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IFN-γ Is Critical to the Control of Murine Autoimmune Encephalomyelitis and Regulates Both in the Periphery and in the Target Tissue: A Possible Role for Nitric Oxide

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NO and IFN-γ have normally been considered cytotoxic and proinflammatory molecules, respectively, in the setting of the central nervous system inflammatory disease autoimmune encephalomyelitis (EAE). Using mice lacking the ligand binding chain of the IFN-γ receptor (IFNγR−/−), we have previously shown that IFN-γ is not essential for myelin oligodendrocyte glycoprotein peptide (MOG35–55) induced EAE expression but is in fact essential for its down-regulation. Here we examined the downstream molecular and cellular mechanism(s) of IFN-γ regulation and demonstrate that neither IL-4 nor IL-10 appear to play a role in down-regulation nor do various lymphoid cell populations. Cells of the macrophage lineage are key to down-regulation as evidenced by the fact that peritoneal exudate cells from IFNγR−/− mice inhibit Ag-driven proliferation of IFNγR−/− lymphocytes, whereas IFNγR−/− peritoneal exudate cells do not. High levels of reactive nitrogen intermediates are detected in the former cultures but not the latter, and the inhibition of proliferation is reversible with an inhibitor of inducible NO synthase, indicating a key role for NO in down-regulation. Studies with bone marrow chimeras indicate that down-regulation occurs not only systemically but also within the target tissue. These data suggest that IFN-γ down-regulates EAE by inducing inducible NO synthase and subsequently NO production, both by macrophages in the periphery and, by inference, microglia and astrocytes in the target tissue. The Journal of Immunology, 1999, 163: 5278–5286.

E xperimental autoimmune encephalomyelitis (EAE) is an induced cell-mediated autoimmune inflammatory disease of the CNS. The pathology of EAE is characterized by lymphocytic and mononuclear cell infiltration of the CNS, an increase in blood-brain barrier permeability, astrocytic hypertrophy, and often demyelination, all of which contribute to the observed clinical expression of disease (1, 2). The clinical presentation is of one hindlimb and occasionally forelimb weakness progressing to flaccid paralysis and may be acute monophasic or chronic relapsing, depending on the type of Ag and species of animal used. Because the clinical and pathological aspects of this disease bear significant similarities to the human disease multiple sclerosis (MS) it has been used as a model of that human demyelinating disease.

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3 Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; iNOS, inducible NO synthase; l-NAME, N-methyl-l-arginine acetate; RNI, reactive nitrogen intermediates; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; IFNγR−/−, IFN-γ receptor knockout; WT, wild type; IFNγR−/−, IFN-γ receptor knockout; KO, knockout; PEC, peritoneal exudate cells.

With the exception of a study in immunocompromised animals (3), EAE has been described as being a Th1 T cell-mediated disease (4–6), and therefore a pivotal role for the Th1 proinflammatory cytokine IFN-γ in EAE pathology has been suggested. Numerous effects of IFN-γ in promoting inflammation have been described, such as: macrophage activation; up-regulation of both class I and class II MHC molecules necessary for (self) Ag presentation; induction of adhesion molecules such as ICAM-1 and VCAM, perhaps promoting homing of inflammatory cells; and induction of expression of receptors for other cytokines, in particular TNF receptors (for a review, see Ref. 7). Therefore, the implication has been that IFN-γ is an essential contributor to EAE pathology.

We have previously shown, however, that mice lacking the ligand binding chain of the IFN-γ receptor (IFNγR−/−) and therefore unable to respond to IFN-γ, develop severe and usually fatal EAE when immunized with human myelin oligodendrocyte glycoprotein peptide 35–55 (MOG35–55), whereas control mice expressing the gene are resistant to disease induction, indicating that IFN-γ is not essential for disease induction (8). Furthermore, passive transfer of disease with MOG35–55-specific lymphoid cells from IFNγR−/− mice produces, in knockout (IFNγR−/−) mice, severe EAE from which the recipients fail to recover. The same cells produce equally severe disease in IFNγR−/− control mice, but importantly all the recipients recover fully. These results provide definitive evidence that IFN-γ is not essential for the generation or function of anti-MOG35–55 effector cells, but that it does play an obligatory role in down-regulating the disease.

We have suggested that, at least in the current model, this down regulation must act indirectly through a secondary mediator. IFNγR−/− effector cells produce extremely high levels of IFN-γ (8) but cannot respond to it because of the lack of the receptor. When these cells are transferred into wild-type (WT) recipients the
recipient cells can and do respond to IFN-γ with the production of some mediator(s) which ultimately feeds back and down-regulates the effector cells.

