Interleukin-12 Receptor/STAT4 Signaling Is Required for the Development of Autoimmune Myocarditis in Mice by an Interferon-γ–Independent Pathway

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Background—Interleukin (IL)-12 exerts a potent proinflammatory effect by stimulating T-helper (Th) 1 responses. This effect is believed to be mediated primarily through the activation of STAT4 and subsequent production of interferon (IFN)-γ.

Methods and Results—We examined the role of IL-12 receptor (IL-12R) signaling in the development of murine experimental autoimmune myocarditis (EAM) induced by cardiac myosin immunization. Both IL-12Rβ1–deficient mice and STAT4-deficient mice were resistant to the induction of myocarditis. Treatment with exogenous IL-12 exacerbated disease. We questioned whether IFN-γ is required for the disease-promoting activity of IL-12. On the contrary, we found that IFN-γ suppresses EAM. Lack of IFN-γ due to either depletion with an antibody or a genetic deficiency exacerbated myocarditis. Spleens from IFN-γ–deficient mice immunized with cardiac myosin showed increased cellularity; greater numbers of CD3+, CD4+, CD8+, and IL-2–producing cells; and heightened ability to produce cytokines on stimulation in vitro. Treatment of mice with recombinant IFN-γ suppressed the development of myocarditis.

Conclusions—IL-12/IL-12R/STAT4 signaling promotes the development of EAM. In contrast, IFN-γ plays a protective role. The disease-limiting effects of IFN-γ might be explained by its ability to control the expansion of activated T lymphocytes. (Circulation. 2001;104:3145-3151.)

Key Words: immunology ■ interleukins ■ myocarditis ■ myosin ■ inflammation

Autoimmunity plays an important role in human myocarditis and contributes to the progression to cardiomyopathy and heart failure.1 Autoimmune response to cardiac myosin (CM) is associated with progressive forms of myocarditis.2 To explore the mechanisms of the immune system–mediated damage to the heart in this disease, we previously established a murine model of experimental autoimmune myocarditis (EAM) that is induced by immunization with CM.3,4 Interleukin (IL)-12 is produced mainly by antigen-presenting cells.5 IL-12 exerts potent proinflammatory effects, protects against intracellular infections, induces T-helper (Th)1 immune responses, and stimulates production of interferon (IFN)-γ by natural killer and T cells. The IL-12 receptor (IL-12R), composed of IL-12Rβ1 and IL-12Rβ2 chains, mediates signal transduction, which involves the recruitment of Janus family tyrosine kinase 2 and signal transducer and activator of transcription (STAT)4,6–8 The proinflammatory effects of IL-12 are believed to be mediated primarily by IFN-γ.5 The STAT4 pathway is important for IFN-γ induction through the direct interaction of STAT4 with the IFN-γ promoter.9 IFN-γ stimulates Th1 T-cell development, activates macrophages, induces major histocompatibility complex class I and II expression, promotes delayed-type hypersensitivity reactions, recruits Th1 T cells to the site of inflammation, plays an important role in clearing intracellular bacteria and intracellular parasites, and exhibits antiviral activity.10 In the present report, we examine the role of IL-12 and IFN-γ in EAM. We find that IL-12 signaling through IL-12Rβ1 and STAT4 promotes EAM. We then show that IFN-γ has a protective role in EAM and is not responsible for the disease-promoting action of IL-12.

Methods

Mice

All mice were on a BALB/c background and were obtained from the Jackson Laboratory, Bar Harbor, Me. The animal work was approved by the Animal Care and Use Committee of Johns Hopkins

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University. Invasive procedures were performed under anesthesia by inhalation of methoxyflurane.

Induction of EAM and Histopathology
On days 0 and 7, 5- to 7-week-old female mice received subcutaneous injections of 200 µg of purified murine CM emulsified in complete Freund’s adjuvant (Sigma). On day 0, mice received 500 ng IP of pertussis toxin (List Biological Laboratories). Mouse hearts were scored grossly on the basis of the extent of white discoloration by 2 independent investigators. Hearts were then fixed in 10% phosphate-buffered formalin and embedded in paraffin. Sections 5 µm thick were cut from base to apex and stained with hematoxylin and eosin. Five sections from each heart were examined by 2 independent investigators in a blinded fashion. Histological assessment of myocarditis was performed on a scale from 0 to 5 based on the estimated percentage of the heart section affected by infiltration with the aid of a background grid: grade 0, no disease; grade 1, up to 10% of the heart section; grade 2, 11% to 30%; grade 3, 31% to 50%; grade 4, 51% to 90%; and grade 5, 90% to 100%.

