>
<th>Accumulative Score (Mean ± SD)</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard MOG dose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>11.3 ± 1.0</td>
<td>4.3 ± 0.7</td>
<td>14 ± 1.5</td>
<td>67.6 ± 14.4</td>
<td>1/10 (10)</td>
</tr>
<tr>
<td>Ifnar1−/−</td>
<td>12.6 ± 2.3</td>
<td>4.2 ± 0.5</td>
<td>15.7 ± 1.6</td>
<td>69 ± 15.8</td>
<td>0/10 (0)</td>
</tr>
<tr>
<td>Ifngr1−/−</td>
<td>15.6 ± 4.1a</td>
<td>4.8 ± 0.7b</td>
<td>23.6 ± 4.3c</td>
<td>62.1 ± 17.7</td>
<td>2/9 (22.2)</td>
</tr>
<tr>
<td>Ifnagr1−/−</td>
<td>14.9 ± 2.09</td>
<td>3.9 ± 1.0</td>
<td>24 ± 2.9d</td>
<td>50.8 ± 22.1</td>
<td>1/8 (12.5)</td>
</tr>
<tr>
<td>High MOG dose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>12.6 ± 2.0</td>
<td>3.8 ± 0.6</td>
<td>15.9 ± 2.7</td>
<td>53.2 ± 12.66</td>
<td>0/11 (0)</td>
</tr>
<tr>
<td>Ifnar1−/−</td>
<td>12.4 ± 1.7</td>
<td>4.2 ± 0.7</td>
<td>16.4 ± 2.7</td>
<td>64.2 ± 13.4</td>
<td>1/9 (11.1)</td>
</tr>
<tr>
<td>Ifngr1−/−</td>
<td>13.4 ± 1.1</td>
<td>5.1 ± 0.6b</td>
<td>19.2 ± 1.5c</td>
<td>72.7 ± 10.1a</td>
<td>6/10 (60)b</td>
</tr>
<tr>
<td>Ifnagr1−/−</td>
<td>14.5 ± 0.9a</td>
<td>4.3 ± 1.3</td>
<td>18.5 ± 1.8c</td>
<td>56.1 ± 17.7</td>
<td>2/8 (25)</td>
</tr>
<tr>
<td>Low MOG dose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>14.6 ± 2.7e</td>
<td>3.6 ± 0.5c</td>
<td>18.1 ± 3.6d</td>
<td>46.5 ± 14.4c</td>
<td>0/14 (0)</td>
</tr>
<tr>
<td>Ifnar1−/−</td>
<td>14 ± 1.6</td>
<td>4.3 ± 0.3b</td>
<td>18.1 ± 1.7d</td>
<td>60.6 ± 11.6</td>
<td>0/13 (0)</td>
</tr>
<tr>
<td>Ifngr1−/−</td>
<td>15.9 ± 1.6</td>
<td>4.3 ± 1.0c</td>
<td>22.4 ± 3.0d</td>
<td>53.7 ± 17.5</td>
<td>2/14 (14.3)</td>
</tr>
<tr>
<td>Ifnagr1−/−</td>
<td>16.7 ± 3.1</td>
<td>3.8 ± 0.8</td>
<td>22.1 ± 3.2c</td>
<td>48.7 ± 20.2</td>
<td>1/11 (9.1)</td>
</tr>
</tbody>
</table>

MOG doses were 150 µg/mouse (standard), 50 µg/mouse (low), and 300 µg/mouse (high).

*p < 0.05, comparisons between each IFNR KO mice versus WT mice within an inducing MOG dose.

*p < 0.05 for comparison between WT, Ifnar1−/− and Ifnagr1−/− mice between days 21 and 30, and for Ifnagr1−/− versus WT, Ifnar1−/− mice, and Ifngr1−/− mice between days 1 and 30.

