Sustained Nitric Oxide Synthesis Contributes to Immunopathology in Ongoing Myocarditis Attributable to Interleukin-10 Disorders

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Ongoing coxsackievirus B3 (CVB3) myocarditis is characterized by persistence of viral RNA and chronic inflammation primarily mediated by macrophages and T cells. Activated macrophages produce anti-viral effector molecules comprising reactive nitrogen intermediates; however, reactive nitrogen intermediates also contribute to host tissue damage. Controlled activation of macrophages depends on interferon (IFN)- γ and interleukin (IL)-10. To evaluate mechanisms involved in CVB3-induced pathogenesis of myocarditis, we determined the relationship of inducible nitric-oxide synthase (iNOS) mRNA expression with IFN- γ and IL-10 secretion during CVB3 infection in different mouse strains. We found in susceptible A.BY/SnJ mice that develop ongoing myocarditis, a low and delayed IFN- γ secretion and highly diminished IL-10 production compared with resistant C57BL/6 mice. Consequently, iNOS mRNA synthesis was delayed but clearly prolonged in susceptible mice. IL-10 gene-deficient mice confirmed the regulatory role of IL-10 in the outcome of CVB3 myocarditis. These mice did not establish a persistent cardiac infection and revealed IFN- γ secretion kinetics similar to resistant mice but showed a slightly elongated cardiac iNOS mRNA expression resulting in extended myocarditis. We conclude that coordinated secretion of IFN- γ and IL-10 is crucial for the effective resolution of CVB3 myocarditis. Moreover, lack of regulatory IL-10 leads to uncontrolled iNOS mRNA production, thus contributing to ongoing myocardial injury. (*Am J Pathol* 2006, 169:2085–2093; DOI: 10.2353/ajpath.2006.060350)

carditis with congestive heart failure. Susceptible patients may develop a chronic inflammatory heart disease leading to dilated cardiomyopathy, which in its end stage can be treated effectively only by heart transplantation.^{1,2} Myocarditis is often caused by infection with coxsackieviruses of group B (CVB), which are members of the enterovirus genus of the *Picornaviridae*.³ Divergent courses of acute and chronic CVB3 myocarditis can be successfully mimicked in mouse models, which are either resistant or susceptible with regard to the development of chronic disease.⁴ During the acute phase, resistant mice as well as susceptible mice suffer from CVB3-induced myocarditis characterized by infiltrating macrophages, CD4⁺ T cells, and CD8⁺ T cells. After the acute phase of infection, CVB3 is successfully eliminated in resistant mice, and inflammatory infiltrates decline. In contrast, in susceptible mice a persistent type of infection of cardiac cells, characterized by restricted virus replication in the absence of detectable infective virus progeny, sustains chronic inflammation.⁴

The balance between virus replication and host immune responses determines the outcome and severity of enterovirus myocarditis.⁴ However, immunological factors and mechanisms leading to successful virus elimination and limited immunopathology, thus defining the resistant phenotype, are still only partially identified. CVB3 infection elicits both humoral and cellular immune responses.⁵ Innate immune responses are characterized by the production of type I interferons (IFN- α and IFN- β), type II interferon (IFN- γ), and further proinflammatory cytokines such as tumor necrosis factor- α or interleukin (IL)-1, and by infiltration of monocytes/macrophages into the infected tissue.⁶ The adaptive immune response in CVB3 myocarditis is characterized by specific antibodies, which remove CVB3 from the circulation, and by T

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The natural course of human enterovirus myocarditis varies from a subclinical infection to fulminant acute myo-

cells, which mediate protection via secretion of IFN- γ , thereby activating macrophages.^{7–11} Activated macrophages produce effector molecules, such as reactive nitrogen intermediates (RNI), that possess anti-viral activities. In the murine model of CVB3 myocarditis, mice deleted in the inducible nitric-oxide synthase (iNOS) gene have been described to exhibit higher susceptibility to CVB3 infection than their heterozygous littermates.¹² As IFN- γ induces iNOS, sustained or overwhelming IFN- γ secretion leads to ongoing activation of macrophages, to sustained synthesis of RNI, such as nitric oxide (NO), and finally to immunopathology.¹³

