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TRIF Is a Critical Survival Factor in Viral Cardiomyopathy

Alexander Riad,* Dirk Westermann,† Christin Zietsch,‡ Konstantinos Savvatis,† Peter M. Becher,‡ Stefan Bereswill,§ Markus M. Heimesaat,‡ Olga Lettau,† Dirk Lassner,† Andrea Dörner,† Wolfgang Poller,† Matthias Busch,* Stephan B. Felix,* Heinz P. Schultheiss,‡ and Carsten Tschöpe‡

TRIF is a member of the innate immune system known to be involved in viral recognition and type I IFN activation. Because IFNs are thought to play an important role in viral myocarditis, we investigated the role of TRIF in induced myocarditis in mice. Whereas C57BL/6 (wild-type) mice showed only mild myocarditis, including normal survival postinfection with coxsackievirus group B serotype 3 (CVB3), infection of TRIF−/− mice led to the induction of cardiac remodeling, severe heart failure, and 100% mortality (p < 0.0001). These mice showed markedly reduced virus control in cardiac tissues and cardiomyocytes. This was accompanied with dynamic cardiac cytokine activation in the heart, including a suppression of the antiviral cytokine IFN-β/2, pathway. TRIF is a part of the innate immune system that acts as a critical survival factor in viral cardiomyopathy.

Viral infection of the heart is a major cause of unexpected and sudden death in patients under 40 y of age (1). The clinical course of viral myocarditis varies from limited cardiac disease to fulminant cardiac injury and severe heart failure leading to increased morbidity and mortality in those patients (2). The endemic single-stranded coxsackievirus group B serotype 3 (CVB3) is among other things cytopathic for cardiac cells in both humans and mice and can cause severe myocarditis (3). Especially in infants and adolescents, CVB serotypes including type 3 are often implicated as being present in up to 45% in patients with acute myocarditis or dilatative cardiomyopathy (4, 5). Intense inflammatory response, including proinflammatory cytokine activation and immune cell infiltration, is often observed under those conditions and are thus suggested to be involved in cardiac damage such as left ventricular (LV) dysfunction and remodeling. Additionally, we and others have shown previously 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 entero visceral or adenoviral persistence and LV dysfunction (6–8).

The production of IFN-β, especially during its early activation, is substantially under control of the intracellular TRIF-dependent pathway. TRIF is a part of the innate immune system that acts as an intracellular adaptor molecule of TLR3 and TLR4 (TLR signaling is reviewed in Ref. 9). In brief, TLR3 and TLR4 are members of 13 functional mammalian TLRs that have been discovered so far. They are localized subcellulary either on the cell surface or in intracellular vesicular compartments (10). Although TLRs are expressed at low levels in a large number of cells, including nonimmune cells, the highest expression is generally on immune cells such as macrophages or lymphocytes, indicating a critical role of TLRs in function of those cells, including cytokine production (11). In the case of TLR4, this receptor has been demonstrated to modulate virus load and cardiac function in viral myocarditis (12, 13). However, the physiological role of TRIF in cardiac diseases, including viral myocarditis, still remains unclear. In the present study, we investigated whether TRIF affects the development of CVB3-induced myocarditis in a mouse model.

Materials and Methods

Study design

TRIF−/− mice with the genetic background of C57BL/6 were generated and maintained as described previously (14). Mutant mice homozygous for the disrupted Trif allele were born at the expected Mendelian ratio and grew to be healthy in specific pathogen-free conditions. Twelve-week-old male C57BL/6 wild-type (WT, n = 60) and TRIF knockout mice (TRIF−/−, n = 83) were obtained from the breeding stocks of the Max-Planck-Institut für Immunologie (Freiburg, Germany) (14). All mouse strains were bred in the Forschungsinstitut für Experimentelle Medizin (Berlin, Germany). This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Bethesda, MD; Publication No. 85-23, revised 1996). WT (n = 52) and TRIF−/− (n = 75) mice were inoculated i.p. with CVB3 (Nancy strain) on day 0 as described previously (7). Saline-treated WT (n = 8) and TRIF−/− (n = 8) mice served as controls.

Animals were euthanized 12, 24, 48, and 72 h (n = 6/group) as well as 7d (n = 8/group) after CVB3 infection. Cardiac tissue was prepared for immunohistological and molecular biological analyses. Seven days after CVB3 infection, left heart function was measured before the animals were euthanized. In a mortality study, CVB3-infected WT (n = 20) and TRIF−/− (n = 11) mice were observed for 60 d. Furthermore, CVB3-infected TRIF−/− mice were treated with murine IFN-β (100 kg U i.p., every second day until day 14; Bayer-Scheringer) (n = 20) and the survival rate was then documented. This time frame for IFN-β treatment was used because the acute

Abbreviations used in this article: CAR, coxsackievirus-associated receptor; ΔCp, comparative Cq; CVB3, coxsackievirus group B serotype 3; LV, left ventricular; WT, wild-type.