Includes three moribund mice that were sacrificed.
Table II. Incidence of atypical EAE in IFNGR-deficient mice

<table>
<thead>
<tr>
<th>Group of Mice</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard MOG dose</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>0/10 (0)</td>
</tr>
<tr>
<td>Ifnar1−/−</td>
<td>0/10 (0)</td>
</tr>
<tr>
<td>Ifngr1−/−</td>
<td>8/9 (89)</td>
</tr>
<tr>
<td>Ifnagr1−/−</td>
<td>5/8 (63)</td>
</tr>
<tr>
<td>High MOG dose</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>0/11 (0)</td>
</tr>
<tr>
<td>Ifnar1−/−</td>
<td>0/9 (0)</td>
</tr>
<tr>
<td>Ifngr1−/−</td>
<td>4/10 (40)*</td>
</tr>
<tr>
<td>Ifnagr1−/−</td>
<td>6/8 (65)</td>
</tr>
<tr>
<td>Low MOG dose</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>0/14 (0)</td>
</tr>
<tr>
<td>Ifnar1−/−</td>
<td>0/13 (0)</td>
</tr>
<tr>
<td>Ifngr1−/−</td>
<td>12/14 (86)</td>
</tr>
<tr>
<td>Ifnagr1−/−</td>
<td>8/11 (73)</td>
</tr>
</tbody>
</table>

*These mice rapidly developed severe EAE, complicating the ability to resolve atypical from classical EAE. The percentage of atypical EAE is a conservative estimate; however, the actual incidence is most likely >90%.

FIGURE 2. Dual role of IFN-γ in EAE. Clinical scores from EAE in C57BL/6 mice that were treated with PBS, 400 ng or 1 μg IFN-γ daily from (A) day 1 to day 10 or (B) day 10 to day 19 after EAE induction (n = 9–13 mice/group). (C) Clinical scores from EAE in WT and Ifnar1−/− mice that were treated with PBS or 1 μg IFN-γ daily from day 10 to day 19 after EAE induction (n = 8 mice/group). (D) Clinical scores from EAE in WT and Stat1−/− mice treated with PBS or 1 μg IFN-γ daily from day 10 to day 19 after EAE induction (n = 7–12 mice/group). Results are pooled from two or three experiments. **p < 0.001 for comparisons between (A) 1 μg IFN-γ-treated WT mice versus PBS-treated WT and 400 ng IFN-γ-treated WT between days 16 and 30; (B) PBS-treated WT versus 400 ng and 1 μg IFN-γ-treated WT mice between days 10 and 30; (C) PBS-treated Ifnar1−− mice versus IFN-γ-treated Ifnar1−− between days 16 and 30.

IFN-γ and IL-17 (Th1IFN-γ+IL-17) (Fig. 3). The proportion of Th1, Th17, and Th1IFN-γ+IL-17+ cells in cultures from WT and Ifnar1−/− mice was similar. Likewise, nonpolarized cultures from Ifngr1−/− and Ifnagr1−/− mice contained equal proportions of the three Th effector subpopulations with respect to each other, but greater than Th effector subpopulations from WT or Ifnar1−/− mice (Fig. 3). The elevated numbers of Th17 cells in nonpolarized and Th17-polarized cultures from Ifngr1−/− mice are expected and reflect the inability of IFN-γ to suppress Th17 differentiation (44, 45). However, the finding that lack of IFNGR signaling led to several fold greater numbers of Th1 cells is unexpected because it opposes the dogma that IFN-γ is a feed-forward signal for Th1 differentiation (46).

Cells cultured under Th1- or Th17-polarizing conditions predominantly contained IFN-γ- or IL-17-expressing cells, respectively, independent of the genotype of the donor (Fig. 3). This reflects a good efficiency in the generation of encephalitogenic Th1 cells or Th17 cells.

We found that WT encephalitogenic T cells induced a more severe disease in naive recipients than did Ifnar1−/− donor T cells, but with similar onset (11 d) (Fig. 4A, Table V). Ifngr1−/− or Ifnagr1−/− donor encephalitogenic T cells induced disease with earlier onset and peak of severity than did disease induced by WT or Ifnar1−/− encephalitogenic cells (Table V). A feature common to recipients of Ifngr1−/− or Ifnagr1−/− cells is the partial remission of disease to a severity less than that induced by WT donors and equivalent to that induced by Ifnar1−/− donors (Fig. 4A).

