Lethal Autoimmune Myocarditis in Interferon-γ Receptor–Deficient Mice
Enhanced Disease Severity by Impaired Inducible Nitric Oxide Synthase Induction

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Background—Interferon-γ (IFN-γ) is an essential cytokine in the regulation of inflammatory responses in autoimmune diseases. Little is known about its role in inflammatory heart disease.

Methods and Results—We showed that IFN-γ receptor–deficient mice (IFN-γR–/–) on a BALB/c background immunized with a peptide derived from cardiac α-myosin heavy chain develop severe myocarditis with high mortality. Although myocarditis subsided in wild-type mice after 3 weeks, IFN-γR–/– mice showed persistent disease. The persistent inflammation was accompanied by vigorous in vitro CD4 T-cell responses and impaired inducible nitric oxide synthase expression, together with evidence of impaired nitric oxide production in IFN-γR–/– hearts. Treatment of wild-type mice with the nitric oxide synthetase inhibitor N-nitro-L-arginine-methyl-ester enhanced in vitro CD4 T-cell proliferation and prevented healing of myocarditis.

Conclusions—Our data provide evidence that IFN-γ protects mice from lethal autoimmune myocarditis by inducing the expression of inducible nitric oxide synthase followed by the downregulation of T-cell responses. (Circulation. 2001; 103:18-21.)

Key Words: interferons ■ mice ■ autoimmunity ■ myocarditis ■ myosin ■ nitric oxide synthase

Cardiomyopathy may result from a long-term autoimmune reaction to cardiac tissue.1 Experimental autoimmune myocarditis can be induced in mice of the BALB/c strain by immunization with α-myosin-heavy-chain–derived peptides, and it is mediated mainly by CD4+ T-helper cells.2,3 Inflammatory cytokines like interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) are critically involved in the pathogenesis of various CD4+ T-cell–mediated autoimmune diseases. Mice lacking the receptor for TNF-α are protected from autoimmune myocarditis,4 but the role of IFN-γ in autoimmune myocarditis is, as yet, unknown.

Nitric oxide (NO) is an immune regulator and an effector molecule mediating tissue injury.5,6 Its formation is catalyzed by NO synthases (NOS). NOS are constitutively expressed in neuronal (nNOS) and endothelial (eNOS) cells, but they also exist as inducible isoforms (iNOS). IFN-γ and TNF-α can trigger iNOS expression in macrophages, cardiac endothelial cells, and cardiac myocytes.5,6 Enhanced iNOS expression has been found in the myocardium of patients with dilative cardiomyopathy.7 However, the role of iNOS in the pathogenesis of murine autoimmune myocarditis is not clear.6

To understand the role of IFN-γ in experimental autoimmune myocarditis, we compared disease prevalence, severity, immune responses, and iNOS expression in IFN-γ receptor–deficient (IFN-γR–/–) mice with wild-type (WT) control mice.

Methods

Mice

Breeding pairs of IFN-γR–/– mutant mice8 backcrossed for >10 generations on BALB/c background were a generous gift from Prof Jacques Louis (Institut de Biochimie, Lausanne, Switzerland). BALB/c mice were purchased from Biological Research Laboratories Ltd, Füllinsdorf, Switzerland. Male mice were immunized at 8 to 10 weeks of age. All experiments involving mice were performed in accordance with Swiss federal legislation and were approved by the local authorities.

Induction of Experimental Autoimmune Myocarditis

Mice were immunized with a heart muscle–specific, α-myosin-heavy-chain–derived peptide (myhα 614 to 634: Ac-SLKLMLFSTYASADTGDSKGGKGKGK KG-OH; designated Mα30) together with complete Freund’s adjuvant. Control mice received complete Freund’s adjuvant only.3

Histopathology and Immunohistochemistry

Hematoxylin- and eosin-stained heart sections were evaluated on a semiquantitative scale using severity scores from 0 to 4 (0, no...
inflammatory infiltrates; 1, small foci of inflammatory cells between myocytes; 2, larger foci of inflammatory cells; 3, >10% of a cross-section involved; and 4, >30% of a cross-section involved). Immunohistochemical staining was performed with an anti-iNOS antibody (1:200 rabbit polyclonal, Cat No N32030, Transduction Laboratories) and an anti-nitrotyrosine antibody (1:100 rabbit polyclonal, Cat No 06 to 284, Upstate) according to standard procedures.