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phase in this model ends on day 14 (15, 16). Additionally, CVB3-infected TRIF−/− mice were treated with murine IFN-β (100 kg U i.p. at days 2, 4, and 6) (n = 6) and obtained for 7 d. Then, heart tissue was prepared for analyses according to those techniques used for CVB3-infected WT and TRIF−/− mice.

Tissue preparation
After euthanization, LV tissues were excised and then snap frozen immediately in liquid nitrogen and stored at −80°C for molecular biological and immunohistological analyses.

Surgical procedures
Seven days after CVB3 infection, WT, TRIF−/−, WT CVB3, and TRIF−/−CVB3 mice were anesthetized (thiopental 125 μg/g i.p.), intubated, and artificially ventilated (n = 6/group). Heart rate (in beats per minutes), end-diastolic volume (μl), end-systolic volume (μl), stiffness index (mmHgμl), isovolumetric relaxation (ms), stroke volume (μl), cardiac output (μl/min), and ejection fraction (%) as indices of LV function were recorded via a microconductance catheter (1.4F system in closed chest animals as described previously (17).

RNA isolation from heart tissue and isolated cardiomyocytes
Total RNA was extracted from LV sections by the TRIzol method (Invitrogen, Carlsbad, CA) as described previously (18). Additional purification was performed using the RNeasy Mini kit (Qiagen, Hilden, Germany). The yield of purified total RNA was determined by measuring the ultraviolet absorbance at 260 nm on the NanoDrop ND-1000 spectrophotometer (Agilent Technologies, Boeblingen, Germany). RNA was reverse transcribed (LV tissue, 1 μg up to a final volume of 100 μl cDNA; cardiomyocytes, 0.25 μg to a final volume of 60 μl) using the High Capacity cDNA Archive kit (Applied Biosystems, Darmstadt, Germany).

TaqMan low-density arrays in heart tissue
The relative quantification of targets was performed using the comparative CΔCT (ΔΔCt) method on an Applied Biosystems 7900HT system (19, 20). Wells (n = 384) of TaqMan Arrays were preloaded with TagMan Gene Expression Assays (two replicates per assay). Then, 1000 ng of total RNA converted to cDNA, and mixed with an equal volume of TaqMan Universal PCR Master mix, was loaded into fill reservoirs of TaqMan Arrays. The real-time PCR was performed according to the manufacturer’s instructions.

Each TaqMan Gene expression Assay contains a forward and reverse primer for each of the genes chosen (collagen I, collagen III, 18S ribosomal, CVB3, coxsackievirus-associated receptor (CAR), IFN-β, TRIF-α, IL-1β, IL-10, and IL-18). 18S RNA was incorporated into our customized TaqMan Low-Density Array as internal standards.

Quantification of protein contents of cytokines
As previously described (21, 22), we quantified protein expression levels of IG-18, IL-1β, and TNF-α in cardiac tissues from CVB3-infected and noninfected controls using FlowCytomix Pro (Bender MedSystems) and commercially available kits (TNF-α lot 59408009, IL-18 lot 58777002, IL-1β lot 59270003; Bender MedSystems). For the measurement, 50 μg protein was used per heart.

Immunostaining of cardiac-infiltrating immune cells and collagen I and III
As described previously (18, 23, 24), immunostaining of infiltrating immune cells and collagen I and III in LV tissue was carried out with specific Abs using commercially available kits ( Vectastain). Specific primary goat anti-CDS (Santa Cruz Biotechnology; 1:75), rat anti-CD4 (BD Pharmingen; 1:50), rat anti-CD68 (Abcam; 1:350), and Armenian hamster anti-CDS (BD Biosciences; 1:50) Abs were used in conjunction with the secondary biotinylated goat anti-rat (Dako; 1:200), biotinylated goat anti-Armenian hamster (Dianova; 1:200), peroxidase-conjugated goat anti-Armenian hamster Ab (Dianova; 1:100), peroxidase-conjugated goat anti-rabbit Ab (Dako; undiluted), and biotinylated anti-Armenian hamster (Dianova; 1:1000), respectively. Quantification was performed by digital image analyses as described previously (18). In brief, the ratio between heart tissue area and the specific chromogen positive area was calculated (depicted here as area fraction, in percentage). The amount of infiltrating cells was calculated by measuring the number of cells per area of heart tissue (cells/mm²).

