Interleukin-6–Deficient Mice Resist Development of Autoimmune Myocarditis Associated With Impaired Uregulation of Complement C3

Urs Eriksson, MD; Michael O. Kurrer, MD; Nicole Schmitz; Stephan C. Marsch, MD; Adriano Fontana, MD; Hans-Pietro Eugster, PhD; Manfred Kopf, PhD

Background—Interleukin (IL)-6 regulates various aspects of the immune response. In the context of heart diseases, it has been recognized as a prognostic factor for dilated cardiomyopathy, which often results from myocarditis.

Methods and Results—Using IL-6–deficient mice, we studied the role of IL-6 in a model of autoimmune myocarditis resulting from immunization with a peptide derived from cardiac α-myosin. Prevalence and severity of myocarditis were markedly reduced in the absence of IL-6. CD4+ T cells from immunized IL-6–deficient mice proliferated poorly on restimulation with specific antigen in vitro and did not mediate disease on adoptive transfer into IL-6–competent RAG-2–deficient mice, which otherwise lack B cells and T cells. Production of complement C3, a crucial factor for the development of myocarditis, was strongly upregulated in IL-6-deficient but not in IL-6–deficient mice after immunization.

Conclusions—Our results demonstrate that IL-6 is required for the expansion of autoimmune CD4+ T cells and the pathogenesis of autoimmune myocarditis, possibly by upregulation of complement C3. (Circulation. 2003;107:320-325.)

Key Words: interleukins • myocarditis • cardiomyopathy

Infections, surgery, myocardial infarction, and the systemic inflammatory response syndrome can cause cardiac inflammation. Acute myocarditis may initiate a chronic immune-mediated inflammation of the heart and result in idiopathic cardiomyopathy.

Experimental autoimmune myocarditis is a model for CD4+ T cell–mediated inflammatory heart disease and can be induced in susceptible mice (eg, BALB/c, A/J) by immunization with cardiac myosin and peptides derived from it. Cytokines like tumor necrosis factor (TNF)–α/TNFRI and interleukin (IL)-12/45 are essential for disease induction, whereas interferon (INF)–γ protects from myocarditis.

IL-6 is a multifunctional cytokine that controls viral, bacterial, and fungal infection and the pathogenesis of various diseases. Furthermore, in the context of autoimmunity, IL-6 is involved in development of experimental allergic encephalitis and experimental arthritis.

Several studies point toward a role for IL-6 in heart disease. It has been shown that elevated plasma concentrations of IL-6 predict a poor prognosis in congestive heart failure secondary to idiopathic dilated cardiomyopathy. Furthermore, its serum levels are associated with an increased future risk of myocardial infarction among healthy men. We addressed the role of IL-6 in cardiac inflammation by using the experimental autoimmune myocarditis model and knockout mice.

Methods

Mice, Immunization, and Treatment Protocols

IL-6–deficient mice (generated by M. Kopf7), RAG-2–deficient mice lacking both B cells and T cells (purchased from BRL, Fuellinsdorf, Switzerland), and µMT-deficient mice lacking B cells (generated by K. Rajewsky et al9) (all >8 generations backcrossed to BALB/c) and IL-6−/− BALB/c mice (wild-type; purchased from BRL, Fuellinsdorf, Switzerland) were immunized at 8 to 10 weeks of age with a peptide derived from α-myosin heavy chain (Myhc-β614-634, Ac-SLKLMAFLSTYASAD-OH), as described.2 No deaths were observed after immunization. For TNF-α treatment, mice were injected intraperitoneal with 300 ng of synthetic TNF-α peptide (ICN) every second day until day 21. Controls received saline only.

All experiments were performed in accordance with Swiss federal legislation and were approved by the local authorities.

Histopathology and Immunohistochemistry

Hearts were assessed on routine paraffin sections and scored on a semiquantitative scale (0 indicated no inflammatory infiltrates; 1, small foci of inflammatory cells between myocytes; 2, larger foci of >100 inflammatory cells; 3, >10% of a cross-section involved; 4, >30% of a cross-section involved).