Th1-polarized encephalitogenic T cells from Ifngr1−/− and Ifnagr1−/− mice induced a very rapid and acute disease followed by a nearly complete remission (Fig. 4B, Table V). In contrast, WT Th1-polarized cells induced a slightly delayed but a nonremitting chronic disease. This rapid remission in the recipients that received IFNGR-deficient Th1-polarized encephalitogenic T cells was impressive considering that the transferred cell population contained up to 6-fold greater IFN-γ-expressing CD4+ T cells than in the WT Th1-polarized population. We observed that Ifnar1−/− encephalitogenic Th1-polarized cells induced EAE with onset and peak of severity equivalent to WT Th1-polarized cells, but this was followed by a partial remission of disease (Fig. 4B). The results suggest that whereas there is a T cell–intrinsic requirement for type I IFN signals to maintain severity of Th1 EAE, type II IFN signals are essential for sustaining disease. The rapid recovery in recipients of IFNGR-deficient Th1-polarized cells does not represent increased numbers of regulatory T cells (Supplemental Fig. 1B), but it may reflect the ability of the innate cells to respond to IFN-γ stimulation. IFN-γ induces inducible NO synthase in innate cells and production of NO, leading to their death and consequently disease attenuation. This property is likely to be more pronounced in recipients of Ifngr1−/− Th1 cells because the donor populations contain greater numbers of Th1 cells expressing high levels of IFN-γ (Fig. 3). To test this possibility, we performed a converse experiment in which WT encephalitogenic Th1 cells were transferred into naive WT and Ifngr1−/− recipients. We observed that WT and Ifngr1−/− recipients of encephalitogenic WT Th1 cells induced nonremitting EAE that ultimately reached equal severity (Fig. 4C). The Ifngr1−/− recipients did exhibit a delay to reach peak of disease. We suggest that this delay in reaching peak of disease mechanistically reflects the delayed onset of active EAE in Ifngr1−/− mice (Fig. 1A); however, this requires additional investigation.

In opposition to Th1 EAE, recipients of Th17-polarized encephalitogenic T cells from all IFN receptor–deficient mice developed EAE that was significantly rapid in onset followed by a chronic phase showing no recovery during the 30 d of observation.
in the recipients of WT or Ifngr1
in recipients. We transferred equivalent numbers of WT or CD4+ T cells were determined 14 and 25 d following transfer.

hyperproliferated in response to MOGp stimulation compared under nonpolarizing or Th1-polarizing conditions significantly to proliferate in the recipient mice. We type II IFNR-deficient Th1 cells to sustain disease is not due to the differential ability of Th1 and Th17 cells from the IFNR-deficient mice to proliferate in response to Ag restimulation. To

The rapid remission or development of chronicity may also reflect inducible factors, such as CXCL9 and CXCL10 (47, 48), in the absence of stimulation or under Th17-polarizing condition, the nonpolarizing, Th1-polarizing, or Th17-polarizing conditions. In cephalitogenic T cells stimulated with MOGp and cultured under

deficient with type II IFN receptor–deficient Th1- or Th17-polarized cells developed atypical EAE. This supports the premise that IFN-

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Table III. Disease stage specific activity of IFN-γ in EAE