CVB3 plaque assay
As described previously (25), heart tissue samples were weighed and homogenized in 2 ml serum-free DMEM. Serially 10-fold–diluted aliquots of 1 ml homogenized tissue were added to each well of six-well plates, which were plated with confluently grown HeLa cells. Cells were incubated for 30 min at 37°C and 5% CO2. Then, the inoculated and uninfected cells were overlaid with 2 ml Eagle’s overlay medium. After 3 d, cells were stained with 0.025% neutral red in PBS for 4 h. The staining solution was removed, virus plaques were counted, and the titer was calculated in regard to tissue net weight.

Cell culture experiments
Isolation and cultivation of primary mouse embryonic cardiomyocytes. For the purposes of isolation and cultivation of primary mouse embryonic cardiomyocytes, WT and TRIF−/− mice have been used. The pregnant mice were put to death by CO2 asphyxiation. The embryos (12 d old) were isolated from the uterus and then transferred into a dish with ice-cold sterile PBS without Ca2+ and Mg2+ (PAA, Paschau, Austria). The embryos were released from the yolk sacs and then transferred into another dish with the same solution as described above. By the use of surgical instruments, every single heart was isolated under a microscope. The heart was incubated at 4°C for 20 h and subsequently at 37°C for 15 min in a solution of 0.05% trypsin and 0.02% EDTA (w/v) (Biochrom, Berlin, Germany). The tissue/trypsin/EDTA mix has been included in 1 ml DMEM (high glucose, 4.5 g/l) without l-glutamine (PAA Laboratories, Linz, Austria) with 10% FCS and 1% each penicillin and streptomycin (Biochrom). To dissociate the heart, tissue was gently pipetted several times. The mix gained by this procedure contains fibroblasts and cardiomyocytes. The cells within the medium from each heart of the same genotype were transferred into a cell culture flask (TPP, Trasadingen, Switzerland) to seed the fibroblast. The cultures were maintained for 1 h at 37°C in a humidified atmosphere with 5% CO2. The supernatant containing

Table 1. Hemodynamic parameters 7 d after CVB3 infection

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>CVB3</th>
<th>TRIF−/−</th>
<th>TRIF−/−CVB3</th>
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<tbody>
<tr>
<td>Heart rate, beats per minute</td>
<td>423.5 ± 18.23</td>
<td>394.1 ± 25.69</td>
<td>384.9 ± 10.90</td>
<td>364.8 ± 1632</td>
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<td>LV pressure, mmHg</td>
<td>76.50 ± 5.268</td>
<td>68.07 ± 4.865*</td>
<td>74.42 ± 2.757</td>
<td>47.53 ± 3.282</td>
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<td>dP/dt maximum, mmHg/s</td>
<td>4529 ± 328.4</td>
<td>4132 ± 299.7*</td>
<td>4700 ± 579.1</td>
<td>2631 ± 260.5</td>
</tr>
<tr>
<td>Stroke volume, μl</td>
<td>16.02 ± 1.861</td>
<td>14.41 ± 1.797*</td>
<td>17.01 ± 2.028</td>
<td>9.361 ± 1.059</td>
</tr>
<tr>
<td>Cardiac output, ml/min</td>
<td>6.835 ± 0.591</td>
<td>5.480 ± 0.916*</td>
<td>6.549 ± 0.756</td>
<td>3.282 ± 0.401</td>
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<td>Ejection fraction, %</td>
<td>66.36 ± 3.141</td>
<td>62.72 ± 5.265*</td>
<td>69.06 ± 4.264</td>
<td>36.52 ± 1.466</td>
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<td>End-systolic volume, μl</td>
<td>12.73 ± 3.201</td>
<td>11.59 ± 4.291*</td>
<td>11.34 ± 2.833</td>
<td>17.72 ± 1.676</td>
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<td>LV end-diastolic pressure, mmHg</td>
<td>6.8 ± 2.6</td>
<td>5.8 ± 0.47</td>
<td>6.3 ± 0.48</td>
<td>5.2 ± 0.40</td>
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<td>dP/dt minimum, mmHg/s</td>
<td>−3857 ± 252.2</td>
<td>−3312 ± 363.0*</td>
<td>−3911 ± 383.7</td>
<td>−1949 ± 222.5</td>
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<td>Isovolumetric relaxation, ms</td>
<td>19.41 ± 0.8588</td>
<td>16.01 ± 1.217</td>
<td>17.27 ± 0.8704</td>
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<td>Stiffness index, mmHg/μl</td>
<td>0.08 ± 0.015*</td>
<td>0.15 ± 0.021*</td>
<td>0.09 ± 0.016</td>
<td>0.36 ± 0.057</td>
</tr>
</tbody>
</table>

Hemodynamic parameters were measured in vivo using a 1.2F microconductance catheter to assess pressure-volume 7 d after CVB3 infection (n = 8/group). Data are expressed as means ± SEM.

