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TLR3 deficiency induces chronic inflammatory cardiomyopathy in resistant mice following coxsackievirus B3 infection: role for IL-4

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1Department of Environmental Health Sciences, Johns Hopkins University, Bloomberg School of Public Health and School of Medicine, Baltimore, Maryland; 2Department of Pathology, Johns Hopkins University, Bloomberg School of Public Health and School of Medicine, Baltimore, Maryland; and 3W. Harry Feinestone Department of Molecular Microbiology and Immunology, Johns Hopkins University, Bloomberg School of Public Health and School of Medicine, Baltimore Maryland

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Abston ED, Coronado MJ, Bucek A, Onyimba JA, Brandt JE, Frisancho JA, Kim E, Bedja D, Sung Y, Radtke AJ, Gabrielson KL, Mitzner W, Fairweather D. TLR3 deficiency induces chronic inflammatory cardiomyopathy in resistant mice following coxsackievirus B3 infection: role for IL-4. Am J Physiol Regul Integr Comp Physiol 304: R267–R277, 2013. First published December 19, 2012; doi:10.1152/ajpregu.00516.2011.—Recent findings indicate that TLR3 polymorphisms increase susceptibility to enteroviral myocarditis and inflammatory dilated cardiomyopathy (iDCM) in patients. TLR3 signaling has been found to inhibit coxsackievirus B3 (CVB3) replication and acute myocarditis in mouse models, but its role in the progression from myocarditis to iDCM has not been previously investigated. In this study we found that TLR3 deficiency increased acute (P = 5.9 × 10⁻⁶) and chronic (P = 6.0 × 10⁻⁷) myocarditis compared with WT B6.129, a mouse strain that is resistant to chronic myocarditis and iDCM. Using left ventricular in vivo hemodynamic assessment, we found that TLR3-deficient mice developed progressively worse chronic cardiomyopathy. TLR3 deficiency significantly increased viral replication in the heart during acute myocarditis from day 3 through day 12 after infection, but infectious virus was not detected in the heart during chronic disease. TLR3 deficiency increased cytokines associated with a T helper (Th)2 response, including IL-4 (P = 0.03), IL-10 (P = 0.008), IL-13 (P = 0.002), and TGF-β1 (P = 0.005), and induced a shift to an immunoregulatory phenotype in the heart. However, IL-4-deficient mice had improved heart function during acute CVB3 myocarditis by echocardiography and in vivo hemodynamic assessment compared with wild-type mice, indicating that IL-4 impairs cardiac function during myocarditis. IL-4 deficiency increased regulatory T-cell and macrophage populations, including FoxP3⁺ T cells (P = 0.005) and Tim-3⁺ macrophages (P = 0.004). Thus, TLR3 prevents the progression from myocarditis to iDCM following CVB3 infection by reducing acute viral replication and IL-4 levels in the heart.

cytokines; innate immunity; inflammation; viral; myocarditis

MYOCARDITIS RESULTS IN AROUND 46% OF DILATED CARDIOMYOPATHY (DCM) CASES (53), WHICH IS THE MOST COMMON FORM OF CARDIOMYOPATHY RESPONSIBLE FOR NEARLY HALF OF ALL HEART TRANSPLANTS (12, 59). THE LIFE EXPECTANCY AFTER DIAGNOSIS OF DCM IS ONLY 50% AT 4 YEARS. CHRONIC MYOCARDIAL INFLAMMATION, PARTICULARLY FOLLOWING VIRAL MYOCARDITIS, HAS BEEN TERMED INFLAMMATORY DCM (iDCM) BY THE WORLD HEALTH ORGANIZATION’S CLASSIFICATION OF CARDIOMYOPATHIES (35, 44). COXSVIRUS B3 (CVB3), A COMMON ENTEROVIRUS, IS A MAJOR CAUSE OF MYOCARDITIS LEADING TO iDCM IN WESTERN POPULATIONS (9, 10, 20). INTERFERONS (IFNs) LIKE IFN-β AND IFN-γ REDUCE MYOCARDITIS AND IMPROVE HEART FUNCTION IN PATIENTS AND ANIMAL MODELS BY REDUCING VIRAL REPLICATION, SUGGESTING THAT VICAL INFECTIONS ARE AN IMPORTANT CAUSE OF MYOCARDITIS CASES THAT LEAD TO iDCM AND HEART FAILURE (18, 19, 39, 57).