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day of Onset (Mean ± SD)</th>
<th>Maximum Score (Mean ± SD)</th>
<th>Time to Peak (d) (Mean ± SD)</th>
<th>Accumulative Score (Mean ± SD)</th>
<th>Incidence (%)</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before onset</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>WT PBS</td>
<td>16.1 ± 1.9</td>
<td>3.8 ± 0.7</td>
<td>19.5 ± 2.9</td>
<td>46.1 ± 7.0</td>
<td>13/13 (100)</td>
<td>1/3 (7.7)</td>
</tr>
<tr>
<td>WT 400 ng IFN-γ</td>
<td>15.8 ± 4.0</td>
<td>4.1 ± 0.2</td>
<td>21.8 ± 4.5</td>
<td>46.6 ± 11.5</td>
<td>5/5 (100)</td>
<td>0/5 (0)</td>
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<tr>
<td>WT 1 μg IFN-γ</td>
<td>16.4 ± 2.7</td>
<td>4.7 ± 1.1</td>
<td>20.6 ± 3.0</td>
<td>58.1 ± 26.6</td>
<td>9/9 (100)</td>
<td>3/9 (33.3)</td>
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<tr>
<td>After onset</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>WT PBS</td>
<td>14.5 ± 3.4</td>
<td>4.4 ± 1.2</td>
<td>21.6 ± 4.9</td>
<td>56.2 ± 26.9</td>
<td>10/10 (100)</td>
<td>3/10 (30)</td>
</tr>
<tr>
<td>WT 400 ng IFN-γ</td>
<td>24.3 ± 4.3</td>
<td>3.6 ± 0.5</td>
<td>26.5 ± 4.0</td>
<td>22.1 ± 17.2</td>
<td>4/4 (100)</td>
<td>0/4 (0)</td>
</tr>
<tr>
<td>WT 1 μg IFN-γ</td>
<td>19.4 ± 5.8</td>
<td>3.9 ± 0.5</td>
<td>27 ± 2.4</td>
<td>32.8 ± 12.1</td>
<td>9/9 (100)</td>
<td>0/9 (0)</td>
</tr>
</tbody>
</table>

*p < 0.05, comparison between WT treatment and PBS treatment.

A higher mortality was observed within the PBS treated group, perhaps an outcome of the stress induced by daily injections.

<table>
<thead>
<tr>
<th>Fig. 2D</th>
<th>Day of Onset (Mean ± SD)</th>
<th>Maximum Score (Mean ± SD)</th>
<th>Time to Peak (d) (Mean ± SD)</th>
<th>Accumulative Score (Mean ± SD)</th>
<th>Incidence (%)</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT PBS</td>
<td>11.7 ± 2.6</td>
<td>3.8 ± 1.1</td>
<td>13.7 ± 2.9</td>
<td>55.7 ± 25.5</td>
<td>10/10 (100)</td>
<td>1/10 (30)</td>
</tr>
<tr>
<td>WT IFN-γ</td>
<td>18.9 ± 3.5</td>
<td>4.4 ± 0.8</td>
<td>25 ± 4.0</td>
<td>34.1 ± 12</td>
<td>7/7 (100)</td>
<td>1/7 (14.2)</td>
</tr>
<tr>
<td>Stat1+/− PBS</td>
<td>13.6 ± 4.2</td>
<td>4.6 ± 0.5</td>
<td>18.9 ± 5.4</td>
<td>69.3 ± 25.4</td>
<td>8/8 (100)</td>
<td>0/8 (0)</td>
</tr>
<tr>
<td>Stat1− IFN-γ</td>
<td>14.9 ± 3.6</td>
<td>5.1 ± 0.9</td>
<td>21.9 ± 3.5</td>
<td>65.5 ± 20.5</td>
<td>7/7 (100)</td>
<td>3/7 (42.9)</td>
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</tbody>
</table>

*p < 0.05, comparison between treatments in WT mice.

INFR signaling does not affect IFN-γ-regulated migration to the brain

The infiltration of mononuclear cells into the CNS is dependent on the Th1/Th17 encephalitogenic T cell ratio and is regulated by IFN-γ (47, 49). To determine whether IFNAR signaling altered IFN-γ–regulated migration of leukocytes into the CNS, we evaluated infiltration and demyelination in brain stem, cerebellum, and SC at the peak of disease (day 20). We observed detectible mononuclear infiltration and demyelination of brain stem and cerebellum only in sections from Ifngr1−/− and Ifngr1−/− mice (Fig. 6A, 6B). The levels of activated microglia/macrophages and the extent of infiltration of neutrophils and CD4+ T cells were similar in the CNS of Ifngr1−/− mice and Ifngr1−/− mice. The results show that type I IFN signaling does not alter IFN-γ–dependent entry of inflammatory cells into the brain.