*p < 0.05 versus TRIF−/−CVB3 mice. dP/dt, rate of LV pressure rise.
the cardiomyocytes was removed gently, counted, and then plated out to an amount of $1 \times 10^5$ cardiomyocytes in a 12-well plate (TPP) coated with fibronectin (Biochrom). The growth medium was changed after 16 h. Twenty-four hours later, BrdU (Sigma-Aldrich, Steinheim, Germany) was added to a final concentration of $10^{-2}$ M BrdU. After a further 24 h, the cells were stimulated or infected.

FIGURE 1. A mortality study was performed in CVB3-infected C57BL/6 mice (WT, n = 20), TRIF$^{-/-}$ mice (n = 11), and TRIF$^{-/-}$ CVB3 mice treated with murine IFN-β in the acute phase of myocarditis ($^*p < 0.001$ versus TRIF$^{-/-}$ CVB3 mice) (A). Hemalaun and eosin staining (n = 6/group) showed mild deterioration of LV tissue from WT CVB3 mice (C) when compared with noninfected WT (B) and TRIF$^{-/-}$ (D) mice. In contrast, TRIF$^{-/-}$ CVB3 mice (F) displayed extensive destruction of the heart and cell infiltration. Collagen I and III content was quantified by real-time RT-PCR ($^*p < 0.001$ versus WT CVB3 mice at 7 d, $^*p < 0.001$ versus WT CVB3 mice at 72 h) and by immunostaining (n = 6/group; $^*p < 0.001$ versus WT CVB3 mice at 7 d) (F). Representative images of immunostaining of collagen I and III in LV tissue 7 d after CVB3 infection are shown in G. Collagen-specific epitopes are colored red (G). Data are expressed as means ± SEM.
Stimulation with LPS. Cardiomyocytes were incubated in DMEM plus 10% FCS containing the TLR4 agonist LPS at a final concentration of 1 μg/ml for 4 h and 24 h at 37°C in a humidified atmosphere with 5% CO₂. Stimulation was stopped by aspiration of medium and cell lysis for RNA isolation.

Infection with CVB3. CVB3 used in this study was of the Nancy strain (ATCC VR-30; provided by Karin Kingel). The embryonic cardiomyocytes were inoculated with CVB3 at a multiplicity of infection of 1 PFU per cell in DMEM without FCS, penicillin, and streptomycin at 37°C for 30 min. After 30 min, the medium containing CVB3 was removed and replaced by DMEM containing 10% FCS and 1% each penicillin and streptomycin.

Statistical analysis
Statistical analysis was performed using SPSS version 12.0. Data are expressed as the mean ± SEM. Statistical differences were assessed by using the Kruskal–Wallis test in conjunction with the Mann–Whitney U test. Survival curves after CVB3 infection were created by utilizing the Kaplan–Meier method and compared by using the log-rank test. Differences were considered to be statistically significant at a value of p < 0.05.

Results
Exacerbation of myocardial damage in TRIF⁻/⁻ mice after CVB3 infection
To study the physiological consequences of TRIF regarding the development of viral myocarditis, we infected 12-wk-old C57BL/6 mice, which showed a mildly impaired LV function when compared with healthy WT mice, which was not statistically significant. In contrast, TRIF⁻/⁻ CVB3 mice displayed significantly impaired LV function when compared with healthy TRIF⁻/⁻ mice, which were indexed by decreased stroke volume (9 ± 1 versus 17 ± 2 μl; p < 0.05), cardiac output (2.9 ± 0.4 versus 6.3 ± 0.8 ml/min; p < 0.05), and ejection fraction (36 ± 1 versus 69 ± 4%; p < 0.05), whereas heart rate measurements did not differ significantly (Table I). As seen in clinical and experimental settings regarding the acute phase of myocarditis, the end-diastolic volume did not differ. In line with signs of severe heart failure in TRIF⁻/⁻ CVB3 mice, hemalaun and eosin staining showed severe myocardial damage, including marked cell infiltration (Fig. 1B–E). Additionally, immunostaining and RT-PCR measurements of the left heart 12, 24, 48, and 72 h as well as 7 d after CVB3 infection showed a significant induction of cardiac fibrosis in TRIF⁻/⁻ CVB3 indexed by increased mRNA and protein contents of collagen I and III when compared with WT CVB3 mice. Whereas mRNA differences rose within 72 h post-infection (p < 0.001), protein production of collagens 7 d post-infection was seen to be statistically considerably different (collagen I, 36-fold; collagen III, 9.7-fold; p < 0.001). Cardiac deterioration in the acute phase of the disease led further to 100% mortality of the TRIF⁻/⁻ CVB3 mice after 70 d, whereas none of the WT CVB3 mice died (p < 0.0001) (Fig. 1A).