In this paper we examine possible mechanisms by which IFN-γ down-regulates disease. We provide evidence that neither IL-4 nor IL-10 appear to be involved nor are CD8+ T cells. In fact, lymphoid cells do not appear to play a role as evidenced by the fact that transfer of large numbers of IFNγR−/− spleen cells into IFNγR−/− animals fails to confer on the IFNγR−/− mice the ability to recover from passively induced disease. In vitro studies of inhibition of Ag-specific proliferation of IFNγR−/− lymphoid cells show that while lymphoid cells from WT mice do not inhibit proliferation, peritoneal exudate cells (PEC) readily do so in a dose-dependent manner and high levels of reactive nitrogen intermediates (RNI) can be detected in such cultures. Inhibition of proliferation is reversible with an inhibitor of inducible NO synthase (iNOS), indicating a key down-regulatory role for NO. Furthermore, the use of chimeric mice indicates that down-regulation occurs not only systemically but also at the level of the target tissue, the CNS.

Materials and Methods
Mice and induction of EAE
129/Sv, H2b mice of either sex, homozygous for disrupted gene (IFNγR−/−) and for null mutations (IFNγR−/γ−), were obtained from Dr. Michel Aguet (University of Zurich, Zurich, Switzerland). Disruption of the IFNγR gene was verified using PCR and primers as described (9). Mice were maintained in pathogen-free conditions and used between the ages of 8 and 14 wk. All animal experimentation was approved by the Animal Ethics Committee of the Australian National University.

The peptide corresponding to amino acids 35–55 of human MOG (Met-Glu-Val-Gly-Trp-Tyr-Arg-Pro-Pro-Phe-Ser-Arg-Val-Val-His-Leu-Tyr-Arg-Asn-Gly-Lys) was synthesized by standard Fmoc chemistry, and the purity was determined by reverse-phase HPLC (Biomolecular Resource Facility, John Curtin School of Medical Research, Australian National University). MOG35–55 (1.67 mg/ml in saline) was emulsified in an equal volume of CFA containing 0.5 mg/ml Mycobacterium butyricum and 4 mg/ml Mycobacterium tuberculosis (H37Ra). Each mouse received 120 μl emulsion distributed in footpads of both hind feet and in the nape of the neck. The total dose of MOG35–55 was 100 μg/mouse. Immediately before and 2 days after injection of emulsion the mice received an i.v. injection of 300 ng pertussis toxin (List Biological Laboratories, Campbell, CA) in 250 μl PBS.

Scoring of clinical disease
Disease severity was scored on a scale of 0 (asymptomatic) to 5 (morbidity, paralysis). For each of the chimeras, the score was assigned 0: slight weakness of the tail, 1: definite tail and partial hind limb paralysis, 2: tail paralysis and moderate hind limb paralysis, 3: complete paralysis of the tail and hind limbs often associated with incontinence, 3.5: paralysis of tail and hind limbs with moderate forelimb weakness, 4, and total paralysis of hind and forelimbs, 5. The mean maximal clinical score and mean day of onset was calculated for each group of animals.

Abs and treatment schemes
The anti-murine IL-4 was the rat IgG1 mAb 1B11 (10) and the anti-IL-10 (2A5) was also an IgG1 mAb and was a gift of Dr. K. Moore (DNAX, Palo Alto, CA). Control Abs was an IgG1 anti-β-galactosidase, a gift from Dr. John Abrams (DNAX). Anti-IFN-γ was R46A2 from the American Type Culture Collection (ATCC, Manassas, VA). All Abs were given on days −1, 0, +1, 2, 4, 6, 8, 10, and 12 in relation to passive transfer of cells on day 0. Depletion of CD8+ cells was done using an anti-CD8 Ab (ATCC TIB-210) kindly donated by Dr. Guu Karupiah (John Curtin School Medical Research, Canberra, Australia). Mice were given 1 mg of Ab i.p., i.e., at 3-day intervals for a total of four doses. Treatment was begun on day 6 following passive transfer. Spleen and lymph nodes assayed 2 days after the last treatment had ≤2% CD8+ cells as assessed by FACS analysis.

Passively transferred EAE
IFNγR−/− mice were immunized with MOG35–55 as above. Fourteen days later they were killed, and spleen cell suspensions were prepared and cultured in RPMI 1640 + 10% FCS with 10 μg/ml MOG35–55 for 4 days at 37°C. Cultures were harvested, cells washed three times with HBSS and transferred at 5 × 106 cells i.v./recipient.

Cell culture and proliferation assay
Spleens were removed from mice 15 days after sensitization, and proliferation assays were performed in 96-well microtiter plates. Cells, 1 × 106/ml (200 μl/well), were cultured with RPMI 1640 + 10% FCS, 5 × 10−3 M 2-ME, penicillin, streptomycin, and neomycin (PSN), and 10 μg/ml MOG35–55 (γ-H)Thymidine was added at 72 h and cultures harvested 18–20 h later. When inhibiting cells were added 48 h after induction of cultures, lymphoid cells were washed extensively (three times) before addition of the PEC. N-methyl-β-arginine acetaete (t-NMA) was prepared essentially according to the method of Pathy et al. (11) and added to cultures at a final concentration of 50 μM.