Although the clinical score was similar to WT (3.5) and Ifnar1−/− (3.5) mice, the SC sections from Ifngr1−/− mice (3.0) showed the greatest extent of mononuclear cell infiltration, demyelination, presence of neutrophils, CD4+ T cells, and activated microglia/macrophages (Fig. 6C). There was no difference between WT and Ifnar1−/− for all parameters evaluated. The extent of mononuclear cell infiltration and demyelination in SC from Ifngr1−/− mice was the least, and this correlated with the lower classical EAE score of 1.0. However, this mouse had severe atypical EAE (3.5).
in IFNAR-deficient mice than in WT mice, but this difference was essentially absent when IFNGR signaling was ablated. IL-13 was comparably reduced in CNS tissues, there was a trend for lower IFN-γ in IFNGR-deficient mice, and mice lacking both IFN receptors had the lowest levels of the chemokine. In fact, the expression of CXCL10 in Ifngr1−/− mice was comparable to that in unimmunized mice. The results indicate that both IFNAR and IFNGR independently induce CXCL10, and combined loss of both IFN receptors has an additive effect. Mice lacking only IFNAR had levels of CXCL9 (MIG) or CCL5 (RANTES) similar to WT mice (Fig. 7E, 7F). However, ablation of IFNGR resulted in complete absence of CXCL9. IFNGR-deficient mice also had dramatically reduced levels of CCL5 that were decreased to levels equivalent to those in unimmunized mice when both IFNGR and IFNAR were absent (Fig. 7F). IFNAR signaling–dependent expression of CCL5 has been observed previously (50), and our data suggest that this occurs only in the absence of IFN-γ signaling.

Higher levels of G-CSF were observed in SC and brains of mice lacking IFNGR compared with IFNAR-deficient or WT mice (Fig. 7G). This observation is consistent with the elevated infiltration of neutrophils in the CNS of Ifngr1−/− mice (Fig. 6). In the SC, the levels of G-CSF were significantly higher in IFNAR-deficient mice compared with WT mice, but this was not associated with any significant increase in neutrophil infiltration (Fig. 7G). We found no significant effect of IFN receptor deletion on the remaining 24 cytokines (see Materials and Methods and data not shown).

Type I and II IFN signals regulate threshold of response to development of EAE

Two previous studies using the same Ifnar1−/− mouse as this study showed that loss of IFNAR signaling exacerbates EAE, a finding that differs from our result (Fig. 1A) (10, 11). We noticed that in both of these reports EAE was induced with a greater amount of MOGp than used in our protocol. To determine whether the dif-
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Discussion

This study reveals a remarkable cooperative relationship between type I and II IFNs in regulating pathogenesis of EAE. The above results suggest that type I and II IFNs have a role in regulating the threshold or response to immunization dose and consequently affecting disease course. We therefore examined the effect of low-dose MOGp (50 µg) immunization on development of EAE in all groups of mice. The severity of EAE in all of the IFNR-deficient mice was similar to each other but greater than that in WT mice (Fig. 8A, Table I). Importantly, immunization with lower amounts of peptide did not significantly alter onset, incidence, or severity of disease in any of the IFNR-deficient mice. In contrast, in WT mice immunized with 50 µg MOGp, EAE was significantly less severe than in mice immunized with 150 µg MOGp (Supplemental Fig. 2, Table I). The severity of atypical EAE in Ifngr1−/− mice was not significantly altered with 300 µg peptide immunization (Supplemental Fig. 3). However, in mice immunized with 50 µg peptide, Ifngr1−/− mice exhibited lower atypical disease severity than did Ifnar1−/− mice. Overall the results show that type I (IFN-α and/or IFN-β) and IFN-γ signals are each independently involved in setting the thresholds for susceptibility to EAE.

continues to be highly expressed in the CNS of mice lacking IFN-γ (56). This local expression of IFN-β is a protective response in EAE and is the principle behind using it as therapy for the treatment of MS (10–12, 57). Our results now show that in the absence of IFN-γ cosignaling, endogenous IFN-α/β drives inflammation rather than immunosuppression. This is consistent with our recent finding that administration of IFN-β to IFNGR-deficient mice with EAE further exacerbates disease (36). This Janus-like property of IFNAR signaling is also evident for IFNγ signaling. During the initiation phase of EAE, IFN-γ drives pathogenesis, as suggested by the delayed onset of disease in IFNGR-deficient mice. Indeed, we found that administration of IFN-γ during this period leads to exacerbation of disease. In contrast, type I IFN signaling seems to have a limited role in disease initiation. In comparison with EAE in WT mice, the lack of IFNAR or IFN-β does not alter disease onset (10–12). The disease onset in mice with combined absence of IFNAR and IFNγ was not different from those lacking only IFNγ, indicating that type I IFN signaling begins to participate in EAE at a time point later than IFN-γ signaling. In fact, IFN-β in EAE is first detected in the CNS only after onset of clinical symptoms (11).