Therefore, after successful elimination of pathogens, effector mechanisms need to be down-regulated to limit inflammatory processes and to prevent consecutive immunopathology. Such a modulatory cytokine is IL-10, which inhibits cytokine synthesis in bacterial and viral infections, down-regulates expression of MHC molecules on macrophages, and prevents NO-dependent microbicidal activities by macrophages.¹⁴ In CVB3 myocarditis, IL-10 mRNA could be detected in the hearts of infected mice and human monocytes and macrophages were found *in vitro* to produce IL-10 after infection with CVB3.^{15,16}

In this study, our aim was to elucidate the role of IFN- γ and IL-10 in acute and chronic CVB3 myocarditis with regard to regulation of NO production. We show that in CVB3-infected susceptible A.BY/SnJ mice, IFN- γ secretion is delayed and IL-10 secretion is highly diminished. In addition, there is firm evidence that disorders in IL-10 secretion in A.BY/SnJ mice and IL-10 gene-deleted mice result in a sustained iNOS mRNA expression, thus contributing to immunopathology in the heart.

Materials and Methods

Virus and Viral Antigens

CVB3 used in this study was derived from the infectious cDNA copy of the cardiotropic Nancy strain, and virus stocks were prepared as previously described.¹⁷ Viral antigen preparation was performed by heat-inactivation of CVB3 at 60°C for 15 minutes.

Mice and Infection

C57BL/6 mice, A.BY/SnJ mice, and IL-10^{-/-} mice (C57BL/6 background) (all H-2^b) were kept under specific pathogen-free conditions at the animal facilities of the Department of Molecular Pathology, University Hospital Tübingen, and experiments were conducted according to the German animal protection law. Four- to 5-week-old mice ($n = 10$ per time point) were infected intraperitoneally with 1×10^5 plaque-forming units of purified CVB3 as described.⁴ At different time points after infection, mice were sacrificed, and hearts, spleens, and serum were collected for analysis. For enzyme-linked immunosorbent assay (ELISA) experiments, spleen cells were cultured, and parts of the hearts were snap-frozen and stored until homogenized. For reverse transcriptase-

polymerase chain reaction (RT-PCR) experiments, parts of the hearts were snap-frozen and stored at -80°C until RNA extraction. For histology, part of the hearts were fixed in 4% paraformaldehyde and embedded in paraffin.

Detection of Virus-Specific Antibodies by ELISA

Detection of virus-specific serum IgG immunoglobulins was performed by an indirect ELISA, as previously described.¹¹ In brief, microtiter plates were coated with purified CVB3 antigen (200 ng/well), incubated overnight at room temperature, blocked with 5% bovine serum albumin (Sigma, Seelze, Germany) in phosphate-buffered saline for 1 hour, and subsequently incubated with twofold serial dilutions of sera obtained from infected mice at indicated time points after infection for 2 hours. Plates were washed and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (Dianova, Hamburg, Germany) for 1 hour. Antibody binding was detected with 3,5,3',5'-tetramethylbenzidine, and the absorbance at 450 nm was measured with a plate reader (Sanofi, Berlin, Germany). CVB3-specific antibody titers are presented as the highest dilution of serum showing an optical density greater than the mean optical density of sera obtained from naive mice plus threefold the SEM.

In Vitro Neutralization Assay

To define the neutralization titers of sera taken from CVB3-infected mice, we performed an *in vitro* neutralization assay as previously described.¹¹ In brief, infectious virus corresponding to 100 TCID₅₀/well was incubated with twofold serial dilutions of sera on a microtiter plate and incubated for 2 hours at 37°C. Cells (10^4 Vero cells/well) were added and plates were incubated for an additional 48 hours. Cells were stained with trypan blue, and neutralization titers were defined as the highest serum dilutions leading to complete protection against CVB3-induced cytolysis.

Determination of Cytokines by ELISA

Frozen heart tissues were homogenized in 500 μ l of Dulbecco's modified Eagle's medium, and supernatants were screened for cytokines by sandwich ELISA. Single-cell suspension of spleen cells from CVB3-infected mice were prepared at different time points after infection and restimulated with viral antigen preparation for 4 days, and culture supernatants were screened for cytokine production by a sandwich ELISA. Determination of IFN- γ and IL-10 levels was performed by an OptEIA kit (Becton-Dickinson, Heidelberg, Germany) according to the manufacturer's instructions, with the modification that 50 μ l of supernatants were incubated overnight at 4°C. The absorbance at 450 nm was measured with a plate reader (Sanofi).

