Myeloid differentiation factor-88 contributes to TLR9-mediated modulation of acute coxsackievirus B3-induced myocarditis in vivo

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VIRAL MYOCARDITIS belongs to one of the important causes of acute and chronic heart failure (23). Especially in young patients who are otherwise healthy, myocarditis may lead to severe left ventricular (LV) dysfunction and dilated cardiomyopathy (16). Coxsackievirus B3 (CVB3) remains a typical cause of viral myocarditis (10, 43). CVB3-induced myocarditis is characterized by the viral infection of cardiomyocytes followed by a cardiac inflammatory response including cell infiltration (18, 30, 44). In this regard, immune activation leading to proinflammatory signaling constitutes a hallmark of viral myocarditis and is believed to trigger LV dysfunction and chronic heart failure in this disease (18).

The innate immune system is mainly responsible for the immediate inflammatory response after viral infection (6). This system recognizes pathogen-associated patterns with the help of a broad panel of pattern recognition receptors, including Toll-like receptors (TLRs) (6). The activation of TLRs via a variety of endogenous and exogenous ligands results in the gene activation of a variety of cytokines in several cell types (1). Some TLRs, namely, TLR2 and TLR4, have been shown in experimental studies to sufficiently trigger LV dysfunction under ischemic hypertrophic, toxic, and septic conditions (9, 17, 27, 33, 35–38, 41). Furthermore, TLR4 has been demonstrated to regulate viral load and cardiac function in CVB3-infected myocarditis (6). However, the role of TLR9, known to be involved in viral infections as well, has not been investigated under these conditions (46). This receptor subtype is endosomally located and can be activated, among other things, by bacterial and viral patterns as well as danger proteins derived from cell damage processes as apoptosis (24, 40). When activated, TLR9 uses (as do all other mammalian TLRs except TLR3) an intracellular signaling pathway that is dependent on the adapter protein myeloid differentiation factor 88 (MyD88) (1, 10). This has recently been shown to decrease cardiomyocyte contractility (19). Whereas the subtypes TLR2 and TLR4 as well as MyD88 have been demonstrated to influence the development of infectious and/or noninfectious cardiovascular diseases, the role of TLR9 in viral myocarditis is still unknown. Therefore, we investigated whether or not TLR9 modulates the development of virus-induced cardiac damage and which mediators are involved in experimental CVB3-induced myocarditis.

MATERIALS AND METHODS

Experimental animals. Male C57BL/6 wild-type (WT) mice and TLR9-deficient [knockout (KO)] mice, which carrying a deletion of the TLR9 gene, were obtained from the breeding stocks of the Max-Planck-Institut für Immunologie (Freiburg, Germany) (15). All mouse strains were bred in the Forschungsinstitut für Experimentelle Medizin (Berlin, Germany). WT and KO mice, aged 8 to 10 wk, were randomly selected into four groups for inoculation with CVB3 (Nancy strain, 5 × 107 plaque-forming units ip) diluted in 0.2 ml saline as previously described (16). Sham infection (n = 8 mice/group) consisted of an injection of 0.2 ml of saline intraperitoneally together with LPS on days 0 and 4. Seven days (n = 8 mice/group) and 28 days (n = 8 mice/group) after viral infection, mice were hemodynamically characterized. Finally, hearts were excised and prepared for molecular biological and immunohistochemical analyses as described below. This investigation conformed with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996). This study was approved by the Landesamt für Gesundheit und Soziales (Berlin, Germany; No. G 0277).

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function was measured by $dP/dt$.

**Biotechnology**, goat anti-CD4 (1:50, Pharmingen), rat anti-CD8a (1:

sections were incubated with goat anti-CD3 (1:25, Santa Cruz

munohistological analyses were performed in transversely dis-

immediately snap frozen in liquid nitrogen and stored at

netek). For molecular biological analyses, isolated heart tissues were

tissues were embedded in OCT compound (Tissue Tec, Sakura Fi-

by stroke volume (SV; in

loops in an open chest model (34, 47, 48). Systolic function was

the LV for the continuous registration of LV pressure-volume (P-V)

ventilated. As previously described, a 1.4-Fr microconductance pres-

output (CO; in ml/min).