**Proliferation Assays**

CD4+ T-cells were purified from splenocytes by depletion with commercially available specific antibodies coupled to magnetic beads (MACS, Miltenyi Biotech GmbH). CD4 T-cells and irradiated (2000 rad) syngenic antigen-presenting cells were pulsed with 50 μg/mL MA30 or 100 μg/mL ovalbumin as unspecific control antigen. Proliferative responses were assessed by measuring [3H]methylthymidine incorporation after 72 hours of culture in serum-free medium with 1 mmol/L of the NOS inhibitor N-nitro-L-arginine-methyl-ester (L-NAME; Sigma, No N5751) or the biologically inactive D-NAME. To generate NO in cell cultures, we used the NO donor S-nitroso-N-acetyl-penicillamine (SNAP; Sigma, No. N3398) at 0.5 mmol/L.

**In Vivo Blocking of NO Production**

NO production was blocked in vivo by treating mice intraperitoneally with 10 mg/kg body weight of L-NAME from day 0 until they were killed. Control mice received phosphate-buffered saline only.

**Statistics**

The Mann-Whitney U test was used to evaluate severity scores and heart weights. Proliferative responses were compared using 2-way ANOVA followed by the unpaired t-test with Bonferroni’s correction. Dichotomous data were analyzed by Fisher’s exact test. Differences for which P < 0.05 are indicated in the tables and figures.

**Results**

Disease prevalence and severity of autoimmune myocarditis were higher in IFN-γR−/− mice than WT controls (Table 1). Hearts from IFN-γR−/− mice were enlarged and entirely infiltrated with histiocytes, lymphocytes, and numerous neu-

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**TABLE 1. Myocarditis Prevalence and Severity Scores in WT, IFN-γR−/−, and L-NAME–Treated WT Mice 21 Days After the First Immunization**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment</th>
<th>Myocarditis Prevalence</th>
<th>Severity Score, Median (Range)</th>
<th>Heart Weight, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>None</td>
<td>16 (25)*</td>
<td>1.5 (1–4)†</td>
<td>0.15±0.03 (n=7)‡</td>
</tr>
<tr>
<td>IFN-γR−/−</td>
<td>None</td>
<td>10 (10)*</td>
<td>3.0 (2–4)†</td>
<td>0.25±0.04 (n=6)‡</td>
</tr>
<tr>
<td>WT</td>
<td>L-NAME</td>
<td>7 (9)</td>
<td>2.0 (1–3)</td>
<td>0.18±0.02 (n=9)</td>
</tr>
</tbody>
</table>

*P=0.0363, †P=0.0011, ‡P=0.0027 for WT vs IFN-γR−/−.

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**TABLE 2. Myocarditis Prevalence in WT, L-NAME–Treated WT, and Surviving IFN-γR−/− Mice at Various Days After the First Immunization**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Day 28</th>
<th>Day 35</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>None</td>
<td>2 (5)</td>
<td>16 (25)*</td>
<td>1 (9)†</td>
<td>0 (15)‡§</td>
</tr>
<tr>
<td>IFN-γR−/−</td>
<td>None</td>
<td>1 (5)</td>
<td>10 (10)*</td>
<td>5 (5)†</td>
<td>3 (3)‡</td>
</tr>
<tr>
<td>WT</td>
<td>L-NAME</td>
<td>...</td>
<td>7 (9)</td>
<td>...</td>
<td>3 (3)§</td>
</tr>
</tbody>
</table>

*P=0.0363, †P=0.0030, and ‡P=0.0012 for WT vs IFN-γR−/− mice. §P=0.0012 for WT vs L-NAME–treated WT.