Reduced control of CVB3 virus in cardiac tissue form TRIF⁻/⁻ mice
To assess the development of cardiac virus control, we analyzed the cardiac mRNA contents of CVB3 by RT-PCR and CVB3 replication using a plaque assay in LV tissues from WT and TRIF⁻/⁻ mice 12, 24, and 48 h and 7 d after CVB3 infection (Fig. 2A,2B). Both CVB3

FIGURE 2. CVB3 mRNA contents were measured using real-time RT-PCR in LV tissues (n = 6/group) (A). Up to 24 h postinfection, the virus could be detected in both WT and TRIF⁻/⁻ mice, and up to 48 h, there was a significant increase of CVB3 mRNA in TRIF⁻/⁻ mice (*p < 0.001 versus WT CVB3 mice at 48 h). Additionally, LV virus replication, determined by using a plaque forming assay, was detectable in both animal groups 24 h postinfection, and a significant increase of virus replication in TRIF⁻/⁻ CVB3 mice was assessed 7 d postinfection (n = 6/group) (*p < 0.001 versus WT CVB3 mice at 7 d) (B). Cardiac mRNA expression of CAR (C) determined by real-time RT-PCR 7 d postinfection was significantly suppressed in WT CVB3 mice when compared with WT controls (n = 6/group) (*p < 0.05), but there was no statistical difference between WT CVB3 and TRIF⁻/⁻ CVB3 mice. Data are expressed as means ± SEM.
mRNA and replication were detectable up to 24 h after CVB3 infection. Whereas mRNA expression of CVB3 reached a significant increase in TRIF−/− mice when compared with WT mice 48 h postinfection (48 h, 2.6-fold; 7 d, 16.3-fold; \( p < 0.001 \)), this significant difference was also seen after 7 d on viral replication level determined by a plaque assay (13.6-fold; \( p < 0.0001 \)). Additionally, the cardiac mRNA expression of the receptor for CVB3, the CAR (26), was significantly decreased in WT CVB3 mice when compared with WT mice (\( p < 0.05 \)). However, CAR expression did not differ significantly between WT CVB3, TRIF−/− CVB3, and TRIF−/− mice (Fig. 2C).

**TRIF-dependent cytokine activation after cardiac CVB3 infection**

We measured cytokine activation in hearts from WT and TRIF−/− mice from those cytokines that were associated with viral myocarditis and under control of TLR signaling (27, 28) by RT-PCR 12, 24, 48, and 72 h and 7 d after CVB3 infection to evaluate the development of TRIF-dependent cardiac cytokine activation in response to viral infection (Fig. 3). As expected, at least low LV cytokine levels were detectable in all animal groups, including healthy mice. We observed 7 d postinfection a general exacerbation of cardiac cytokines in TRIF−/− hearts indexed by increased mRNA levels of TNF-α (3.5-fold), IL-1β (3.9-fold), IL-10 (4.1-fold), and IL-18 (3.4-fold) (\( p < 0.001 \)). In contrast, in the very early phase of cardiac viral infection, cytokine levels showed significant differential expression patterns up to 72 h after CVB3 infection when compared TRIF−/− and WT mice. In the case of IL-18, we observed also a significant regulation up to 24 h after CVB3 infection among the groups. As shown in Fig. 4, we also quantified the protein expression of the cytokines IL-18, IL-1β, and TNF-α in cardiac tissue. In line with the mRNA expression profile, all three cytokines were significantly increased in TRIF−/− CVB3 mice 7 d after CVB3 infection when compared with WT CVB3 mice at the same time point (\( p < 0.05 \)). Additionally, in the case of IL-1β, we observed also a significant increased upregulation in TRIF−/− CVB3 mice 3 d after CVB3 infection when compared with WT CVB3 mice at the same time point (\( p < 0.05 \)).

**Infiltration of immune cells after CVB3 infection**

To identify possible sources for TRIF-dependent cytokine regulation, we investigated cardiac immune cell infiltration accordingly to the observation time points of cytokine expression patterns by immunohistochemical analyses (Fig. 5). In line with cytokine expression patterns, 7 d after CVB3 infection, WT CVB3 mice displayed significantly increased cardiac amounts of CD3+, CD4+, CD8+, and CD11b+.
CD68⁺ and CD80⁺ cells (p < 0.001) compared with those of healthy WT mice. Furthermore at this time point, cardiac immune cell infiltration in TRIF⁻/⁻ CVB3 mice was even significantly higher when compared with WT CVB3 mice. This was indexed by increased amounts of CD3⁺ and CD4⁺ T lymphocytes (5.3-fold and 3.9-fold, respectively; p < 0.001), CD68⁺ macrophages (3.2-fold; p < 0.001), and CD80⁺ activated B cells (4.1-fold; p < 0.001).