Measurement of RNI
The concentration of nitrate plus nitrite ions was used as an indirect measure of the amount of NO being produced. Measurement was performed using a microplate assay essentially according to the method of Rockett et al. (12). Briefly, nitrite was measured by addition of 100 μl of Griess reagent to 30 μl of test sample. Protein was removed by addition of 100 μl of trichloroacetic acid followed by centrifugation, and the OD of the sample read at 540 nm with a reference at 650 nm using a microplate reader (Molecular Devices, Menlo Park, CA). For nitrate measurements, the nitrate was first converted to nitrite by incubation with nitrate reductase and NADPH (Boehringer Mannheim, Mannheim, Germany) for 20 min. The results were quantified by reading against appropriate nitrite and nitrate standard curves.

Generation of radiation bone marrow chimeras
Bone marrow cells were harvested from the long bones of either IFNγR−/− or IFNγR−/γ− mice by flushing with cold RPMI. Cells were disaggregated by gently cycling through a 26-gauge needle and then through a 400 mesh stainless steel strainer. Cells were washed three times, and nucleated cells were counted and transferred, i.e., at 7 × 106/recipient. Recipients had been prepared by two doses of whole body irradiation with 5.5 Gy/dose from a Cobalt 60 source, given 48 h apart. Bone marrow cells were transferred within 5 h of the last dose of irradiation. Mice were maintained on antibiotics via the drinking water for the first 4 wk posttransfer. At 8 wk posttransfer, all mice were tail bled and the extent of engraftment was determined. Initially we attempted to define the genotype of peripheral leukocytes using Abs to the IFN-γ receptor and flow cytometric analysis. Interesting, using two different Abs (rabbit polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA) and hamster monoclonal IgG (Genzyme Diagnostics, Cambridge, MA)), we were unable to detect more than 25% positive cells in the circulation of IFNγR−/− control mice even if the cells had been previously activated with mitogen in vitro. This was unexpected in light of all reports stating that all cells express the IFN-γ receptor (13). We did not feel that 25% was an adequate window with which to examine for chimerism, so we therefore chose to do cellular PCR on peripheral blood of each of the chimeras and for comparison three animals each of the control WT or KO mice.

Three drops of peripheral blood were obtained from the tails of mice and DNA extracted following standard techniques. Using primers described previously (9), DNA was amplified by 25 cycles of PCR under conditions that result in a single amplification product of the correct size: 1 min denaturation at 94°C, 1 min annealing at 54°C, and 2 min extension at 72°C. Amplified DNA was analyzed by gel electrophoresis. Under these conditions 9 of 12 WT recipients of IFNγR−/− control mice even if the cells had been previously activated with mitogen in vitro. This was unexpected in light of all reports stating that all cells express the IFN-γ receptor (13). We did not feel that 25% was an adequate window with which to examine for chimerism, so we therefore chose to do cellular PCR on peripheral blood of each of the chimeras and for comparison three animals each of the control WT or KO mice.
Materials and Methods

The inability of splenic lymphoid cells from WT mice to confer recovery indicates the essential nature of IFN-\(\gamma\) at day 8. The mice then recovered by day 18 (data not shown). This process we treated the IFN-\(\gamma\)R\(^{+/-}\) recipients with neutralizing Ab to either of the cytokines beginning the day before cell transfer and observed the mice for the possible loss of the ability to recover. Table I shows that inhibiting IL-4 or IL-10 had no effect on the ability of IFN-\(\gamma\)R\(^{+/-}\) animals to recover. Another group of recipient mice were treated with anti-IFN-\(\gamma\) Abs, and these animals failed to begin to recover until the Ab treatment was discontinued at day 8. The mice then recovered by day 18 (data not shown). This again indicates the essential nature of IFN-\(\gamma\) in the recovery process.

CD8\(^+\) T cells are not required in the recovery process

To examine the function of CD8\(^+\) cells in recovery, IFN-\(\gamma\)R\(^{+/-}\) mice were depleted of CD8\(^+\) cells according to the treatment described in Materials and Methods. This treatment of mice with EAE resulted in \(<2\%\) CD8\(^+\) cells in spleen and lymph nodes at day 17 post-cell transfer. The data in Table II, representing one of these experiments, demonstrate that such depletion of CD8\(^+\) cells did not alter the ability of IFN-\(\gamma\)R\(^{+/-}\) mice to recover from disease.