The innate immune response to viral infection is mediated at least in part by Toll-like receptors (TLRs), including TLR3, TLR7, and TLR9 (37). TLR3 binds to dsRNA, an intermediate product of viral ssRNA, in endosomes and inhibits viral replication by upregulating IFN-α/β and IFN-γ (37, 46). Previously, TLR3-deficient mice were found to have reduced survival following infection with encephalomyocarditis virus (EMCV), CVB3, or CVB4 that was associated with increased viral replication and inflammation in the heart (31, 46, 52). Negishi et al. (46) found that CVB3 levels were significantly increased in the spleen, sera, and heart of TLR3-deficient mice at day 3 postinfection, while IFN-γ was significantly lower in the heart at day 3. Although Negishi et al. (46) observed that CVB3 myocarditis was increased in TLR3-deficient mice at day 12 postinfection, they did not quantify disease, examine cardiac cytokine profiles, or characterize cardiac function. They also did not determine the effect of TLR3 deficiency on the progression from myocarditis to DCM. In contrast to CVB3 or CVB4, EMCV-induced myocarditis was significantly reduced in TLR3-deficient hearts at days 3 and 5 postinfection, indicating that TLR3 increases EMCV-induced myocarditis (31). TLR3 polymorphisms in myocarditis patients were recently found to be associated with an increased susceptibility to enteroviral myocarditis and DCM (30), suggesting that TLR3 may be important in protecting against the progression from myocarditis to iDCM. Recently, we showed that TLR3-deficient mice develop a Th helper (Th)2-skewed immune response during acute CVB3 myocarditis and that an IL-4-driven Th2
response has less severe consequences than an IL-33-driven Th2 response on cardiac function (1, 2). In this study, we further examined the mechanisms involved in protection mediated by TLR3 in the progression from myocarditis to IDCM.

METHODS

Ethical approval. All animal procedures were submitted to and approved by the Animal Care and Use Committee of the Johns Hopkins School of Medicine. Mice were housed at the animal facility at Johns Hopkins School of Medicine. Eight- to ten-week-old male mice were inoculated with 10^3 PFU of heart-passaged CVB3 containing infectious virus and heart tissue diluted in sterile PBS or PBS alone intraperitoneally, and tissues were collected at various time points after infection (20).

Histology. Hearts were fixed in 10% buffered formalin and stained with hematoxylin and eosin to assess inflammatory cells or Masson’s trichrome to detect collagen deposition. Myocarditis was assessed as the percentage of the heart section (i.e., ventricles) with hematoxylin staining, necrosis, and/or fibrosis compared with the overall size of the heart section at low power (×25 magnification) using a microscope eyepiece grid, as has been done previously (1, 2, 11, 18). Sections were scored by at least two individuals blinded to the treatment group.

Cardiac function. Cardiac function was determined by transthoracic echocardiography in conscious mice using the Sequoia Acuson C256 ultrasound machine (Malvern, PA) equipped with a 15-MHz linear transducer, as previously described (1, 11). In separate experiments, cardiac function was assessed by pressure-volume catheter (1.2F Scisense, London, Ontario) placed in the left ventricle via the apex in open-chest mice anesthetized with 3% isoflurane, as previously described (1, 2, 29, 48).

PLAQUE ASSAY AND ELISA. Hearts were homogenized at 10% wt/vol in 2% MEM, and individual supernatants were used in ELISA to measure cytokines or in plaque assays to determine the level of infectious virus, as previously described (17, 20). Cytokines were determined using R&D Systems ELISA kits (Minneapolis, MN), according to the manufacturer’s instructions. Levels were expressed as mean plaque-forming units (PFU)/g of tissue for plaque assays and as picograms per gram of heart for cytokines.