RT-PCR

RNA of murine heart tissue was isolated by using Trizol reagent (Invitrogen, Heidelberg, Germany) according to

the manufacturer's instruction. RNA (500 ng) was used for RT-PCR using a one-step RT-PCR kit (Qiagen, Hilden, Germany). PCR was done with an annealing temperature for CVB3-PCR at 60°C and for mGAPDH-PCR at 58°C at 40 cycles. The primers specific for CVB3 and the murine housekeeping gene GAPDH were purchased from MWG Biotech (Ebersberg, Germany). Primers were CVB3 forward: 5'-TATCCGGCCAACTACTTCGAA-3'; CVB3 reverse: 5'-TGCGGTGACTCATCGACCT-3'; mGAPDH forward: 5'-AATGCCTCCTGCACCACC-3'; and mGAPDH reverse: 5'-ATGCCAGTGAGCTTCCCG-3'. We performed RT-PCR with RNA from hearts of individual mice as well as from RNA, which was pooled on equal amounts from single mice.

Histology

Paraffin-embedded hearts were cut in 5- μ m-thick tissue sections and stained with hematoxylin and eosin (H&E) to assess myocardial injury and inflammation. For determination of histological severity score of myocarditis, 5- μ m tissue sections covering the right and the left ventricle, which were stained with H&E, were examined for evidence of myocarditis. To quantify myocardial damage comprising cardiac cell necrosis, inflammation, and scarring, we applied a myocarditis score from 0 to 4 according to Eriksson and colleagues¹⁸ with slight modifications (score: 0, no inflammatory infiltrates; 1, small foci of inflammatory cells between myocytes; 2, larger foci >100 inflammatory cells; 3, $\leq$ 10% of cross-section involved; 4, 10 to 30% of a cross-section involved).

In Situ Hybridization

CVB3-positive-strand genomic RNA in tissues was detected using single-stranded ³⁵S-labeled RNA probes, which were synthesized from the dual-promoter plasmid pCVB3-R1.⁴ Control RNA probes were obtained from the vector pSPT18. ³⁵S-labeled anti-sense and sense RNA probes for detection of iNOS were synthesized by *in vitro* transcription from the dual promoter plasmid pSPT18 containing a 725-bp murine iNOS cDNA fragment.¹⁹ Pre-treatment, hybridization, and washing conditions of dewaxed 5- μ m paraffin tissue sections were performed as described previously.⁴ Slide preparations were subjected to autoradiography, exposed for 3 weeks at 4°C, and counterstained with H&E. Quantitative analysis of autoradiographic signals obtained by *in situ* hybridization representing CVB3-infected myocardium were done as previously described.¹¹ Areas of hybridization-positive heart tissue were referred to the total area of tissue sections and expressed as area fractions of infection (μ m²/mm²). To quantify iNOS mRNA-positive lesions in the myocardium of the three mouse strains, we referred the inflammatory lesions containing iNOS mRNA-expressing cells to the total number of inflammatory lesions. Results are expressed in percent iNOS mRNA-positive (% iNOS⁺) lesions.

Statistical Analysis

Data are presented as mean $\pm$ SEM. Differences between experimental groups were analyzed for statistical significance ($P < 0.05$) by unpaired *t*-test or by one-way analysis of variance followed by Bonferroni's comparison as indicated in the figure legends, respectively.

Results

Uniform Humoral Response in Resistant and Susceptible Mice

An efficient antibody production is essential for the control of infection with lytic viruses. From our studies with β_2 -microglobulin-deficient mice, which are susceptible to chronic CVB3 myocarditis, it is known that these mice suffer from a deficiency in IgG production and have reduced neutralization titers. To investigate whether differences in the CVB3-specific humoral immune response determine the outcome of myocarditis, we compared the CVB3-specific IgG titers in resistant C57BL/6 mice, susceptible A.BY/SnJ mice, and IL-10^{-/-} mice. In all three mouse strains, the CVB3-specific IgG titers were congruent with an increase in IgG levels during the acute phase and high amounts of virus-specific antibodies at later stages of infection (Figure 1A). To further assess the effectiveness of the humoral response, we compared the amount of neutralizing antibodies. In all three mouse strains, the titers of CVB3-specific neutralizing antibodies were increased but not significantly different in the course of infection (Figure 1B).