Cytokine Production
Splenocytes were collected on day 21; red blood cells were lysed, and live cells were counted by trypan blue exclusion. The proportions of dead cells did not vary in different mouse groups. Splenocytes were then cultured for 48 hours at an initial cell density of 5x10⁶ cells/mL in complete RPMI 1640 medium (Life Technology) in the presence of media alone or with different concentrations of CM or concanavalin A (Sigma). Levels of cytokines in the splenocyte culture supernatants were measured with Quantikine murine cytokine ELISA kits (R&D Systems).

Flow Cytometry
On day 21, splenocytes were collected and red blood cells were lysed. Cells were stimulated for 4 hours with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) (Life Technology) and 1 µmol/L ionomycin (Sigma). Monensin was added for the last 2 hours of culture. After centrifugation with Lympholyte (Cedarlane), live cells were collected, washed in staining buffer (1% FCS, 0.1% sodium azide in PBS), and preblocked with anti-FcRIII/II mAb (2.4G2) for 5 minutes. Cells were then stained for 30 minutes with Cy-Chrome-labeled anti-CD4 (clone H129.19), anti-CD8a (clone 536.7), or anti-CD3e (clone 1452C11). The cells were fixed overnight with Cytofix/Cytoperm buffer, washed with Perm/Wash buffer, stained for 30 minutes with phycoerythrine-labeled anti-IL-2 (clone S4B6), washed, and resuspended in staining buffer. The specificity of the cytokine staining was confirmed by preincubation with unlabeled anti-IL-2 (clone S4B6). For the surface staining only, after red blood cells had been lysed, splenocytes were washed, preblocked with anti-FcγRIII/II mAb, stained for 30 minutes, and then fixed. All procedures were performed at 4°C. All reagents were purchased from BD PharMingen unless specified otherwise. Cell fluorescence was measured with a FACScan flow cytometer (BD Biosciences).

Statistical Analyses
The Mann-Whitney U test was used to compare EAM severity scores between treatment groups. Normally distributed data were analyzed with the 2-tailed Student’s t test. Values of P<0.05 were considered statistically significant.

Results
To examine the role of IL-12 signaling in EAM, IL-12Rβ1−/− knockout (KO)11 mice and wild type (WT) BALB/c mice were immunized with CM. IL-12Rβ1−/−KO mice were completely resistant to the development of myocarditis (Figure 1, a and b). Activation of STAT4 is an important signaling pathway in IL-12–induced responses. To examine whether the STAT4 pathway is necessary for the IL-12 receptor–
mediated development of EAM. STAT4-KO12 mice were immunized with CM. STAT4-KO mice were also resistant to the induction of EAM (Figure 1, a and b). One STAT4-KO mouse, however, did develop a moderately severe myocardial infiltrate that was rich in eosinophils, indicating that the requirement for STAT4 is not absolute. To confirm the role of IL-12 in mediating the disease-promoting effects of IL-12R signaling, we treated BALB/c mice with rIL-12 during the course of EAM induction. IL-12 treatment exacerbated disease by gross examination of the hearts (Figure 1c) and increased such commonly used measures of myocarditis severity as heart weight and heart weight-to-body weight ratio (Figure 1, e and f). IL-12 treatment, however, did not significantly change the percentage of myocardial infiltration as assessed by histological examination (Figure 1d).

On day 21, both IL-12Rβ1 and STAT4-KO mice had lower spleen cellularity than the WT mice (Table); STAT4-KO mice had a more pronounced decrease in the number of splenocytes. We examined the patterns of cytokine production by splenocytes cultured in the presence of either CM or a T-cell mitogen, concanavalin A. Production of IFN-γ, IL-1β, and IL-6 were consistently reduced in both IL-12Rβ1–KO and STAT4-KO groups in response to either CM or concanavalin A (Figure 2, a through f). There was an increase in IL-2 production in IL-12Rβ1–KO mice (Figure 2, a and c); however, such an increase was not observed in the STAT4-KO group (Figure 2, d and f). As assessed by flow cytometry, the frequency of IL-2– and IFN-γ–producing T cells in IL-12Rβ1–KO mice after stimulation with ionomycin and PMA was not different from that in WT mice (data not shown). IL-12 treatment resulted in a mild suppression of a number of cytokines, especially those of a Th2 type (IL-4, IL-5, IL-13).

**Table**  
Spleen Cellularity (×10⁶)  

<table>
<thead>
<tr>
<th>Group</th>
<th>IL-12Rβ1</th>
<th>STAT4</th>
<th>Unimmunized</th>
<th>Immunized</th>
</tr>
</thead>
<tbody>
<tr>
<td>KO</td>
<td>7.8±0.7</td>
<td>6.0±0.7*</td>
<td>12.7±0.1*</td>
<td>24.6±2.4†</td>
</tr>
<tr>
<td>WT</td>
<td>8.8±0.7</td>
<td>9.6±0.4</td>
<td>4.7±0.4</td>
<td>10.9±1.1</td>
</tr>
</tbody>
</table>

Data are mean±SEM.  
*P<0.01, †P<0.001 vs WT group.