The inability of splenic lymphoid cells from WT mice to confer the ability to recover on IFN-\(\gamma\)R\(^{-/-}\) recipients

In an attempt to identify a cell type responsible for contributing to the recovery process we transferred into IFN-\(\gamma\)R\(^{-/-}\) mice one spleen equivalent of lymphoid cells (\(1 \times 10^8\)) from naive IFN-\(\gamma\)R\(^{+/-}\) animals 24 h before the induction of EAE passively with IFN-\(\gamma\)R\(^{-/-}\) effector cells. These animals developed severe EAE as did IFN-\(\gamma\)R\(^{-/-}\) animals receiving no cells or cells from naive IFN-\(\gamma\)R\(^{-/-}\) mice (to control for possible inhibition of disease due to crowding); none of the recipients recovered from disease by day 14 and some had in fact died indicating the absence or insufficient numbers of a cell population in the IFN-\(\gamma\)R\(^{+/-}\) spleen lymphoid population capable of orchestrating recovery (data not shown).

In vitro Ag-specific proliferation of IFN-\(\gamma\)R\(^{-/-}\) lymphoid cells and its inhibition by IFN-\(\gamma\)R\(^{+/-}\) cells

No inhibition with IFN-\(\gamma\)R\(^{+/-}\) lymphoid cells. Splenic lymphoid cells from MOG\(_{35-55}\)-immunized IFN-\(\gamma\)R\(^{-/-}\) mice proliferate readily in vitro when stimulated with specific Ag. Using this in vitro assay system we attempted to define a cell population in WT animals able to inhibit this proliferation. Neither spleen nor lymph node lymphocytes from IFN-\(\gamma\)R\(^{-/-}\) mice, when mixed at various concentrations with IFN-\(\gamma\)R\(^{-/-}\) spleen cells, inhibited Ag-specific proliferation of the latter (data not shown).

Inhibition with IFN-\(\gamma\)R\(^{+/-}\) PEC. Naive mice were given i.p. injections of thioglycollate and PEC harvested 3 days later. These PEC were added at various concentrations to IFN-\(\gamma\)R\(^{-/-}\) spleen cells from MOG\(_{35-55}\) immunized mice; MOG\(_{35-55}\) was also added and proliferation determined 4 days later (Table III). At high concentrations (\(8 \times 10^6\)) PEC from both IFN-\(\gamma\)R\(^{-/-}\) and IFN-\(\gamma\)R\(^{+/-}\) mice gave inhibition of proliferation, indicating perhaps a nonspecific crowding effect. At lower concentrations however (\(4 \times 10^6\) and \(2 \times 10^6\)) IFN-\(\gamma\)R\(^{-/-}\) PEC gave 100% and 75% inhibition, respectively. The same number of PEC from naive IFN-\(\gamma\)R\(^{-/-}\) mice had no effect. Similar results were also obtained when the addition of IFN-\(\gamma\)R\(^{-/-}\) PEC to cultures of IFN-\(\gamma\)R\(^{-/-}\) spleen cells plus MOG\(_{35-55}\) was delayed until 48 h. after the initiation of such cultures (Table IV).

Production of NO in cultures containing IFN-\(\gamma\)R\(^{+/-}\) PEC

IFN-\(\gamma\)R\(^{-/-}\) spleen cells from immunized mice were cultured with IFN-\(\gamma\)R\(^{+/-}\) or IFN-\(\gamma\)R\(^{-/-}\) PEC in the presence or absence of specific Ag (MOG\(_{35-55}\)) for 3 days at which time supernatants were harvested and assayed for nitrate and nitrite. Proliferation of spleen cells in cultures containing IFN-\(\gamma\)R\(^{-/-}\) PEC were inhibited by 80% (data not shown).

Results

No apparent role for IL-4 or IL-10 in down-regulation of EAE

IFN-\(\gamma\)R\(^{+/-}\) mice receiving MOG\(_{35-55}\)-specific effector cells from IFN-\(\gamma\)R\(^{-/-}\) mice develop severe disease from which they recover. To determine whether IL-4 or IL-10 play a role in this recovery process we treated the IFN-\(\gamma\)R\(^{+/-}\) recipients with neutralizing Ab to either of the cytokines beginning the day before cell transfer and observed the mice for the possible loss of the ability to recover. No apparent role for IL-4 or IL-10 in down-regulation of EAE resulted in treatment of mice with neutralizing Ab to IL-4 or IL-10 on days 1–3 and 7–9 post-infection. The same number of PEC from naive IFN-\(\gamma\)R\(^{-/-}\) mice, when mixed at various concentrations with IFN-\(\gamma\)R\(^{-/-}\) spleen cells, inhibited Ag-specific proliferation of the latter (data not shown).