Reduced and Delayed IFN- γ Secretion in Mice with Ongoing CVB3 Myocarditis

IFN- γ is the marker cytokine for a Th1 response that promotes cellular immunity. Moreover, presence of IFN- γ is a prerequisite for the activation of macrophages, which then may produce anti-viral effector molecules such as RNI. To determine the capacity of susceptible A.BY/SnJ mice, resistant C57BL/6 mice, and IL-10^{-/-} mice to respond with cytokine secretion on CVB3 infection, we monitored cardiac IFN- γ secretion in the course of infection in the heart by preparing heart homogenates at different time points after infection. In the heart, IFN- γ secretion was significantly lower between day 4 and day 16 after infection in susceptible mice compared with resistant C57BL/6 mice and IL-10^{-/-} mice ($P < 0.001$) (Figure 2A). However, there was a continuing increase of IFN- γ level in hearts of A.BY/SnJ mice until day 28 after infection. For analysis of CVB3-specific IFN- γ production, spleen cells of infected mice and uninfected controls from different time points after infection were used because they represent the pool of immunological cells capable of infiltrating infected tissues. Remarkably, in resistant C57BL/6 mice and IL-10^{-/-} mice, the CVB3-specific IFN- γ secretion of spleen cells peaked during

Figure 1. CVB3-specific IgG levels and neutralizing antibody titers in susceptible, resistant, and IL-10^{-/-} mice. Sera from CVB3-infected A.BY/SnJ, C57BL/6, and IL-10^{-/-} mice were collected at indicated time points, and titers of virus-specific IgG antibodies (A) as well as *in vitro* neutralization activity (B) were determined by a CVB3-specific ELISA and neutralization assays, respectively. No significant difference was observed in the three mouse strains in A and B. Experiments were repeated twice with five mice per group, and values are given as mean titers (log2) $\pm$ SEM.

the acute phase of myocarditis, whereas in susceptible A.BY/SnJ mice IFN- γ secretion was retarded (Figure 2B).

Absence of IL-10 in CVB3-Infected Mice Revealing Chronic Myocarditis

The opponent of IFN- γ in regulation of immunity is IL-10, which is an important factor for modulation of immune responses and especially of macrophage activities. To analyze the role of IL-10 in chronic CVB3 myocarditis, we determined IL-10 production in the hearts in the course of CVB3 infection in resistant C57BL/6 mice and susceptible A.BY/SnJ mice. In susceptible mice, IL-10 secretion was weakly induced and showed invariable expression in the course of CVB3 infection. In contrast, in the hearts of resistant C57BL/6 mice, a significant increase of IL-10 was observed during acute myocarditis (Figure 3A). These findings are supported by our observations that in spleen cells of resistant mice CVB3-specific IL-10 secretion peaked during the acute phase of infection, whereas

Figure 2. Reduced IFN- γ levels of susceptible CVB3-infected A.BY/SnJ mice compared with resistant C57BL/6 mice and IL-10^{-/-} mice. A.BY/SnJ mice were compared with C57BL/6 and IL-10^{-/-} mice for levels of IFN- γ in heart (A) and in spleen cell (B) cultures in the course of CVB3 infection. Mice were infected with 1×10^5 plaque-forming units of CVB3; control mice remained uninfected. At indicated time points the hearts and spleens were collected, hearts were homogenized, and supernatants were analyzed for IFN- γ levels using ELISA. Single cell suspensions of spleen cells were restimulated with heat-inactivated CVB3; after 4 days supernatants were collected and tested for IFN- γ levels by ELISA. Experiments were repeated twice with five mice per group, and data are presented as mean $\pm$ SEM. Kinetics of cardiac and splenic IFN- γ production in C57BL/6 mice and IL-10^{-/-} mice versus A.BY/SnJ mice differed significantly; $P < 0.001$ (Student's *t*-test).

in susceptible mice CVB3-specific splenic IL-10 secretion was completely absent (Figure 3B).

Resistance of IL-10^{-/-} Mice to Ongoing CVB3 Myocarditis

As shown in Figure 2, CVB3-infected IL-10^{-/-} mice exhibited IFN- γ secretion patterns comparable with those of resistant C57BL/6 mice. Therefore, the question arose whether IFN- γ is sufficient to control virus replication in these mice or whether IL-10 is required to mediate antiviral defense mechanisms and deficiency of IL-10 promotes the development of ongoing myocarditis. To answer this question, we isolated RNA from heart tissue at different time points after infection and performed RT-PCR to detect CVB3 RNA. In accordance with our findings in resistant mice, in IL-10^{-/-} mice CVB3 RNA could be detected only until day 12 after infection; thereafter, no viral RNA was assessed in the heart muscles, whereas in