For immunohistochemistry, samples were incubated with antibodies against CD106 (vascular cellular adhesion molecule [VCAM], Serotec, Oxford, UK), CD54 (intracellular adhesion molecule [ICAM], Serotec), CD11c (N418), MHC class II, MHC class I (M1/42), CD3 (KT3-1.1), CD4 (YTS 191), and CD8 (YTS169), followed by goat anti-rat Ig (Caltag, South San Francisco, Calif) or
rabbit anti-hamster Ig (Jackson ImmunoResearch) and appropriate alkaline phosphatase (AP)-labeled third antibodies.

Measurement of Specific Antibody Responses and Complement C3
Antibody responses directed against Myhc-α1,431 were assessed by ELISA as described4 using AP-labeled goat anti-mouse IgG1, IgG2a, and IgG2b antibodies (Southern Biotechnology Associates). Serum C3 levels were measured by ELISA as described.20 Titers were determined at half maximum OD405nm.

Cytokine Measurements
Twenty-one days after immunization, cytokines were measured in supernatants of lymph node cells cultured with 20 μg/mL of Myhc-α1,431 for 20 hours (TNF-α) or 48 hours (IL-2, IL-4, IL-10, IL-1β) using commercially available ELISA kits. For intracellular staining, cells were restimulated with Myhc-α1,431 before adding PMA (10−7 mol/L) and ionomycin (1 μg/mL) for 4 hours. Two hours before harvesting, Brefeldin A (10 μg/mL) was added. Cells were stained with APC-labeled anti-CD4 mAb and fixed with 2% paraformaldehyde. Subsequently, cytokines were stained with FITC-labeled anti-IFN-γ and PE-labeled anti–IL-4 in a 1% BSA/PBS solution containing 0.5% saponin for permeabilization before analysis by flow cytometry.

CD4+ Cell Proliferation Assays
CD4+ T cells were purified from spleens using magnetic beads as described4 and restimulated on irradiated syngeneic splenocytes pulsed with 10 μg/mL of Myhc-α or ovalbumin as unspecific control antigen. Proliferation was measured by [3H]methyl-thymidine incorporation.

Adoptive Transfer
CD4+ T cells were isolated and cultured for 96 hours on antigen-pulsed APC in the presence of 50 U/mL of recombinant murine IL-2. T cells 1×10^5 (>90% CD4+) were transferred intravenously into RAG-2-deficient mice. Recipients were killed after 14 days.

Statistics
The Mann-Whitney U test was used for the evaluation of severity scores and heart weights. Proliferation responses and cytokine levels were compared using ANOVA and the unpaired t test. Dichotomous data were analyzed by Fisher’s exact test. Myocarditis prevalence refers to the number of diseased mice compared with the number of immunized mice still alive at the days indicated.

Results
IL-6−/− Deficient Mice Are Protected From Autoimmune Myocarditis
IL-6−/− and IL-6+/+ mice were immunized with Myhc-α1,431. IL-6−/− mice developed severe myocarditis, which peaked at day 21, as described previously.3,6 In contrast, disease prevalence and severity were strongly reduced in IL-6−/− mice (Figure 1, Table 1). Moreover, immunohistochemistry showed reduced expression of MHC class II, VCAM-1, and ICAM-1 molecules (Figure 1) and low numbers of CD3-positive cells (not shown) in the hearts of IL-6−/− deficient mice compared with controls.

Reduced Myosin Autoantibodies in IL-6−/− Deficient Mice
IL-6−/− mice showed high autoantibody levels against Myhc-α1,431 of the subclasses IgG1>IgG2b>IgG2a. In contrast, IgG1, IgG2b, and IgG2b autoantibody levels were significantly reduced in IL-6−/− deficient mice (Figure 2). Thus, disease severity correlated with the magnitude of the autoantibody response in IL-6−/− and IL-6+/+ mice. Because IL-6 promotes B cell responses, we asked whether impaired antibody responses contribute to disease resistance. We therefore immunized B cell–deficient (μMT) mice with Myhc-α. As shown in Table 1, mice lacking B cells develop myocarditis indistinguishable from IL-6−/− controls, demonstrating that antibodies play no role in this disease model. In addition, there was no difference in the course of myocarditis between IL-6+/+ and B cell–deficient mice up to 4 weeks after immunization (not shown). Moreover, our data suggest that the reduced autoantibody response was not responsible for protection from myocarditis in IL-6−/− deficient mice.