Animals were anesthetized (125 $\mu$g/g ip thiopental), intubated, and artificially

ventilated. The animals were subsequently assessed 7 and 28 days after

infection; †

Postinfection; $P < 0.05$ vs. WT and KO mice.

LV function after CVB3 infection. WT, wild type; KO, Toll-like receptor 9-deficient (knockout) mice. * $P < 0.05$ vs. WT; CVB3: coxsackievirus type 3. †

<table>
<thead>
<tr>
<th></th>
<th>WT Mice KO Mice</th>
<th>WT Mice KO Mice</th>
<th>WT Mice KO Mice</th>
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<tbody>
<tr>
<td>Heart rate, beats/min</td>
<td>431 ± 34</td>
<td>423 ± 14</td>
<td>215 ± 47*</td>
</tr>
<tr>
<td>LV pressure, mmHg</td>
<td>92 ± 3</td>
<td>98 ± 1</td>
<td>59 ± 6*</td>
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<tr>
<td>$dP/dt_{max}$, mmHg/s</td>
<td>7,557 ± 232</td>
<td>7,741 ± 947</td>
<td>3,014 ± 701*</td>
</tr>
<tr>
<td>LV end-diastolic pressure, mmHg</td>
<td>6 ± 1</td>
<td>5 ± 2</td>
<td>8 ± 2*</td>
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<tr>
<td>$dP/dt_{min}$, mmHg/s</td>
<td>−5,417 ± 440</td>
<td>−5,374 ± 398</td>
<td>−2,076 ± 662*</td>
</tr>
<tr>
<td>Cardiac output, ml/min</td>
<td>11.1 ± 4</td>
<td>11.7 ± 1.6</td>
<td>4.5 ± 2.1</td>
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<tr>
<td>Stroke volume, ml</td>
<td>44 ± 4</td>
<td>46 ± 7</td>
<td>17 ± 2</td>
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Values are means ± SE; $n = 8$ mice/group. Left ventricular (LV) function was assessed using a microconductance catheter 7 and 28 days after coxsackievirus B3 (CVB3) infection. WT, wild type; KO, Toll-like receptor 9-deficient (knockout) mice. * $P < 0.05$ vs. WT, KO, and CVB3-infected KO mice at 7 days postinfection; † $P = 0.05$ vs. WT and KO mice.
Statistical analyses. Statistical analysis of animal experiments was performed using ANOVA, a nonparametric Kruskal-Wallis test, and a Mann-Whitney U-test to compare groups. Values are means ± SEM (n = 8 per group). WT: wild-type, CVB3: coxsackievirus type 3, d: days post infection; *P < 0.05 vs. WT, KO, KO-CVB3 7d, WTCVB3 28d and KO-CVB3 28d, respectively.

RESULTS

TLR9 expression in cardiac tissue. As shown in Fig. 1, CVB3-infected WT mice displayed significant upregulation of TLR9 mRNA only in the acute phase of 7 days after CVB3 infection but not in the chronic phase of 28 days after CVB3 infection compared with WT control mice (n = 8 mice/group).

LV function after CVB3 infection in mice. Hemodynamic data are shown in Table 1 (n = 8 mice/group). No differences in the parameters of systolic, diastolic, and global LV function were found between WT and KO mice under basal conditions. In agreement with previous findings, CVB3-infected WT mice displayed significant impaired systolic (LVESP: −36% and...
levels compared with uninfected KO mice (after CVB3 infection. In CVB3-infected KO mice, there were same time point (after 7 days compared with CVB3-infected WT mice at the /H9252 contrast, collagen type I protein and mRNA as well as TGF-

CVB3 by RT-PCR (expression.

/H9252 there were no significant differences between CVB3-infected

Twenty-eight days after CVB3 infection, the viral load in both

in left ventricular tissues. Data are expressed as mean ± SEM. WT: wildtype, CVB3: coxsackievirus type 3, KO: TLR9 deficient mice. N. s. = non significant.

Fig. 3. Cardiac mRNA expression of CVB3. Murine cardiac CVB3 mRNA expression were measured 7 days after CVB3 infection by real-time RT PCR in left ventricular tissues. Data are expressed as mean ± SEM. WT: wildtype, CVB3: coxsackievirus type 3, KO: TLR9 deficient mice. N. s. = non significant.