Figure 3. Increased IL-10 production in resistant C57BL/6 mice compared with susceptible A.BY/SnJ mice after CVB3 infection. A.BY/SnJ mice were compared with C57BL/6 mice for levels of IL-10 in the heart (A) and in spleen cell (B) cultures in the course of CVB3 myocarditis. Removed hearts were homogenized, and supernatants were analyzed for IL-10 levels by ELISA. Single cell suspensions of spleen cells were restimulated with heat-inactivated CVB3; supernatants were collected after 4 days and evaluated for cytokine production. Experiments were repeated twice with five mice per group, and data were presented as mean $\pm$ SEM. Kinetics of cardiac and splenic IL-10 production in C57BL/6 mice versus A.BY/SnJ mice were significantly different; $P < 0.001$ (Student's *t*-test).

hearts of susceptible A.BY/SnJ mice CVB3 RNA could be detected up to day 28 after infection (Figure 4A). To quantify the virus infection in the hearts, we determined the area fractions of infection by measuring the extent of autoradiographic signals obtained by *in situ* hybridization, representing CVB3-infected myocardium.¹¹ As expected, the highest area fractions of infection were detected at day 8 after infection in A.BY/SnJ, C57BL/6, and IL-10^{-/-} mice; thereafter, there was a decline in all mouse strains (day 12 after infection), and CVB3 RNA was not measurable any more in the myocardium of C57BL/6 and IL-10^{-/-} mice at day 16 and later in infection (Figure 4B). In contrast, in A.BY/SnJ mice CVB3 RNA could be detected up to day 28 after infection, which is a characteristic finding in chronic myocarditis of susceptible mice (Figure 4B). Histopathological analysis of CVB3-infected hearts from IL-10^{-/-} mice revealed a slightly extended myocarditis (until day 16 after infection) compared with C57BL/6 mice; thereafter, no ongoing inflam-

Figure 4. Time course of CVB3 RNA expression and quantitation of CVB3-infected tissue in hearts of A.BY/SnJ, C57BL/6, and IL-10^{-/-} mice. **A:** At indicated time points the presence of viral RNA was investigated in hearts of infected and control mice by RT-PCR. To document equal amounts of total cellular RNA, RT-PCR was performed for the detection of the mRNA from the housekeeping gene GAPDH. **B:** Quantitation of area fractions of infection (μm²/mm²) representing *in situ* hybridization-positive tissue within transverse cardiac tissue sections of A.BY/SnJ, C57BL/6, and IL-10^{-/-} mice in the course of myocarditis. CVB3 RNA is present later than 12 days after infection only in susceptible A.BY/SnJ mice. Results are shown as mean $\pm$ SEM ($n = 5$ for each time point).

mation such as in susceptible mice was detected. These findings are illustrated in Figure 5 by typical H&E staining of the heart muscle after CVB3 infection at days 4, 8, 16, and 28 after infection. Whereas at day 4 after infection no damage of myocytes or infiltration is obvious, we found numerous focal areas of mononuclear infiltrates and lysed myocytes in the hearts at day 8 after infection. In the course of the disease, a significant reduction of inflammation and an increase of replacement fibrosis was observed (day 16 after infection), and at day 28 after infection only few small areas of scarring in absence of notable inflammation were detectable. Quantitative analysis of H&E stains strengthened the observations that IL-10^{-/-} mice still have measurable amounts of cardiac inflammatory lesions at day 16 after infection, whereas at day 28 after infection the histopathological findings in the hearts of both mouse strains were restricted to small areas of fibrosis. Ongoing cardiac inflammation (until day 28 day after infection) is, however, present in the susceptible mouse strain A.BY/SnJ (Table 1).

Extended iNOS Generation in IL-10^{-/-} Mice

The role of IL-10 as a contributor to immunopathology was further clarified by analysis of RNI generation in the course of myocarditis. To investigate this, we analyzed cardiac iNOS mRNA distribution by *in situ* hybridization. We compared patterns of virus infection and expression of iNOS mRNA in the hearts of CVB3-infected C57BL/6

Figure 5. Histology of CVB3-infected hearts from IL-10^{-/-} mice in the course of infection. H&E stains of hearts from CVB3-infected IL-10^{-/-} mice illustrate mononuclear infiltrates at day 8 after infection and persisting myocardial damage at day 16 after infection. At day 28 after infection, only small areas of scarring but no inflammation was detected, pointing to resistance against chronic myocarditis.