Inefficient Induction of Autoreactive CD4+ T Cells in the Absence of IL-6
Because autoimmune myocarditis is CD4+ T cell–mediated, we investigated the role of IL-6 for the induction and

<table>
<thead>
<tr>
<th>Mice</th>
<th>Treatment</th>
<th>Prevalence (Day 21)</th>
<th>Severity Grade (at Day 21, Median (range))</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6+/+</td>
<td>None</td>
<td>11/14</td>
<td>2 (0 to 4)</td>
</tr>
<tr>
<td>IL-6−/−</td>
<td>None</td>
<td>2/14*</td>
<td>0 (0 to 2)</td>
</tr>
<tr>
<td>μMT</td>
<td>None</td>
<td>10/10†</td>
<td>2 (2 to 4)</td>
</tr>
<tr>
<td>IL-6−/−</td>
<td>TNF-α fragment</td>
<td>0/6</td>
<td>...</td>
</tr>
<tr>
<td>IL-6−/−</td>
<td>Saline</td>
<td>0/8</td>
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*p<0.0018 IL-6+/+ vs IL-6−/−.
†P<0.2391 μMT vs IL-6−/−.
Th1/Th2 subset polarization of antigen-specific CD4+ T cells. Twenty-one days after immunization, IL-6–/– CD4+ T cells showed reduced proliferation compared with IL-6+/+ CD4+ T cells on in vitro stimulation (Figure 3A). Intracellular staining of IL-4 and IFN-γ in CD4+ T cells of restimulated lymph node cells revealed a similar Th1/Th2 cell ratio with only a minimal increase of IL-4 producing CD4+ T-cells in IL-6–/– mice (Figure 3B). Furthermore, cytokine measurement in the supernatants of these cell cultures showed no significant differences in IL-4 and IL-10 production, although IFN-γ levels were slightly reduced for IL-6–/– deficient lymph node cells. (Figure 3C). Interestingly, supernatants of IL-6–/– cultures contained reduced TNF-α, whereas levels of IL-1β were comparable to IL-6+/+ cultures (Figure 3D). To additionally investigate the function of autoimmune CD4+ T cells in vivo, we purified CD4+ T cells from immunized IL-6–/– and IL-6+/+ mice at day 21, restimulated with Myhc-α614-634, and injected them into recipients devoid of endogenous B cells and T cells (RAG-2–/– deficient mice). Adoptive transfer of CD4+ T cells from diseased IL-6–/– mice induced myocarditis in RAG-2–/– recipients (Table 2). In contrast, transfer of IL-6–/– CD4+ T cells failed to induce disease in RAG-2–/– mice, which are otherwise competent to produce IL-6. Thus, protection from myocarditis in IL-6–/– deficient mice results from inefficient induction of autoreactive CD4+ T cells. Additionally, reduced TNF-α production may prevent myocarditis development in the absence of IL-6.

**TNF-α Does Not Restore Disease Susceptibility of IL-6–Deficient Mice**

TNFR1 is crucial for the development of autoimmune myocarditis. Given the markedly reduced TNF-α levels within lymphocyte cultures from IL-6–/– deficient mice, we speculated that TNF-α treatment may reverse myocarditis resistance of IL-6–/– deficient mice. Immunized IL-6–/– deficient mice were injected with a synthetic peptide fragment mediating TNF-α bioactivity according to an established protocol. Compared with saline, injection of the TNF-α-derived peptide upregulated MHC class II and adhesion molecules in the hearts of IL-6+/+ and IL-6–/– deficient mice within 24 hours (not shown). However, TNF-α peptide treatment failed to restore disease susceptibility in IL-6–/– deficient mice (Table 1). Moreover, there was no change in the cytokine production pattern of in vitro restimulated lymphocytes (not shown) after
TNF-α treatment. These data suggest that the impaired production of TNF-α is not responsible for myocarditis resistance of IL-6−/− mice.

**IL-6 Is Required for C3 Upregulation After Immunization**

Because complement C3 is essential for development of myocarditis and IL-6 is a major regulator of the acute phase response including C3,7,23 we assessed C3 levels in sera of immunized mice. Immunization with Myhc-α16 in adjuvant resulted in a strong increase of C3 serum levels in IL-6−/− mice within 24 hours (Figure 4). In contrast, no increase was observed in IL-6−/− deficient mice. Thus, impaired upregulation of complement is associated with protection against myocarditis in IL-6−/− mice.