**Figure 2.** Effects of IL-12 signaling on cytokine production by splenocytes. Splenocytes were collected from IL-12Rβ1–KO mice (a through c), STAT4-KO mice (d through f), mice treated with rIL-12 (g through i), and corresponding controls on day 21 and cultured for 48 hours in presence of either 10 μg/mL of CM (a, d, and g) or concanavalin A (b, c, e, f, h, and i). Cytokine levels in supernatants were measured by ELISA. Data are mean±SEM for ≥4 individual mice per group. *P<0.05, **P<0.001 vs controls. ND, not detectable.
IL-13, IL-10, and IL-6), reaching significance for IL-4 and IL-10 responses to concanavalin A (Figure 2, g through i).

Because the IL-12R/STAT4 pathway leads to production of IFN-\(\gamma\),13 we explored the role of IFN-\(\gamma\) in our model by blocking IFN-\(\gamma\) with an mAb in both A/J and BALB/c mice. Treatment with anti–IFN-\(\gamma\) exacerbated disease in both strains as assessed by gross (Figure 3, a and c) and histological (Figure 3, b and d, and Figure 4b) examination and significantly increased heart weights and heart weight-to-body weight ratio compared with the controls (data not shown). Anti–IFN-\(\gamma\)-treated A/J mice had more prominent cardiomyopathy on day 29, although myocardial infiltration was less intense than on day 21. Our findings suggested that IL-12 and IFN-\(\gamma\) have opposing roles in EAM. To ensure that such a dichotomy was not simply due to the use of 2 different systems (knockout model versus mAb), we immunized IFN-\(\gamma\)-KO mice on a BALB/c background. Consistent with the results obtained in anti–IFN-\(\gamma\) experiments, IFN-\(\gamma\)-KO mice developed more severe pathology than WT mice (Figure 3, e and f, and Figure 4a).

To better understand how the disease can be enhanced in the absence of IFN-\(\gamma\), we studied cytokine responses by culturing splenocytes in the presence of CM. On anti–IFN-\(\gamma\) treatment, we observed significantly greater production of almost all cytokines tested, including IFN-\(\gamma\), in both A/J (Figure 5a) and BALB/c mice (Figure 5b). Similar increases in cytokine production were observed in the culture superna-

tants of splenocytes from IFN-\(\gamma\)-KO mice. Naturally, IFN-\(\gamma\) was undetectable in this setting (data not shown). Both immunized and unimmunized IFN-\(\gamma\)-KO mice had an increased spleen cellularity compared with their age-matched WT counterparts (Table). Flow cytometric analysis showed that spleens from IFN-\(\gamma\)-KO mice had greater percentages of

![Figure 3](image-url) Exacerbation of EAM in absence of IFN-\(\gamma\). Mice were immunized with CM and euthanized at indicated days. EAM in anti–IFN-\(\gamma\) or isotype control treated A/J (a and b) and BALB/c (c and d) mice. e and f, EAM in IFN-\(\gamma\)-KO and WT BALB/c mice. Myocarditis scores were assessed by gross (a, c, and e) and histological (b, d, and f) examination.

![Figure 4](image-url) Lack of IFN-\(\gamma\) facilitates development of cardiomyopathy in EAM. Mice were euthanized on day 21. a, Gross hearts from immunized IFN-\(\gamma\)-KO mice vs those from immunized and nonimmunized WT BALB/c mice. b, Heart from immunized A/J mouse treated with anti-IFN-\(\gamma\) mAb (top) vs normal A/J heart (bottom). Hematoxylin and eosin stain. Magnification \(\times 5\).
CD3+ T lymphocytes, with both CD4+ and CD8+ subpopulations being increased (Figure 6a). In contrast, the percentage of B220+ B cells was significantly decreased in IFN-γ–KO compared with WT mice (data not shown). A higher frequency of T lymphocytes, especially CD4+ cells, from IFN-γ–KO mice produced IL-2 on stimulation with ionomycin and PMA, indicating a more activated state (Figure 6b).

Next, we examined the effects of the administration of rIFN-γ during the course of disease induction. Treatment with rIFN-γ suppressed disease, with very few mice developing mild to moderate myocarditis (Figure 7), and significantly decreased the in vitro production of TNF-α in response to CM (Figure 5c).