and IL-10^{-/-} mice as well as of A.BY/SnJ mice by *in situ* hybridization on serial heart tissue sections. At day 8 after infection, the number of cardiac cells, most probably representing infiltrating macrophages, expressing iNOS mRNA was more prominent in resistant C57BL/6 mice and IL-10^{-/-} mice than in susceptible A.BY/SnJ mice, indicating minor activation of macrophages in A.BY/SnJ mice during acute infection (Figure 6 and Table 1). When the maximal cardiac inflammation was evident (day 12 after infection), CVB3 RNA and iNOS mRNA expression was balanced in all investigated mouse strains. However,

at day 16 after infection, iNOS mRNA was detected only in IL-10^{-/-} mice and A.BY/SnJ mice, in which IL-10 production is missing. Quantitation of iNOS mRNA-positive (iNOS⁺) lesions in respect to inflammatory lesions demonstrated a significantly higher ratio of iNOS⁺ lesions in cardiac tissue of IL-10^{-/-} mice compared with C57BL/6 mice; however, in A.BY/SnJ myocardium the ratio was even more pronounced (Table 1). In contrast, in resistant C57BL/6 mice, in which IL-10 is secreted, no cardiac iNOS mRNA was observed after day 12 of infection. Interestingly, IL-10^{-/-} mice were found to produce iNOS

Table 1. Myocarditis Severity Score and Ratio of iNOS mRNA-Positive (iNOS⁺) Inflammatory Cardiac Lesions in A.BY/SnJ Mice, C57BL/6 Mice, and IL-10^{-/-} Mice after CVB3 Infection

Days after infection	Strains	Severity score	<i>P</i> value	% iNOS ⁺ lesions	<i>P</i> value
8	A.BY/SnJ	3.50 ± 0.28	} <i>P</i> < 0.05*	66.75 ± 3.4	} <i>P</i> > 0.05
	C57BL/6	2.50 ± 0.20		83.50 ± 8.4	
	IL-10 ^{-/-}	2.00 ± 0.40		91.25 ± 5.9	
12	A.BY/SnJ	3.25 ± 0.25	} <i>P</i> > 0.05	93.75 ± 3.7	} <i>P</i> > 0.05
	C57BL/6	2.25 ± 0.47		78.25 ± 8.8	
	IL-10 ^{-/-}	2.25 ± 0.47		80.25 ± 6.6	
16	A.BY/SnJ	2.50 ± 0.28	} <i>P</i> < 0.001*	81.00 ± 7.2	} <i>P</i> < 0.01*
	C57BL/6	0.37 ± 0.12		0.00 ± 0.0	
	IL-10 ^{-/-}	1.25 ± 0.14		61.00 ± 20.0	
28	A.BY/SnJ	2.25 ± 0.47	} <i>P</i> < 0.01*	60.60 ± 14.6	} <i>P</i> < 0.001*
	C57BL/6	0.25 ± 0.25		0.00 ± 0.0	
	IL-10 ^{-/-}	0.25 ± 0.25		0.00 ± 0.0	

*Differences in severity scores and iNOS⁺ lesions of the three mouse strains at single time points are considered significant as determined by ANOVA. Posttesting of single pairs with Bonferroni revealed *P* < 0.05 between A.BY/SnJ versus IL-10^{-/-} mice at day 8 after infection, between all three strains at day 16 after infection, and between A.BY/SnJ versus C57BL/6, A.BY/SnJ versus IL-10^{-/-}, but not between C57BL/6 and IL-10^{-/-}, at day 28 after infection.

Figure 6. *In situ* hybridization for detection of iNOS mRNA and CVB3 RNA in serial heart tissue sections from susceptible A.BY/SnJ mice, resistant C57BL/6 mice, and IL-10^{-/-} mice during the course of CVB3 myocarditis. At day 8 after infection, iNOS mRNA expression was more prominent in C57BL/6 mice and IL-10^{-/-} mice than in A.BY/SnJ mice, whereas the area fractions of CVB3 infection were higher in susceptible A.BY/SnJ mice compared with resistant C57BL/6 mice and IL-10^{-/-} mice. At day 12 after infection, the patterns of iNOS mRNA expression and CVB3 infection were similar in all strains. At day 16 after infection, iNOS mRNA was detectable at high amounts in susceptible A.BY/SnJ mice and to a lesser degree in IL-10^{-/-} mice. Low levels of CVB3 RNA could only be detected in A.BY/SnJ mice at that time point. Neither iNOS mRNA nor viral RNA could be detected in C57BL/6 mice at day 16 after infection. At day 28 after infection, iNOS mRNA expression was noted in the presence of low levels of viral RNA only in susceptible mice, whereas in resistant C57BL/6 mice and IL-10^{-/-} mice neither CVB3 RNA nor iNOS mRNA expression was detected. Scale bars = 20 μ m.

mRNA until day 16 after infection despite the absence of CVB3 RNA, which was demonstrated by *in situ* hybridization as well as by RT-PCR. At later phases of infection (day 28 after infection) only susceptible A.BY/SnJ mice, revealing CVB3 RNA persistence and ongoing myocarditis as well as sustained IFN- γ production, were found to generate iNOS mRNA.