IFN-γ treatment controls the progression of experimental autoimmune myocarditis (58). In rodents, IFN-γ treatment ameliorated EAE in some studies but induced encephalomyelitis in others (24, 28–31, 59, 60). In MS, IFN-γ treatment led to exacerbations in some patients and no benefit in others, an outcome recapitulated to some extent in a novel marmoset model of EAE (31, 61). Overall, these studies led to ambiguity regarding the role of IFN-γ in EAE and MS (17–26). The present study contributes to clarify this paradoxical evidence and provides insight into the complex role of IFN-γ during EAE pathogenesis. Our results indicate that the previous mixed outcome likely reflects disease stage–specific Janus-like function of IFN-γ. We found that IFN-γ treatment during the initiation phase promoted pathogenesis; in contrast, such treatment during the effector phase was immunosuppressive in EAE. However, this immunosuppressive activity of IFN-γ required functional type I IFN signaling. The composite developing picture reinforces the model that integration of type I and II IFN signaling pathways are necessary to regulate pathogenesis in EAE. Imbalance of either pathway drives pathogenesis.

We found that encephalitogenic Th1 cells require type II IFN signals for inducing persistent and chronic EAE. In comparison, IFN-α/β signals are not necessary for chronicity but are important for disease severity. As reported in this study and shown previ-
viously, MOGp-induced EAE in IFN-γ signaling–deficient mice is severe and chronic, and this seems to conflict with the self-limiting EAE induced by IFNGR-deficient Th1 cells (21–23, 27). We suggest this difference reflects role of IFNGR signaling in Th1 cells versus other effector cells. IFN-γ signaling is needed 1) to restrain Th17 differentiation and expansion (44, 45), 2) for efficient IL-27 induction by dendritic cells and macrophages (62, 63), and 3) for induction of inducible NO synthase and consequently production of NO (23, 28). Loss of these key protective mechanisms would lead to exacerbation of disease.

The absence of persistent EAE induced by IFN-γ signaling–deficient mice is severe and chronic, and this seems to conflict with the self-limiting EAE induced by IFNGR-deficient Th1 cells (21–23, 27). We suggest this difference reflects role of IFNGR signaling in Th1 cells versus other effector cells. IFN-γ signaling is needed 1) to restrain Th17 differentiation and expansion (44, 45), 2) for efficient IL-27 induction by dendritic cells and macrophages (62, 63), and 3) for induction of inducible NO synthase and consequently production of NO (23, 28). Loss of these key protective mechanisms would lead to exacerbation of disease.

The absence of persistent EAE induced by IFN-γ signaling–deficient Th1 donor cells can also be attributed to the protective effects of IFN-γ. Th1-polarized cell cultures from IFNGR-deficient mice contain several fold greater proportion of IFN-γ-expressing cells and these cells also express higher levels of IFN-γ compared with cells from WT or IFNAR-deficient mice. The high levels of IFN-γ can initially promote pathogenesis by promoting activation of APCs and other accessory cells such as upregulation of MHC class II, costimulatory molecules, and adhesion molecules (64). Subsequently, this higher level of IFN-γ can suppress CNS inflammation by inducing expression of NO in activated macrophages (65), inducing apoptosis of CNS-infiltrating lymphocytes and other mononuclear cells (28) and/or production of anti-inflammatory or neuroprotective cytokines/chemokines such as IL-13 and LIF (66–69). Indeed, we did observe that both IL-13 and LIF were barely induced in the CNS of IFNGR-deficient mice with EAE.