**Discussion**

Here, we describe the dependence of EAM on IL-12R signaling. Disease-promoting effects of IL-12R ligation require signaling through STAT4. Yet IFN-γ, a cytokine inducible by activated STAT4, suppresses disease development. EAM was exacerbated when IFN-γ was depleted due to either IFN-γ blockade with an mAb or a genetic deficiency. IFN-γ deficiency led to an exacerbated disease phenotype in both A/J and BALB/c mice, emphasizing the generalizability of the phenomenon. Furthermore, administration of rIFN-γ reduced both prevalence and severity of disease. Lack of IFN-γ led to the expansion of activated T cells, providing a plausible disease-promoting mechanism.
The role of IL-12R signaling in EAM has not previously been elucidated. One study suggested a pathogenic role for IL-12 in a rat model of EAM. IL-12 is a major cytokine that drives Th1 responses and is therefore considered important in initiating organ-specific autoimmune diseases, which are believed to be Th1-driven. In the case of EAM, however, we have shown that a Th2 component is important, in both A/J and BALB/c mice (data not shown). In this regard, it is intriguing that a major Th1 signaling pathway, IL-12/IL-12R/STAT4, promotes the disease. Interestingly, in some systems, IL-12 has been shown to promote Th2-mediated processes. It is still unclear whether human myocarditis is a Th1- or a Th2-driven disease. It is likely that there is a spectrum of forms ranging from a more Th2-like eosinophilic and, possibly, giant cell myocarditis to a more Th1-like lymphocytic myocarditis.

It was recently shown that a novel cytokine, IL-23, also signals through IL-12Rβ1 and STAT4 and shares many biological effects with IL-12, including IFN-γ induction. To distinguish between the roles of IL-12 and IL-23 in EAM, we treated mice with rIL-12 and confirmed its pathogenic role in EAM.

We have demonstrated that IL-12R signaling does not require IFN-γ to promote disease and that IFN-γ has suppressive effects on EAM. Consistent with our observations, Smith et al demonstrated that anti–IFN-γ–blocking antibody exacerbates EAM, although the authors, at that time, could not explain their finding. It was recently shown that EAM is exacerbated in IFN-γ receptor–KO mice. Using more direct approaches, we clearly provide evidence for the suppressive role of this cytokine in EAM. IFN-γ–KO mice have increased spleen cellularity and increased percentages of both CD4+ and CD8+ T cells. These lymphocytes exhibit a more activated phenotype, as judged by the increased production of cytokines and higher cell surface expression of CD44 [data not shown]. These observations are in accord with findings by others that IFN-γ controls the expansion of activated CD4+ and CD8+ T cells. In addition to its disease-limiting effects in a CM-induced model of myocarditis, IFN-γ has been shown to be protective in a number of virus-induced models. Intranasal administration of IFN-γ suppresses viral replication and improves prognosis of murine myocarditis induced by encephalomyocarditis virus. Transgenic expression of IFN-γ in the pancreas protects mice from coxsackievirus B3–induced myocarditis. The majority of cases of autoimmune myocarditis in humans are believed to be due to coxsackievirus infection and consist of viral and autoimmune phases that may be temporally separated or overlapping. Immunosuppressive treatments of myocarditis have produced mixed results, possibly because they lead to the suppression of both antiviral (protective) and self-aggressive (deleterious) immunity. Therefore, the agents that are beneficial regardless of the stage of disease should be explored for the optimal therapeutic intervention. On the basis of the results from both virus- and CM-induced models of myocarditis, IFN-γ may be an appropriate candidate. Miric et al showed the efficacy of IFN-α treatment in patients with biopsy-proven myocarditis and dilated cardiomyopathy.

A number of studies have shown that the IL-12/STAT4 pathway is required for IFN-γ production. Carter et al, however, found that unlike CD4+ T cells, CD8+ T cells produce IFN-γ on engagement of their T-cell receptor for antigen in a STAT4-independent fashion. It has been shown that CD8+ T cells inhibit antigen-induced responses of CD4+ T cells and that this suppression is mediated by IFN-γ produced by CD8+ T cells. In our hands, when we stimulated peripheral blood cells, lymph node cells, or splenocytes from CM-immunized mice with ionomycin and PMA, CD8+ T cells represented the major source of IFN-γ (M.A., unpublished data, 2001). On the basis of these observations, one might speculate that CD8+ T cells play a protective role in EAM through the secretion of IFN-γ. In support of this conjecture, it has been demonstrated that CD8 deficiency in mice leads to exacerbation of both CM-induced and coxsackievirus B3–induced myocarditis.

We show that IL-12R signaling plays a pathogenic role in EAM by an IFN-γ–independent mechanism. Our cytokine data suggest that IL-12 may act via upregulation of proinflammatory cytokines, such as IL-1β and IL-6, levels of which were decreased in the absence of IL-12R signaling. The pathogenic role of IL-1β has been demonstrated in coxsackievirus B3–induced myocarditis.

In summary, we demonstrate that the 2 major components of a Th1 response, IL-12/IL-12R/STAT4 signaling and IFN-γ, play opposing roles in the development of EAM. Further studies on human myocarditis are necessary to determine whether these findings can be translated to the clinical setting.

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References


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