Discussion

Our findings strengthen the notion that self-limitation of CVB3 myocarditis is accomplished by the coordinate expression of the Th1 cytokine IFN- γ and IL-10. After CVB3 infection, IFN- γ levels were found to be raised in hearts of all infected mouse strains. However, while in resistant C57BL/6 and IL-10^{-/-} mice there was a prominent increase of IFN- γ after CVB3-infection with culmination during the acute phase of myocarditis, IFN- γ levels in susceptible A.BY/SnJ mice were considerably lower during acute disease and increased throughout time reaching a maximum only during the chronic phase of CVB3 myocarditis. IFN- γ production in infected hearts is accomplished by the infiltration of specific T cells that encounter CVB3-antigen-presenting cells. Because spleen cells provide a major source of lymphocytes infiltrating the infected heart, our observation that specific IFN- γ secretion by spleen cells is delayed in susceptible A.BY/SnJ mice reflects well the retardation of IFN- γ expression in the heart. We have first evidence that the

decreased levels of IFN- γ in A.BY/SnJ mice are attributable to reduced frequencies of IFN- γ -specific spleen cells (G.S., unpublished results). The importance of IFN- γ as a protective cytokine is supported by previous studies in which IFN- γ -expressing recombinant CVB3 variants were shown to confer protection against viral myocarditis.²⁰ In transgenic mice, which express IFN- γ in pancreatic β cells, no myocardial inflammation was obvious after CVB3 infection and protection from myocarditis was found to be dependent on IFN- γ -mediated reduction of CVB3 replication.²¹ In more recent reports, IFN- γ -deficient mice were shown to develop severe chronic CVB3 myocarditis and dilated cardiomyopathy, whereas in wild-type mice profibrotic cytokines, fibrosis, and mast cell degranulation was reduced, as was the severity of myocarditis.^{22,23} Our results stress the importance of IFN- γ in the outcome of CVB3 myocarditis by pointing out that the protective effect of this cytokine is highly dependent on an accurately timed secretion.

Direct anti-viral capacity can be attributed to IFN- γ activity, and missing IFN- γ supports viral persistence.^{24,25} Therefore, low IFN- γ levels in the hearts of susceptible A.BY/SnJ mice may support CVB3 replication and thus ongoing myocarditis. In addition to direct anti-viral activities, IFN- γ promotes a Th1 immune response and activates macrophages to express anti-viral effector molecules such as RNI (eg, NO). Generation of NO by macrophages is not constitutive but is dependent on the expression of iNOS.²⁶ In previous experiments it

was shown that gene deletion of iNOS renders mice highly susceptible to CVB3 infection.¹² CVB3 infection was found to induce expression of iNOS by macrophages rather than by myocytes during the acute phase of infection in NMRI mice, and iNOS mRNA was detected by PCR during the chronic phase of myocarditis.^{27,28} Our *in situ* hybridization analyses for detection of CVB3 RNA and iNOS mRNA in serial heart tissue sections demonstrate that in resistant C57BL/6 and IL-10^{-/-} mice the extent of CVB3 infection and iNOS mRNA expression in inflammatory infiltrates was concordant during the acute phase of infection. Contrary expression studies in hearts of susceptible mice revealed an imbalance between CVB3 RNA and iNOS mRNA during acute myocarditis pointing to a minor activation of macrophages despite a massive CVB3 infection. At the time point of maximal inflammation, levels of CVB3 RNA and iNOS mRNA were comparable in hearts of resistant and susceptible mice. However, after the acute phase of myocarditis, viral burden also decreased in the susceptible host, yet iNOS mRNA was still present at high levels. The ongoing NO synthesis indicates ongoing activation of macrophages and corresponds with increasing IFN- γ levels detected in the hearts of susceptible mice.