After 3 d, both CVB3-infected WT and TRIF⁻/⁻ mice showed significantly increased cardiac CD4⁺ and CD80⁺ cell infiltration when compared with healthy littermates, but no significantly different regulation among each other was observed except in the case of CD68⁺ macrophages, which were reduced in infected TRIF⁻/⁻ mice at this time point (p < 0.05).

Reduced virus control in TRIF⁻/⁻ mice

To investigate the virus control of myocytes during myocarditis, we determined mRNA expression level in hearts from WT and TRIF⁻/⁻ mice 12, 24, 48, and 72 as well as 7 d after CVB3 infection (Fig. 7A). At least a low-grade expression of IFN-β was observed in all animal groups, including healthy controls. In line with analyses from LV cell infiltration and cytokine activation, which showed a general exacerbation 7 d after CVB3 infection, IFN-β mRNA expression also was significantly increased in infected TRIF⁻/⁻ mice at this time point (11.0-fold; p < 0.001). However, after CVB3 infection 3 d earlier, this IFN-β mRNA expression was significantly decreased in CVB3 infected TRIF⁻/⁻ mice when compared with infected WT mice (−73%; p < 0.001). To investigate whether TLR4 is involved in TRIF-dependent regulation of IFN-β, we isolated cardiac myocytes from WT and TRIF⁻/⁻ mice and performed a TLR4 stimulation with LPS (Fig. 7B).

Stimulation of WT myocytes with LPS led to reduced IFN-β mRNA expression when compared with nonstimulated myocytes (p < 0.05). IFN-β activation in LPS-stimulated TRIF⁻/⁻ myocytes was abrogated as indexed by similar IFN-β mRNA expression levels from stimulated and nonstimulated TRIF⁻/⁻ myocytes. Stimulated WT myocytes showed significantly increased IFN-β mRNA levels when compared with stimulated TRIF⁻/⁻ myocytes (7.3-fold; p < 0.001). Then, we investigated the consequence of IFN-β on virus control and cardiac inflammation in vivo by treatment of infected TRIF⁻/⁻ mice with murine IFN-β, which showed reduced cardiac CVB3 (−72%; p < 0.001) and TNF-α (−41%; p < 0.01) mRNA levels when compared with cardiac expression in untreated CVB3-infected TRIF⁻/⁻ mice (Fig. 7C, 6D). Furthermore, in vivo treatment of TRIF⁻/⁻ mice with murine IFN-β after CVB3 infection led to a significant improvement of survival when compared with untreated CVB3-infected TRIF⁻/⁻ mice (mortality 20 versus 100%; p < 0.0001) (Fig. 1A).

Discussion

In the present study we have shown that the intracellular TLR mediator protein TRIF is essential for cardioprotection against CVB3 infection in mice. Loss of TRIF led to loss of virus control along with LV dysfunction, cardiac remodeling, and an increase in mortality postinfection. We showed, moreover, that this is associated with an impaired early IFN-β response in the heart. This is, to our knowledge, the first study showing physiological effects of TRIF in a cardiac disease.

Markedly increased CVB3 mRNA expression and CVB3 replication in hearts of TRIF-deficient mice clearly showed a loss of virus control. Furthermore, our cell culture experiments demonstrated that TRIF is directly essential for myocyte virus control. TRIF has been identified as an important mediator for antiviral host response to dsRNA and its analog polyinosinic-polycytidylic acid, both of which are recognized by TLR3 (14, 29). Our study showed that TRIF also affects infection with the single-stranded CVB3 virus. Several components of the TLR system have been suggested to trigger viral myocarditis. Thus, there had been found a significant role of TLR4 as an inducer for CVB3 replication (12). In line with these findings, mice deficient of the intracellular adaptor protein MyD88, which is used by all TLRs except TLR3, displayed cardiac protection in a similar CVB3 model as that used in the current study and in a recently published article on autoimmune myocarditis (7, 30). Additionally, others have described with our findings from LV tissue, CAR mRNA expression did not differ significantly among the groups (Fig. 6B).

TRIF relevantly regulates antiviral defense via IFN-β response

To investigate IFN-β response during viral myocarditis, we determined mRNA expression level in hearts from WT and TRIF⁻/⁻ mice 12, 24, 48, and 72 as well as 7 d after CVB3 infection (Fig. 7A). At least a low-grade expression of IFN-β was observed in all animal groups, including healthy controls. In line with analyses from LV cell infiltration and cytokine activation, which showed a general exacerbation 7 d after CVB3 infection, IFN-β mRNA expression also was significantly increased in infected TRIF⁻/⁻ mice at this time point (11.0-fold; p < 0.001). However, after CVB3 infection 3 d earlier, this IFN-β mRNA expression was significantly decreased in CVB3 infected TRIF⁻/⁻ mice when compared with infected WT mice (−73%; p < 0.001). To investigate whether TLR4 is involved in TRIF-dependent regulation of IFN-β, we isolated cardiac myocytes from WT and TRIF⁻/⁻ mice and performed a TLR4 stimulation with LPS (Fig. 7B).