**Discussion**

Together with indirect clinical evidence,17,18 our data demonstrate a critical role for IL-6 in the pathogenesis of inflammatory heart disease.

Immunized IL-6−/− deficient mice showed a reduction in innate and adaptive immune responses linked to myocarditis susceptibility, such as expression of adhesion molecules (ie, ICAM-1 and VCAM-1), inflammatory responses (ie, C3 and TNF-α), levels of autoantibodies, and proliferation of autoreactive CD4+ T cells.

Heart ICAM-1 and VCAM-1 expression can be induced by IL-1β and TNF-α and by bacterial components leading to accumulation of inflammatory cells in the heart and contractile dysfunction.24 Moreover, increased ICAM-1 expression has been implicated in the pathogenesis of viral myocarditis.25 Reduced ICAM-1 and VCAM-1 expression may reflect impaired activation of endothelial cells and result in less-efficient recruitment of autoreactive T cells to the heart of IL-6−/− deficient mice. IL-6 has been suggested to contribute indirectly to the activation of endothelial cells, which lack the IL-6 receptor (IL-6R). Thus, expression of ICAM-1, VCAM-1, and E-selectin on endothelial cells is mediated by transactivation mediated by soluble IL-6R released from stimulated neutrophils.26 Reduced expression of adhesion molecules was also observed in IL-6−/− deficient mice resisting experimental allergic encephalomyelitis.13 Interestingly, triggering of a TNFR1-dependent pathway by staphylococcus enterotoxin B treatment restored adhesion molecule expression and overcame disease resistance of IL-6−/− deficient mice in the experimental allergic encephalomyelitis model.15 Because TNF-α is a critical factor for the development of Coxsackie virus-induced myocarditis23 and TNFR1 is crucial for autoimmune myocarditis,3 we reasoned that impaired TNF-α production may contribute to myocarditis resistance of IL-6−/− mice. However, TNF-α (Table 1) treatment did not render IL-6−/− deficient mice susceptible. Thus, impaired production of TNF-α and expression of adhesion molecules seem not to be responsible for protection from myocarditis in IL-6−/− deficient mice.

IL-6 is required for efficient antibody responses after infection with viruses27 and after immunization with antigen.23 In keeping with these results, we found that IL-6−/− deficient mice showed reduced levels of autoantibodies. Although autoimmune myocarditis is associated with autoantibody formation, B cells were shown to be dispensable for disease development after immunization of BALB/c mice with cardiac myosin whole protein.28 Nevertheless, it remained possible that B cells play an important role after immunization with the myosin peptide used in our immunization regimen, because of their higher density of MHC class II myosin peptide complexes. This possibility was excluded by showing that disease severity and mortality did not differ between B cell–deficient (μMT) mice and IL-6−/− mice after immunization. This finding does not preclude a role of autoantibodies in a delayed cardiac dysfunction, in human cardiomyopathy, or in other mouse models of myocarditis, including viral myocarditis or autoimmune myocarditis in DBA/2 mice, which have been shown to develop disease after transfer of autoantibodies.29,30 Nevertheless, our data suggest that reduced IgG autoantibody production is not responsible for resistance to autoimmune myocarditis in IL-6−/− deficient mice on a BALB/c background.

Autoimmune myocarditis is dependent on CD4+ T cells. We found that the proliferation of autoreactive CD4+ T cells was impaired in IL-6−/− deficient mice. Furthermore, IL-6−/− CD4+ T cells, in contrast to IL-6+/+ CD4+ T cells, failed to mediate disease on adoptive transfer. Defective T cell responses in IL-6−/− deficient mice have also been described in other models of autoimmune disease. In some reports, reduced CD4+ T cell proliferation was associated with a reduction of both IL-4 and IFN-γ responses,15 whereas other studies demonstrated a shifted Th1:Th2 ratio with decreased IFN-γ and increased IL-4 production in the absence of IL-6. The Th1/Th2 paradigm is still widely used to explain autoimmunity with IFN-γ promoting and IL-4 protecting against
CD4+/H11001

tis. Therefore, our data suggest that IL-6 is involved in the respective knockouts, cannot explain resistance to myocarditis in C3-depleted and CR1/2-deficient mice. Protection from inflammation (effector phase) prevented disease. Further-

complement receptors CD21 and CD35.22 Depletion of C3 production locally in the microenvironment of lymphatic tissue.31 Increased expression of CD25 and CD69.22 Thus, the protection from myocarditis observed for IL-6–/– deficient mice infected with Candida albicans. J Exp Med. 1996;183:1345–1355.