In opposition to Th1 cells, type I and II IFN signals restrain encephalitogenic Th17 cells and do so independently of each other. These observations recapitulate in vivo our previous in vitro finding of the ability of IFN-β to directly suppress Th17 cells but not Th1 cells (36). The direct suppression of Th17 cell differentiation by IFN-γ or IFN-β in vitro has been reported in several studies (10, 44, 45, 70).
Prinz et al. (11), using *Ifnar1*−/− conditional mice, demonstrated that development and severity of MOGp-induced EAE were not altered in mice with selective loss of IFNAR in CD4+ T cells. On first examination, our result is inconsistent with their findings. Importantly, however, note that the previous study represents active EAE and therefore the combined result of Th1 and Th17 cells. Our study dissects the relative role of IFNAR in Th1 cells versus Th17 cells. Because loss of IFNAR signaling has opposing outcomes in Th1 and Th17 EAE, the combined effect could be null and therefore it reconciles the inconsistencies between the two studies.

The higher levels of IFN-γ and IL-17 in brains and SC of IFNGR−/− mice are expected because IFN-γ signals inhibit IL-17 gene expression (44, 45). However, the expanded numbers of Th1 cells induced in the absence of IFNAR is counterintuitive when considering the paradigm that IFN-γ provides feed-forward signals for the expression of Tbet (46). This suggests that IFN-γ may also have regulatory effects in Th1 cells. IFNAR-deficient mice showed significantly lower concentration of CXCL10 and IL-13 in brain, whereas higher levels of G-CSF and CCL11 were detected in SC with respect to WT mice. Remarkably, the absence of IFN-γ signaling resulted in a similar effect on these cytokines. These results suggest that the expression of CXCL10, IL-13, G-CSF, and CCL11 is independently regulated by both type I and II IFN signaling and in a tissue-specific manner. The CNS of mice lacking both IFNAR and IFNGR contained lower levels of CXCL10 and higher levels of IL-17 than did the CNS of mice that only lacked IFNAR. This indicates modulation by type I IFN signaling on those IFN-γ-regulated cytokines. The brain and spinal cords of IFNGR-deficient mice with EAE contained high levels of G-CSF and very low expression of the IFN-γ-regulated chemokines CXCL9, CXCL10, and CCL5. The elevated G-CSF might contribute to the enhanced numbers of neutrophils detected in the CNS of IFN-γ signaling–deficient mice. CXCL9 and CXCL10 are chemokines that restrain the entry of leukocytes into the brain, and their downregulation is associated with atypical EAE (47, 48), whereas CCL5 is an important chemokine for firm adhesion of leukocytes to the vasculature in the brain (71). Collectively, these results reveal the independent and dependent action of type I and II IFNs in the CNS-specific modulation of key cytokines and chemokines in the pathogenesis of EAE.

As previously reported, we found high levels of IFN-γ and IL-17 in the brains of IFNGR-deficient mice as they developed atypical EAE (35, 47). Mice with atypical EAE have much greater infiltration of encephalitogenic effector cells into the brain and brain stem than do those with classical EAE (47). Our finding that IFNAR-deficient mice do not develop symptoms or pathological features of atypical EAE indicates that IFN-γ signaling, but not IFNAR signaling, is necessary to restrain encephalitogenic cells from entering the brain. None of the mice receiving IFNGR-deficient Th1 or Th17 cells developed clinical symptoms of atypical EAE. This implies that loss of IFNγ signals in T cells is insufficient to induce atypical EAE and suggests a role of other cells such as macrophages, epithelial cells, and glial cells in this process.

In summary, our data reveal that cooperative signaling from type I and II IFNs is necessary to restrain the pathogenesis in EAE. Loss and perhaps imbalance of either IFN signal aggravates inflammation and results in exacerbated disease. This study also demonstrates that response to IFN signals by the different effector populations involved in the pathogenesis of EAE varies and can have opposite outcomes. Elucidating these mechanisms for the different innate and
adaptive effector cells that contribute to the pathogenesis of MS will enable the development of effective therapeutics that selectively target arms of the IFN signaling pathway.

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Disclosures

The authors have no financial conflicts of interest.

References