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Discussion

In the present study we have shown that the intracellular TLR mediator protein TRIF is essential for cardioprotection against CVB3 infection in mice. Loss of TRIF led to loss of virus control along with LV dysfunction, cardiac remodeling, and an increase in mortality postinfection. We showed, moreover, that this is associated with an impaired early IFN-β response in the heart. This is, to our knowledge, the first study showing physiological effects of TRIF in a cardiac disease.

Markedly increased CVB3 mRNA expression and CVB3 replication in hearts of TRIF-deficient mice clearly showed a loss of virus control. Furthermore, our cell culture experiments demonstrated that TRIF is directly essential for myocyte virus control. TRIF has been identified as an important mediator for antiviral host response to dsRNA and its analog polyinosinic-polycytidylic acid, both of which are recognized by TLR3 (14, 29). Our study showed that TRIF also affects infection with the single-stranded CVB3 virus. Several components of the TLR system have been suggested to trigger viral myocarditis. Thus, there had been found a significant role of TLR4 as an inducer for CVB3 replication (12). In line with these findings, mice deficient of the intracellular adaptor protein MyD88, which is used by all TLRs except TLR3, displayed cardiac protection in a similar CVB3 model as that used in the current study and in a recently published article on autoimmune myocarditis (7, 30). Additionally, others have described with our findings from LV tissue, CAR mRNA expression did not differ significantly among the groups (Fig. 6B).
FIGURE 5. Cardiac expression of CD3$^+$ T lymphocytes, CD4$^+$-activated T lymphocytes, CD68$^+$ macrophages, and CD80$^+$-activated B cells was assessed by immunostaining and quantified by digital image analysis after CVB3 infection ($n = 6$ group). Seven days postinfection, TRIF$^-/-$ mice displayed exacerbated cardiac cell infiltration of CD3$^+$ (A), CD4$^+$ (C), CD68$^+$ (E), and CD80$^+$ (G) cells when compared with CVB3-infected WT mice. Up to 48 h postinfection, there was no significant induction of cell infiltration in both WT and TRIF$^-/-$ mice. Three days postinfection, TRIF$^-/-$ mice displayed, in contrast to that at the later time point, suppressed infiltration of CD68$^+$ macrophages (E) ($p < 0.05$ versus WT CVB3 mice at 72 h). Data are expressed as means ± SEM. Representative images of immunostaining of CD3$^+$ (B), CD4$^+$ (D), CD68$^+$ (F), and CD80$^+$ (H) immune cells show specific epitopes, which are colored red. Original magnification $\times 100$. 

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a significant increase especially of TLR7 and TLR8 in myocytes after CVB3 infection, thus suggesting a regulative function of the innate immune system under these conditions (31). In contrast to those previous studies, which suggested that the TLR system suppresses cardiac virus control of CVB3, the current study provides a novel role for this system since our data provide strong evidence that activation of the TRIF pathway is essential for a sufficient antiviral response against this virus.

This does not seem to be caused by the gene activation of the receptor for CVB3, namely CAR (26), since mRNA expression of CAR, which correlates with its protein levels (26), did not differ between WT and TRIF−/− mice after CVB3 infection. A number of studies suggested that the development of heart failure due to viral infection is determined by myocardial viral persistence (6, 7). In line with these data, increased virus load in the hearts of TRIF−/− mice went along with LV dysfunction. In agreement with previous studies, which demonstrated a relatively mild myocarditis in C57BL/6J mice exposed to CVB3 (15), WT mice in our study displayed no significant LV dysfunction 7 d postinfection, but they showed a deterioration in that direction. In contrast, TRIF deficiency under those conditions led to a reduced LV function after viral infection, which subsequently resulted in a heart rate-independent cardiac output decrease of ~53% when compared with noninfected TRIF−/− mice. Whereas other members of the TLR system, namely TLR2, TLR4, TLR9, and MyD88, have been shown to induce a decrease of contractility in different experimental models of heart failure and in myocyte cell culture, our study demonstrates protective effects of TRIF and therefore a new physiological role at least in regard to a distinct part of the cardiac TLR system (17, 18, 32–39).