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the chronic phase of CVB3 infection, TLR9 seems not to play a role for the cardiac phenotype under these conditions.

Several TLRs have been suggested to be involved in viral myocarditis. Thus, a causal link between TLR4 and CVB3 replication has been found (8). Others (42) have described a significant increase, especially of TLR7 and TLR8, in myocytes after CVB3 infection, suggesting a regulative function of the innate immune system under these conditions. However, the role of TLR9 in viral myocarditis has, to our knowledge, not yet been investigated. It is known that TLR9 can recognize several DNA virus types. For example, it has been shown that both murine cytomegalovirus as well as herpes simplex virus 1 and 2 can stimulate dendritic cells via TLR9 (46). The present study suggests that the acute, but not the chronic, pathogenicity of CVB3 is affected by TLR9. TLR9 is unable to directly recognize RNA viruses, as is indeed the case for CVB3. However, both interactions recently described between TLR9 and TLR7/TLR8 and DNA-bacterial coinfection could be responsible for a potential modulation of CVB3-induced myocarditis by TLR9 (28). Despite coregulation, stimulation by CVB3 in a double-strain manner after replication might be a target. At least a stimulation by CVB3-mediated upregulation of proteins such as high-mobility group box 1 or renal tumor antigen would also be a potential mediator for TLR9-induced myocardial damage in acute myocarditis (40).

To further investigate whether or not cardiac TLR9 expression patterns in hearts from CVB3-infected mice could have a causal link to the development of acute viral myocarditis, we performed experiments in the well-established TLR9 KO mouse model (15). Viral myocarditis caused by CVB3 in mice is characterized by the viral infection of cardiomyocytes followed by a pronounced infiltration of inflammatory cells, similar to pathophysiological mechanisms in humans (22, 23,

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Fig. 4. Infiltration of immune cells in the heart after CVB3-infection. Immunohistological analyses were performed in transversely dissected myocardial samples as previously described (20). Cardiac immunostaining of CD3+ (3A), CD4+ (3B), CD8a+ (3C), CD11b+ (3D) positive cells were quantified 7 and 28 days after CVB3 infection by digital imaging analyses. Data are depicted as mean ± SEM. Fig. 3F shows representative pictures of the immunostaining. Specific immune cells are colored red. Quantification and representative pictures of cardiac contents of ICAM-1 are shown in (E). WT: wildtype, CVB3: coxsackievirus type 3, KO: TLR9 deficient mice. *p < 0.05, n.s. = non significant.
In addition, the chronic phase of this model is characterized by the loss of myocardial CVB3 persistence (10). Moreover, CVB3 infection in mice results in a disease similar to the clinical heart disease observed in humans, with the development of acute myocarditis from day 7 to 14 after infection that later progresses into a chronic, autoimmune phase of the disease (7). In this regard, there is, as similar observed in humans, a high variation in CVB3 susceptibility dependent on the different mouse strains (16, 30). In agreement with previous findings, CVB3-infected WT mice in our study displayed impaired LV function and enhanced cardiac infiltration of immune cells (16, 21). TLR9 deficiency led to improved LV function in the acute phase of myocarditis, which was contributed by decreased infiltration of immune cells, known to be hallmarks in virus-induced cardiac dysfunction (10, 11, 18, 43), at the same time point. Interestingly, these effects were independent of cardiac CVB3 mRNA levels. We also observed, at least in part, increased cardiac cell infiltration 28 days after CVB3 infection compared with the 7-day time point in both WT and KO mice, respectively. In the light of similar and much lower viral load in both groups at the chronic time point, this increase of infiltration is most likely a result from secondary autoimmune reaction as also known in the CVB3 model (31). In contrast to these beneficial effects due to TLR9 deficiency, in the chronic phase of myocarditis, LV dysfunction was not modulated by TLR9, showing that, at least in our model, TLR9 triggers only acute and transient, but not chronic, myocardial damage resulting from CVB3 infection. This is also based on our findings that TLR9 deficiency led to a marked reduction of cardiac fibrosis, a hallmark of heart failure, only in the acute phase of myocarditis. This might also be affected by a suppression of one of the most important profibrotic cytokines, TGF-β, since cardiac regulation is also affected at this time point (29).