The role of NO in virus-induced inflammatory heart diseases is a double-sided sword. On the one hand, expression of NO exhibits direct anti-viral effects in CVB3-infected mice by inhibition of a viral proteinase.²⁹ On the other hand, oxidative stress leads to programmed cell death of cardiomyocytes.³⁰ Therefore, it is likely that unrestricted NO synthesis by activated macrophages contributes to cardiac tissue damage and needs to be regulated. One important factor for down-modulation of macrophage activities is IL-10.¹⁴ We know from our *in vitro* experiments in bone marrow-derived CVB3-infected macrophages that in the presence of IFN- γ , NO production is highly up-regulated but is reduced by more than 50% when the cells are simultaneously treated with IL-10 (data not shown). Our analyses of IL-10 production in the murine model of CVB3 myocarditis support this perception because in resistant mice, a virus-specific increase of IL-10 was observed, whereas only basal IL-10 secretion in susceptible mice was noted in the course of infection.

To confirm further the important role of IL-10 in resistance to CVB3-induced chronic myocarditis, we compared the course of infection and inflammation as well as IFN- γ production and iNOS mRNA expression in IL-10^{-/-} mice with those of immunocompetent mice. CVB3-infected IL-10^{-/-} mice revealed a cardiac pattern of infection and an inflammatory response typical for resistant animals with IFN- γ production and iNOS mRNA synthesis during the acute phase of myocarditis. Interestingly, despite the successful elimination of CVB3 during acute myocarditis, iNOS mRNA expression and myocardial damage continued beyond the acute phase until day 16 after infection, indicating missing deactivation of macrophages due to absent IL-10. The protective role of IL-10 in enterovirus myocarditis is supported by findings of Henke and colleagues²⁰ that infection of mice with recombinant CVB3-expressing IL-10 protects against lethal

disease and prevents tissue destruction. In the mouse model of encephalomyocarditis virus infection, application of rIL-10 or gene therapy transferring IL-10 cDNA decreases iNOS expression, thus ameliorating myocarditis.^{31,32} Moreover, gene transfer of IL-10 protects against experimental autoimmune myocarditis and blocking of IL-10 by monoclonal antibodies increases severity of disease.^{33,34} Now, further support for IL-10 as a protective cytokine in CVB3 myocarditis is presented by this study.

A possible mechanism involved in protection against chronic myocarditis is the accurately timed kinetics of IFN- γ secretion in the heart by infiltrating CVB3-specific T cells, with maximal secretion during the acute phase of CVB3 myocarditis. Thus, IFN- γ can directly inhibit CVB3 replication and activates macrophages to express CVB3-inhibiting RNI.³⁵ As a modulator of macrophage activities, IL-10 functions in this setting as a down-regulator of RNI production, thereby avoiding unrestricted cardiomyocyte destruction. If the induction of CVB3-specific T cells occurs too late and secretion of cardiac IFN- γ is only modest during the acute phase, CVB3 replication is not restricted and cardiomyocyte lysis is evident. Obviously, incomplete CVB3 elimination and delayed IFN- γ secretion in the absence of sufficient amounts of IL-10 favor immunopathology and susceptibility to ongoing myocarditis. Our results together with the findings of Fairweather and co-workers²³ that IFN- γ reduces fibrosis by a diminished mast cell degranulation and of Palaniyandi and colleagues³⁶ that IL-10 inhibits mast cell degranulation in acute myocarditis contribute significantly to the understanding of pathogenic mechanisms in ongoing myocarditis and dilated cardiomyopathy.

In view of human enteroviral myocarditis, evaluation of changes in IFN- γ and IL-10 should be useful as potential diagnostic tools to predict disease progression. First results were published with IL-10 as a predictor for inflammatory heart disease.³⁷ In patients with fulminant myocarditis, elevated IL-10 serum levels were observed,³⁸ which is in agreement with our observation in mice during the acute phase of CVB3 myocarditis. In these patients, IFN- γ levels were not increased,³⁸ so the fatal outcome in one third of the patients, who had the highest IL-10 levels, is plausible. In addition, iNOS activity was found to be present in patients with myocarditis and dilated cardiomyopathy.³⁹ This observation also supports the interpretation of our results that a coordinate secretion of IFN- γ and IL-10 and controlled iNOS activity is indispensable for the resolution of myocarditis. In terms of treatment, it can be assumed that combined administration of IFN- γ and IL-10 might provide a rational therapy of inflammatory cardiomyopathy under the condition that routes of administration and possible side effects are taken under particular consideration.

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