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**Figure 1.** Histological sections of myocardium in immunized WT and IFN-γR−/− mice on day 21. Inflammatory infiltrations in WT (a and b) and IFN-γR−/− mice (d and e). iNOS expression is distinctly localized to inflammatory infiltrate in WT hearts (c) and is totally absent in myocardium of IFN-γR−/− mice (f). Hematoxylin and eosin and anti-iNOS staining was used; final magnification was 13× (a), 9× (d), and 60× (b, c, e, and f).
trophils and eosinophils (Figure 1). In addition, rare giant cells were observed. Although WT mice almost completely recovered from disease, most M₃₀ immunized IFN-$\gamma$R$^{-/-}$ mice died from severe myocarditis within 35 days (Table 2 and Figure 2a). IFN-$\gamma$R$^{-/-}$ control mice immunized with complete Freund’s adjuvant only had neither myocarditis nor were they different from WT mice with respect to mortality.

Given that experimental autoimmune myocarditis is mainly a CD4 T-cell–mediated disease, 1,2 we compared the in vitro CD4$^+$ T-helper cell responses of IFN-$\gamma$R$^{-/-}$ and WT mice. IFN-$\gamma$R$^{-/-}$ CD4$^+$ T-cells showed higher proliferation indices than WT CD4 T-cells (Figure 2b). These proliferative responses persisted in CD4 T-cells isolated from IFN-$\gamma$R$^{-/-}$ mice for up to 35 days after immunization, whereas proliferation of WT CD4$^+$ T-cells decreased after 3 weeks. This suggests that an ongoing reduction in the number of antigen-specific CD4$^+$ T-cells parallels recovery from disease in WT mice.

Given that IFN-$\gamma$ is a potent inducer of iNOS expression and that NO reversibly impairs T-cell proliferation, 9 it may be essential to a mechanism eliminating activated CD4 T-cells by apoptosis.10 Our data suggest that IFN-$\gamma$ downregulates CD4 T-cells in autoimmune myocarditis. This mechanism is probably mediated by NO, as documented by the impaired iNOS expression in IFN-$\gamma$R$^{-/-}$ mice and by prior data published by our group. 9 The observation that WT mice treated with the NO inhibitor L-NAME develop prolonged myocarditis and vigorous CD4 T-cell responses support this hypothesis. However, L-NAME treatment did not significantly increase myocarditis severity. This could be explained by the fact that the L-NAME treatment used in our protocol does not completely inhibit the generation of NO in vivo, despite the fact that iNOS cannot be detected by immunohistochemistry in L-NAME–treated mice (data not shown). To specifically assess the in vivo role of iNOS in myocarditis and T-cell regulation, further experiments with iNOS-deficient mice should be performed. Our findings suggest that IFN-$\gamma$ and NO should be evaluated as therapeutic agents in established myocarditis. In addition, IFN-$\gamma$R$^{-/-}$ mice may be a useful model for the elucidation of inflammation mechanisms in rapidly progressive myocarditis.

Figure 2. a, Survival of WT and IFN-$\gamma$R$^{-/-}$ mice 35 days after first immunization. b, CD4$^+$ T-cell responses of IFN-$\gamma$R$^{-/-}$ and WT mice are shown various days after first immunization. c, Proliferation of CD4$^+$ T-cells from IFN-$\gamma$R$^{-/-}$ L-NAME–treated WT, and WT mice at day 21 in presence of NOS inhibitor L-NAME (1 mmol/L), biologically inactive D-NAME (1 mmol/L), or NO-generating agent SNAP. Stimulation indices were calculated as (counts per minute [cpm] in wells with antigen)/cpm in wells without antigen). Background values were between 600 and 1500 cpm. Each bar represents mean±SD of 5 to 6 mice.
Acknowledgments
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References
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