To better understand the underlying mechanisms of the cardiac phenotype due to TLR9 deficiency, we investigated TLR9 signal-

![MyD88 expression in the heart](image)

![Cardiac TNF-α and IFN-β expression after CVB3-infection](image)

![NF-κB activity](image)
ing. TLR9 activates the adapter protein MyD88 for intracellular signaling, which has been shown to sufficiently modulate CVB3-induced myocarditis in mice (10). In line with this study, markedly increased expression of this protein in CVB3-infected hearts has been found (10). It was also demonstrated that MyD88 can trigger mortality in this disease (10). In addition, MyD88 has been shown to modulate LV function in several types of heart failure (12, 13). TLR9 deficiency in our study led to a significant decrease of cardiac MyD88 expression during the acute phase of CVB3 infection compared with infected WT mice, suggesting that the beneficial effects in infected TLR9-deficient mice are mediated by downregulation of the MyD88-dependent signaling pathway. This hypothesis was also strengthened by our findings of a main target transcription factor of MyD88, namely, NF-κB. In line with MyD88 expression, cardiac NF-κB was activated in WT mice within the acute phase of CVB3 infection. TLR9-deficient mice showed reduced binding activity of this transcription factor at the same time point compared with CVB3-infected WT mice.

In contrast to the findings by Fuse et al. (10), who found improved cardiac viral control in MyD88-deficient mice, TLR9 deficiency did not lead to reduced viral load in CVB3-infected hearts, although MyD88 is known to be the main mediator for TLR9 signaling. Due to the fact that MyD88 is a pivotal adapter protein for all TLRs except TLR3, including TLR4, and is also known to influence cardiac CVB3 infection, it is likely that TLR9 deficiency alone might not suffice to completely inhibit MyD88 signaling (1, 6). This hypothesis is supported by the fact that the expression of MyD88 was not completely normalized in CVB3-infected TLR9-deficient mice. However, TLR9 deficiency was sufficient enough to reduce cardiac inflammation, including cardiac contents of TNF-α, which has been shown to be activated by TLR9 and, furthermore, to be a sufficient cardiodepressive cytokine (2, 4, 19). A number of studies in humans and animals have shown a relevant role for this cytokine regarding the development of heart failure. Circulating levels of TNF-α are independent predictors of mortality in patients with heart failure (26). TNF-α antagonism exerts cardioprotective effects in rats subjected to continuous TNF-α infusion, in mice with cardiac-specific TNF-α overexpression, and in experimental animal models of heart failure (14). Furthermore, it has been shown that TNF-α plays a causal role for CVB3 inoculation (32). In our study, KO mice after 7 days of CVB3 infection showed significantly lower levels of this cytokine in the heart, suggesting that this might be a possible effector cytokine of TLR9 signaling in viral myocarditis. However, in the chronic phase of myocarditis, TNF-α levels of both infected WT and KO mice were similar to uninfected controls, showing a low cardiac inflammation in the chronic phase of viral myocarditis, in at least in our model.

In addition, analyses of further effector cytokines that are possibly involved in the TLR9-mediated cardiac deleterious changes during CVB3 infection revealed a significant upregulation of the antiviral IFN-β in the heart. We and others have previously shown in experimental and clinical settings that the cytokine IFN-β can lead to an elimination of viral genomes and to an improvement of LV function in patients and animals with enteroviral or adenoviral persistence and LV dysfunction (5, 10, 43). Since IFN-β gene induction is more under control of TLR3 and TLR4 through TIR domain-containing adaptor-inducing IFN-β than directly by TLR9-dependent MyD88 activation, the IFN-β activation in TLR9-deficient mice during acute viral infection is most likely a secondary effect possibly due to an interaction with other components of the TLR system (25). Taken together, our data show that TLR9 is a sufficient modulator of LV function in the acute phase of viral myocarditis, but not in the chronic phase, in our CVB3 model. The cardioprotective effects of TLR9 deficiency here, including changed LV function and reduced cardiac inflammation in acute myocarditis, possibly affected by suppressed cardiac TNF-α via suppressed MyD88 as well as activation of IFN-β.

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GRANTS

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DISCLOSURES

No conflicts of interest are declared by the author(s).

REFERENCES