Table I. Abs to IL-4 or to IL-10 do not alter the ability of IFN-\(\gamma\)R\(^{+/-}\) mice to recover from passively induced EAE

<table>
<thead>
<tr>
<th>Mice(^{a})</th>
<th>Treatment(^{b})</th>
<th>No. Sick/ Total</th>
<th>Mean Day of Onset</th>
<th>Mean Maximum Clinical Score</th>
<th>No. Recovering by Day 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-(\gamma)R(^{+/-})</td>
<td>Control Ab</td>
<td>6/6</td>
<td>6.0</td>
<td>4.0</td>
<td>6/6</td>
</tr>
<tr>
<td>IFN-(\gamma)R(^{-/-})</td>
<td>Anti-IL-4</td>
<td>6/6</td>
<td>6.5</td>
<td>3.5</td>
<td>6/6</td>
</tr>
<tr>
<td>IFN-(\gamma)R(^{+/-})</td>
<td>Anti-IL-10</td>
<td>6/6</td>
<td>6.3</td>
<td>4.1</td>
<td>5/5*</td>
</tr>
<tr>
<td>IFN-(\gamma)R(^{-/-})</td>
<td>Control Ab</td>
<td>6/6</td>
<td>7.5</td>
<td>4.5</td>
<td>0/6</td>
</tr>
</tbody>
</table>

\(^{a}\) All mice were given \(5 \times 10^8\) activated spleen cells from MOG\(_{35-55}\) immunized IFN-\(\gamma\)R\(^{-/-}\) donor mice.

\(^{b}\) Ab was given on days -1, 0, +1, 2, 4, 6, 8, 10, and 12 in relation to passive transfer.

\(^{*}\) One mouse in this group died from disease shortly after onset.
cells was also assessed. As shown in Fig. 2A only those cultures containing Ag-stimulated spleen cells plus IFNγR−/− PEC produced high levels of RNI. Cultures without Ag or with IFNγR−/− PEC with or without Ag showed only background levels of RNI. There was also an inverse relationship between increased RNI production and lymphoid cell proliferation (Fig. 2B).

Reversal of the inhibition of proliferation by PEC with an inhibitor of iNOS

Cultures of spleen cells from MOG35–55-immunized IFNγR−/− mice were set up as before with specific Ag in the presence of various numbers of PEC from either IFNγR+/+ or IFNγR−/− mice. L-NMA was also added to some of the cultures with IFNγR+/+ PEC. As seen in Table V IFNγR+/+ PEC totally inhibited proliferation of the spleen cells whereas equal numbers of PEC from IFNγR−/− mice had no inhibitory effect. L-NMA restored the proliferative response to between 25 and 100% depending on the number of IFNγR+/+ PEC that had been added to the cultures. The inhibition seen when IFNγR−/− PEC are added 48 h after initiation of culture was also shown to be reversible by the addition of L-NMA when added at the same time as the PEC (data not shown).

Bone marrow chimeras between IFNγR+/+ and IFNγR−/− mice behave differently than either the IFNγR+/+ or IFNγR−/− mice with respect to recovery from disease

Having shown that IFNγR+/+ mice have cells, probably of the macrophage lineage, that are capable of shutting off IFNγR−/− lymphoid cell proliferation, we addressed the question as to where in the IFNγR−/− mice the down-regulation of disease was occurring. In other words, was it in the peripheral lymphoid organs or in the target tissue or in both? To examine this we generated chimeric mice as described in Materials and Methods. Mice thus constructed have IFNγR−/− (WT) cells in the periphery (i.e., the ability to respond to IFN-γ) and IFNγR−/− (KO cells) in the CNS (the inability to respond to IFN-γ), or KO cells in the periphery and WT cells in the CNS. EAE effector cells were generated in IFNγR−/− mice as before, and 5 × 107 effector cells from four groups of mice: IFNγR−/−, IFNγR−/−, and the two types of chimeras. Fig. 3 shows individual clinical scores for animals in the four groups. As seen previously, IFNγR−/− WT recipients developed severe EAE from which they recovered, whereas IFNγR−/− recipients developed severe disease and died. Interesting, both

<table>
<thead>
<tr>
<th>Mice</th>
<th>Treatment*</th>
<th>No. Sick/Total</th>
<th>Man Day of Onset</th>
<th>Mean Maximum Clinical Score</th>
<th>No. Recovering by Day 13**</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNγR+/+</td>
<td>None</td>
<td>4/4</td>
<td>6.5</td>
<td>4.0</td>
<td>4/4</td>
</tr>
<tr>
<td>IFNγR+/+</td>
<td>Control Ab</td>
<td>6/6</td>
<td>6.7</td>
<td>4.5</td>
<td>5/5</td>
</tr>
<tr>
<td>IFNγR−/−</td>
<td>Anti-CD8</td>
<td>6/6</td>
<td>6.7</td>
<td>4.1</td>
<td>5/5</td>
</tr>
</tbody>
</table>

* Mice received anti-CD8 or control Ab on days 6, 9, 12, and 15 in relation to passive transfer of 5 × 10⁷ effector cells from sensitized IFNγR−/− donors.
** Number of animals recovering by 2 or more clinical score points by day 13.