Lane JR, Neumann DA, Lafond-Walker A, et al. Interleukin 1 or tumor necrosis factor can promote Coxsackie B3-induced myocarditis in resis-


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References


In this study, we provide genetic evidence that IL-6 is crucial for the development of autoimmune heart disease. IL-6 bridges innate and adaptive immune responses by regulation of complement C3. We suggest that elevated IL-6 levels in patients with cardiomyopathy or after myocardial infarction are not merely prognostic surrogate markers but contribute to cardiac damage by triggering pathways resulting in autoimmunity. Therefore, blocking the IL-6 pathway may be beneficial in the treatment of inflammatory heart disease.

Disease. However, it has been shown previously that autoimmune myocarditis is exacerbated in IFN-γ–/– deficient mice, whereas disease prevalence at the peak of the response is reduced in IL-4R–/– deficient mice with an abrogated IL-4 and IL-13 pathway4 or on injection of anti–IL-4 monoclonal antibody.31 IL-6–/– deficient mice display a slightly increased IL-4 to IFN-γ ratio, which, considering the phenotype of the respective knockouts, cannot explain resistance to myocarditis. Therefore, our data suggest that IL-6 is involved in the activation or maintenance of autoimmune CD4+ T cells independent of Th1/Th2 polarization. Interestingly, in experimental colitis induced by adoptive transfer of congenic CD62L−/−CD45RB−/− CD4+ T cells into SCID (severe combined immunodeficiency) mice, neutralization of IL-6 resulted in apoptosis of lamina propria cells and reduced disease severity.12 Our findings of reduced proliferation do not exclude the possibility of enhanced apoptosis of myosin-

Specific IL-6–/– CD4+ T cells.

We have previously demonstrated that impaired complement C3 production is responsible for defective germinal center development and B-cell responses in immunized IL-6–/– deficient mice.21 Moreover, we have reported that C3 is essential for the activation and recruitment of both CD4+ and CD8+ T cells after pulmonary virus infection,23 which is also defective in IL-6–/– deficient mice (N. Schmitz et al., unpublished data, 2001). Consistent with these data, we describe here that IL-6–/– deficient mice fail to upregulate C3 production associated with impaired CD4+ T cell responses after immunization with Myhc−α14.33 Recently, autoimmune myocarditis has been shown to depend on C3 and the complement receptors CD21 and CD35.22 Depletion of C3 during the inductive phase but not during the progression of inflammation (effector phase) prevented disease. Furthermore, the absence of the complement receptors CD21 (CR2) and CD35 (CR1) prevented myocarditis. Protection from myocarditis in C3-depleted and CR1/2-deficient mice was associated with reduced production of proinflammatory cytokines. In addition, CD4+ T cells from CR1/2-deficient mice were less responsive to antigen activation, as indicated by reduced expression of CD25 and CD69.22 Thus, the protection from myocarditis observed for IL-6–/– deficient mice and C3-deficient mice may be related. We suggest that impaired generation of complement is a key factor for disease prevention in the absence of IL-6. Despite the fact that complement levels do not increase in IL-6–/– deficient mice after immunization, baseline concentrations in the serum were in the normal range but seem insufficient to activate CD4+ T cells and autoimmune disease. Thus, it seems that IL-6 regulates C3 production locally in the microenvironment of lymphatic tissue,23 where CD4+ T cells interact with B cells and antigen-presenting cells.

In conclusion, we provide genetic evidence that IL-6 is crucial for the development of autoimmune heart disease. IL-6 bridges innate and adaptive immune responses by regulation of complement C3. We suggest that elevated IL-6 levels in patients with cardiomyopathy or after myocardial infarction are not merely prognostic surrogate markers but contribute to cardiac damage by triggering pathways resulting in autoimmunity. Therefore, blocking the IL-6 pathway may be beneficial in the treatment of inflammatory heart disease.
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