There is a solid body of evidence that LV remodeling, which includes among other things the deterioration of the cardiac extracellular matrix, is an important pathogenetic factor for LV dysfunction in viral myocarditis (18, 23, 40). Viral infection with CVB3 led to an increase of fibrosis in infected WT mice. However, the absence of TRIF led to an exacerbation of fibrosis. To prove whether loss of virus control and cardiac damage due to TRIF deficiency is an initial, reversible situation, as is often seen in clinical settings, or whether it may affect the long-term outcome after CVB3 infection, we performed a survival study (2, 15). Deficiency of this protein led to induction of a 100% mortality within 70 d, whereas none of the WT mice died within this time frame. This clearly demonstrates the emerging role of TRIF in CVB3 defense.

Although the TLRs and their downstream adaptors including TRIF are thought to be nearly ubiquitously expressed in human and murine organisms, it is by far the highest expression levels that display immune cells, and this suggests a relevant role for those cells regarding TLR signaling (9, 41). In this regard, we found it to be of interest to identify possible immune cell populations in the heart that could be responsible for mediating increased inflammatory response in TRIF-deficient mice exposed to CVB3. Therefore, we measured the cardiac amounts of CD68+ macrophages, CD3+ T cells, CD4+ activated T cells, and CD80+ cells (e.g., activated B lymphocytes), which are all known to be a relevant source of cytokines and to be, at least in part, under the control of TLR signaling (42, 43). A marked increase of cardiac infiltration of all these cells in TRIF-deficient mice 7 d after CVB3 infection suggests a pathogenetic role for these cell types in our model. In line with markedly increased cell infiltration in TRIF−/− mice due to CVB3 infection at this time point, we also found at the same time a general exacerbation of cytokines that are regulated by the TLR system, suggesting that this overexpression of TNF-α, IL-1β, IL-10, and IL-18 in our study might be caused by a common effect of different immune cell populations. This could be a result due to activation of the MyD88 pathway as the second distinct signaling pathway of TLR4 in spite of the TRIF pathway, since loss of this important TLR adaptor led to a suppression of cardiac cytokine activation in the same animal model (7). A second cause for cytokine overactivation might be a secondary, MyD88-independent cytokine production as a result from viral infection itself or from cardiac damage. In contrast, in the initial phase of viral infection we observed in the case of IL-18 a TRIF-dependent regulation despite any regulation of cardiac-infiltrating cells. This demonstrates that other TLR-regulated cell types might also be involved in initiating immune response against CVB3 in the heart.

In this regard, we investigated further the antiviral cytokine IFN-β. Its production is initiated during the early stages of the innate immune response and is also a downstream target of TRIF. Therefore, it is thought to be a dominant factor in shaping downstream events in the innate immune responses (7, 44, 45). In the heart, loss of TRIF in our model led to a highly dynamic IFN-β response with significant early suppression of this antiviral cytokine. We then investigated the cardiac influence of TLR4 as a typical receptor of the immune response and is also a downstream target of TRIF. Its production is initiated during the early stages of the innate immune response against CVB3 in the heart.

FIGURE 6. Embryonic cardiomyocytes were isolated from WT and TRIF−/− mice to assess CVB3 mRNA (A) and CAR mRNA (B) expression as determined by real-time RT-PCR after viral infection. TRIF−/− cardiomyocytes displayed increased mRNA levels of CVB3 when compared with WT cardiomyocytes postinfection (*p < 0.01 versus all other groups), whereas CAR mRNA expression was not statistically different among the groups. Data are expressed as means ± SEM.
suggesting that IFN-\(\beta\) might contribute to the beneficial effects of TRIF in viral myocarditis. Moreover, our in vivo experiments showed that treatment of TRIF \(\textsuperscript{-/-}\) mice with IFN-\(\beta\) led to improved virus control and decreased inflammation in the heart. We have further undertaken to prove in the current study whether antiviral IFN-\(\beta\) effects are necessary in regard to TRIF-dependent cardiac protection against CVB3. To this end, we substituted TRIF-deficient mice with murine IFN-\(\beta\) during the viremic phase in our model (15). This led to the survival of TRIF-deficient mice being \(\sim 80\%\), which demonstrates that IFN-\(\beta\) treatment leads to a compensation of TRIF deficiency and shows that TRIF-dependent IFN-\(\beta\) activation belongs to an important protective mechanism in CVB3 myocarditis.

In summary, the current study shows that TRIF is essential in virus control of CVB3 in the heart. Loss of TRIF leads to exacerbation of viral replication, heart failure, and mortality in viral myocarditis. Our data suggest that early impaired IFN-\(\beta\) response may contribute to the consequences of TRIF deficiency seen in CVB3-induced murine myocarditis. To our knowledge, the present study provides the first evidence for TRIF as an essential cardioprotective TLR adaptor in viral myocarditis and anticipates a novel role for the TLR system concerning this disease.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**