Table III. Inhibition of Ag driven proliferation of IFNγR−/− spleen cells by peritoneal exudate cells from IFNγR+/+ mice

<table>
<thead>
<tr>
<th>Cells</th>
<th>CPM*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Responders</strong></td>
<td><strong>Inhibitors</strong></td>
</tr>
<tr>
<td>2 × 10⁷ IFNγR−/−</td>
<td>None</td>
</tr>
<tr>
<td>None</td>
<td>IFNγR+/+ PEC</td>
</tr>
<tr>
<td>2 × 10⁷ IFNγR−/−</td>
<td>8 × 10⁶</td>
</tr>
<tr>
<td></td>
<td>4 × 10⁹</td>
</tr>
<tr>
<td></td>
<td>2 × 10⁹</td>
</tr>
<tr>
<td></td>
<td>1 × 10⁹</td>
</tr>
<tr>
<td>None</td>
<td>IFNγR−/− PEC</td>
</tr>
<tr>
<td>2 × 10⁷ IFNγR−/−</td>
<td>8 × 10⁶</td>
</tr>
<tr>
<td></td>
<td>4 × 10⁹</td>
</tr>
<tr>
<td></td>
<td>2 × 10⁹</td>
</tr>
<tr>
<td></td>
<td>1 × 10⁹</td>
</tr>
</tbody>
</table>

* Peritoneal exudate cells were added to cultures of antigen stimulated spleen cells form MOG35–55 sensitized IFNγR−/− mice at the initiation of culture.
** Cultures were incubated for 72h at which time [³H]thymidine was added and cultures harvested 20–24 h later. Mean cpm of eight wells.
types of chimeras developed severe disease from which they neither died nor recovered. Instead, they all developed a chronic course of disease with some animals showing modest recovery with subsequent relapse.

At the termination of the experiment at day 45, blood was taken from three animals in both chimera groups as well as from three in the WT (IFN-$\gamma R_1^+/1$) group. Sera were pooled (to obtain an adequate volume for assay) in each group, and RNI determinations were performed. The samples from the intact IFN-$\gamma R_1^+/1$ mice and the IFN-$\gamma R_2^+/2$ mice with IFN-$\gamma R_1^+/1$ reconstituted periphery had 7.2 and 8.7 $\mu$M RNI, respectively. There were no detectable RNI in the sample from the IFN-$\gamma R_1^+/1$ mice with an IFN-$\gamma R_2^+/2$ periphery.

Discussion

Mice lacking the ligand binding chain of the IFN-$\gamma$ receptor develop severe EAE and usually die following immunization with human MOG$_{35-55}$ peptide, whereas their WT counterparts possessing the receptor are resistant to EAE induction. This demonstrates that IFN-$\gamma$ is not essential as a proinflammatory molecule in EAE and suggests that it may in fact protect against disease. Evidence for the essential nature of IFN-$\gamma$ in down-regulation of disease is shown by the fact that IFN-$\gamma R^+/+$ mice die following severe disease induced by passive transfer of effector cells from MOG$_{35-55}$ immunized IFN-$\gamma R^+/+$ donor mice whereas IFN-$\gamma R^{+/+}$ mice (which differ only in their ability to respond to IFN-$\gamma$) develop equally severe passive disease but recover fully. We have suggested that recovery occurs as a result of cells in the IFN-$\gamma R^+/+$ mice responding to IFN-$\gamma$ being produced by the IFN-$\gamma R^{+/+}$ effector cells and producing a factor(s) which feeds back to the effector cell and shuts them off (8).

In the current investigation we have examined a number of possible cellular and molecular mechanisms that might contribute to the IFN-$\gamma$ down-regulation of disease. The Th1 and Th2 CD4$^+$ T cell populations cross-regulate one another because their respective cytokines act antagonistically (14), and therefore IL-4 and IL-10 both have been implicated in down-regulation of EAE (15–17). We treated IFN-$\gamma R^{+/+}$ mice with Abs to either IL-4 or IL-10 to determine whether this would alter their ability to recover from passively induced EAE. Neither treatment altered the course of disease in these animals, indicating that neither molecule appears to be the feedback effector shutting off disease. That treatment with an anti-cytokine Ab in such a model can alter the course of disease is seen by the fact that treating the IFN-$\gamma R^{+/+}$ mice with anti-IFN-$\gamma$ Ab resulted in augmented disease until the Ab was discontinued. It could of course be argued that one or both of these cytokines play a role in down-regulation and do so at the level of the target tissue and that the anti-cytokine Abs in these experiments did not cross the blood-brain barrier. However, this seems...
unlikely in light of the results obtained from the chimeric mice suggesting that down-regulation occurs at the level of both the target tissue and the periphery. If the cytokines in question were playing a role, then Ab treatment should have at the very least caused a more chronic disease in the IFN-γ R−/− mice by its ability to block cytokine function in the periphery.

From a cellular perspective we examined the role of CD8+ cells in down-regulation of disease and found that depleting IFNγR−/− mice of CD8+ cells did not impair their ability to recover from passively induced disease suggesting a negligible role for this cell type in regulation. We could not in fact demonstrate any involvement of lymphoid cells in recovery in this model. Transferring one spleen equivalent of lymphoid cells from IFNγR−/− mice into IFN-γ R−/− failed to alter the rapidly lethal outcome of passively induced disease in these animals.

In vitro studies, based on the observation that lymphoid cells from MOG35-55 immunized IFNγR−/− mice proliferate extensively when stimulated with specific Ag, suggested both a cellular and a molecular mechanism for down-regulation of EAE by IFN-γ. PEC harvested from thioglycollate stimulated IFNγR−/− mice and added to IFNγR−/− spleen cultures at the time of addition of Ag inhibited proliferation in a dose-dependent manner. Equal numbers of PEC from IFNγR−/− mice had no such effect, indicating that inhibition was not merely a matter of crowding. Similar inhibition was observed when PEC were added 48 h after the initiation of culture, demonstrating that ongoing proliferation as well as initiation of proliferation is sensitive to down-regulation.

Measurement of RNI in these mixed cultures revealed high levels in those containing PEC from IFNγR−/− mice plus MOG35-55. In the absence of specific Ag, no significant levels of RNI were produced nor were RNI detected when PEC from IFNγR−/− mice were added to spleen cells, with or without Ag. Finally, the inhibition of proliferation, both early and late, was totally reversible by the addition of L-NMA, an inhibitor of NO production via inhibition of iNOS. Taken together, these data provide strong support for a central role for IFN-γ-driven NO production by macrophages in down-regulating effector cell proliferation leading to recovery from disease.

NO mediates a great variety of biological functions. For example, it regulates vascular tone (18), causes platelet activation (19), acts as a neurotransmitter of nonadrenergic, noncholinergic innervation (20), is tumoricidal and microbicidal (21, 22), and plays the role of trans-synaptic retrograde messenger in the brain, thus participating in synaptic plasticity (23, 24). It has also been implicated in a number of immunopathologies including EAE and MS (25–27). In fact, until rather recently the majority of reports concerning

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<th>Table IV. Inhibition of Ag-driven proliferation of IFNγR−/− spleen cells by peritoneal exudate cells from IFNγR−/− mice</th>
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*Peritoneal exudate cells were added to cultures of washed antigen stimulated spleen cells from MOG35-55 sensitized IFNγR−/− mice 48 h after the initiation of culture.

*Cultures were incubated for a total of 96 h with [3H]thymidine being added 20–24 h prior to harvest. Man cpm of eight wells.

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<th>Table V. Inhibition of Ag-driven proliferation of IFNγR−/− spleen cells by PEC from IFNγR−/− mice and its reversal with an inhibitor of NOS</th>
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*PEC were added at the initiation of culture as was the iNOS inhibitor L-NMA.

*Cultures were incubated for a total of 96 h with [3H]thymidine being added 20–24 h prior to harvest. Man cpm of eight wells.
NO and autoimmunity have focused on NO as a contributor to the pathogenesis of disease mainly due to the observations that autoimmune diseases correlate with an overproduction of NO and that often administration of NO inhibitors prevented development of disease, e.g., glomerulonephritis (28), arthritis (29, 30), diabetes (31), and of course EAE (27, 32–34). Contrary evidence is now appearing, however, that despite its demonstrated role in certain tissue pathologies, NO plays a role in down-regulation of adaptive immune responses (35–38). For example, we have shown that in vivo inhibition of iNOS not only exacerbates EAE in the Lewis rat but also converts the relatively resistant PVG rat to high susceptibility (38). Susceptibility in the PVG rats correlated with the reduction of serum NO levels following immunization from 3-fold greater than the Lewis rats to levels similar to or below those found in the Lewis. Furthermore, inhibition of iNOS in vivo led to a 3-fold increase in Ag-specific proliferation by lymphocytes from immunized PVG rats. These data indicate that NO might regulate the development of autoimmune reactivity by limiting proliferation of autoreactive lymphocytes.

Stimuli that induce NO production via iNOS include cytokines and microbial products (22), and the cells that respond to these stimuli are widely distributed and include peripheral macrophages as well as resident CNS cells such as microglia and astrocytes (39). In the context of the present work, the cytokine IFN-γ is of central importance, and this cytokine has been shown to be the major inducer of iNOS (40). IFN-γ induces iNOS in peripheral macrophages as well as cells in the CNS. With respect to CNS cells, IFN-γ is sufficient to induce NO release from microglial cells but astrocytes are provoked into NO production only by a combination of IFN-γ and TNF-α (39).

We have suggested previously (8) that recovery of IFNγR+/− mice from passively induced MOG35–55 EAE is driven by IFN-γ and that this happens as a result of some cell type(s) in the IFNγR+/− mice responding to the high levels of IFN-γ being produced by the transferred IFNγR−/− effector cells with the production of a factor(s) that then feeds back and silences the effector cells. We now suggest that the factor responsible for down-regulation of disease is NO, and that the cells producing NO under the upstream influence of IFN-γ are most likely macrophages in the periphery, either intact WT or chimera mice responding to the high levels of IFN-γ being produced by the transferred IFNγR−/− effector cells with the production of a factor(s) that then feeds back and silences the effector cells. NO is of central importance, and this cytokine has been shown to be the major inducer of iNOS (40). IFN-γ induces iNOS in peripheral macrophages as well as cells in the CNS. With respect to CNS cells, IFN-γ is sufficient to induce NO release from microglial cells but astrocytes are provoked into NO production only by a combination of IFN-γ and TNF-α (39).

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It is interesting to note that both types of chimeras developed a moderately severe chronic disease from which none had recovered at the termination (for ethical reasons) of the experiment 45 days after initiation. Chronicity could be explained on the basis that effector cells in an IFNγR−/− periphery are being down-regulated and/or eliminated by NO production in the periphery, but the effector cells that have entered the IFNγR−/− CNS, where the regulating signal cannot be made, continue to orchestrate disease. Conversely, effector cells in an IFNγR−/− periphery are continually proliferating, recirculating, and entering the IFNγR−/− CNS where they sustain pathology but are also continually eliminated because of the ability of IFNγR−/− cells to respond to IFN-γ with NO production. With respect to the cell type making NO, in this latter case we would suggest that because the effectors are making large quantities of both IFN-γ and TNF (8) that both astrocytes and microglia are making NO (39). This is currently under investigation.

There are a number of possible mechanisms by which NO down-regulates the immune response. Certainly, as demonstrated here and elsewhere (41), NO inhibits lymphocyte proliferation, and the suggestion has been made that this is by preventing the activation of Janus kinases (42), molecules critical in cytokine receptor signal transduction (43). More recent studies have described a NO-induced bias of cytokine gene expression in which NO preferentially down-regulates Th1-type cytokines specifically while up-regulating Th2-associated molecules resulting in a Th2 bias (44). Such bias could shut off the Th1 pathology of EAE. A similar regulatory effect might be exerted by NO at the level of Ag presentation, as it has been shown that NO induces transcription of the IL-12 p40 gene in macrophages but not the p35 gene. Because the p40 homodimer acts as an antagonist for IL-12 (45), this might lead to less Th1 reactivity in the presence of NO.

Expression of adhesion molecules has been found to be down-regulated by NO and hence lymphocyte migration into the CNS might be impaired and contribute to down-regulation of the disease process. Finally, NO can, depending on concentration, be an inducer or suppressor of apoptosis and/or necrosis in a number of different systems (46). Several studies have indicated that elimination of inflammatory T cells and macrophages from the CNS during EAE might be the result of apoptosis (47–49). There is also recent evidence that Th1 cells are more prone to apoptosis than are Th2 cells (50), which would again be beneficial in regulating EAE, which is essentially a Th1-mediated disease.

In the present system it is likely that NO acts at any number of these levels to down-regulate CNS inflammation. The observation that, at least in vitro, rapidly proliferating cells can be shut off when NO-producing PEC are added at the height of proliferation suggests an important role for cell death—either apoptosis or necrosis—in regulation. Because regulation is occurring at both the systemic level and at the level of the target tissue, it will be of interest to determine whether cell death of effector cells is occurring by the same mechanism in both places.

We have demonstrated that the normally considered proinflammatory cytokine IFN-γ is, in the context of MOG-induced EAE, essential in down-regulation of the disease and that reactivity to IFN-γ in the target tissue itself, i.e., the CNS, as well as the periphery plays a critical role in this down-regulation. The beneficial role of IFN-γ in the CNS is in contrast to recent work described by Horwitz et al. (51). These investigators expressed IFN-γ in the CNS of mice under the myelin basic protein (MBP) promoter such that the cytokine was produced by oligodendrocytes. They found demyelination in a proportion of their mice that was both age and gender related. The effect of any cytokine is undoubtedly dose, time and site dependent and these parameters in the work of Horwitz et al. are vastly different from what would be found for IFN-γ produced during an inflammation of the CNS where the cytokine is produced mainly by T cells under control of the TCR, i.e., upon Ag recognition. The prolonged over-expression of IFN-γ in the CNS with subsequent production of demyelination does not preclude the possibility of IFN-γ in more physiological circumstances (an inflammatory episode) contributing to down regulation of inflammation.

With respect to mechanism of action of IFN-γ, we present in vitro data which show clearly that reactivity to IFN-γ results in the production of large amounts of RNI by peripheral mononuclear cells with subsequent inhibition of effector cell proliferation. We suggest that this IFN-γ-driven NO production in the periphery, and by inference in the CNS as well, is responsible for limiting disease. In light of the robust nature of the results presented here, we suggest that perhaps the data on the therapeutic use of IFN-γ in the clinical setting of the human CNS inflammatory disease MS should be re-examined with an eye to the possible retrait of IFN-γ or perhaps another inducer of the downstream molecule NO, in MS.

Acknowledgments

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References