RNA extraction and quantitative RT-PCR. Total RNA from hearts was assessed by quantitative real-time (qRT)-PCR using Assay-on-Demand primers and probe sets and the ABI 7000 Taqman System from Applied Biosystems (Foster City, CA). Data were normalized to hypoxanthine phosphoribosyltransferase 1 (Hprt) and expressed as a relative gene expression according to Onyimba et al. (47).

FACS analysis. Hearts were digested with 1 mg/ml collagenase II (Sigma, St. Louis, MO) and 0.5 mg/ml protease XIV (Sigma), as done previously (11, 24, 26, 27). Immune cells were stained with fluorochrome-conjugated antibodies against CD45, CD3, CD4, CD11b, F4/80, GR1, T-cell immunoglobulin mucin-3 (Tim-3), or forkhead box P3 (FoxP3) (BD Pharmingen/eBiosciences, San Jose, CA). For intracellular cytokine staining, cells were fixed and permeabilized using BD Cytofix/Cytoperm or an anti-mouse FoxP3 staining kit (BD Biosciences) (24, 27).

Statistical analysis. Two groups were analyzed using the Mann-Whitney rank sum test with a P < 0.05 considered significant. We controlled the family-wise error rate (FWER) for experimental stages with repeated measures and multiple pairwise tests. For correlated variables, such as those found in repeated measures and multiple time points, the Bonferroni correction is overly conservative for FWER correction. Thus, we used a permutation procedure that allowed us to generate the empirical distributions of the test statistics of the multiple hypothesis tests. The genotype labels in the data set for the experimental stage were randomly permuted to generate a null-association data set. The association P values from this null data set were used to get an empirical estimate of the lowest P value in multiple null hypothesis tests. The lowest P values of each of 10,000 such null permuted data sets were used to generate the empirical null distributions of the most significant P value. The lowest P value from the observed hypothesis tests was then corrected using this empirical distribution. This empirical P value is corrected for the multiple tests. If the most empirical P value of the most significant association was below the significance threshold (empirical P < 0.05), the permutation procedure was repeated for all variables excepting this empirically significant association. This procedure was continued until the last P value was empirically corrected.

RESULTS

TLR3 mRNA is elevated in response to CVB3 infection. To determine whether TLR3 was upregulated following CVB3 infection in our model, we examined TLR3 mRNA levels by qRT-PCR in the spleen at 12 h postinfection and in the heart during the innate response at day 2 postinfection and during acute CVB3 myocarditis at day 10 postinfection. We found that TLR3 mRNA levels were significantly elevated in the spleen at 12 h postinfection (Fig. 1). Relative gene expression was normalized to hypoxanthine phosphoribosyltransferase 1 (Hprt). Data show the means ± SEM of 7–11 mice/group, PBS and CVB3 groups were evaluated using the Mann-Whitney rank sum test, *P < 0.05; ***P < 0.001.
TLR3 deficiency increases acute and chronic myocarditis. TLR3-deficient mice developed significantly increased acute CVB3 myocarditis at day 10 postinfection compared with WT B6.129 mice ($P = 5.9 \times 10^{-9}$) (Fig. 2, A–C), as previously reported (1, 46). We found that CD45 levels (a marker expressed on all leukocytes) were significantly increased in the heart of TLR3-deficient mice at day 10 postinfection compared with WT controls by qRT-PCR ($P = 0.0005$) (Fig. 3A).

Specific immune cell populations, such as CD11b$^+$ cells (in Fig. 2, A–C), were increased in TLR3-deficient mice compared with WT controls at day 10 postinfection. Representative histology sections show magnification at $\times64$ (B) and $\times260$ (C), $\times40$ (E). Data show the means $\pm$ SE of four separate experiments collapsed together ($n = 32$–$37$ mice/group) (A). B6.129 and TLR3$^--$ groups were evaluated using the Mann-Whitney rank sum test; ***$P < 0.001$.

Fig. 3. TLR3 deficiency (TLR3$^--$) increases immune cells and regulatory cell markers during CVB3 myocarditis. Wild-type B6.129 and TLR3$^--$ mice were infected with CVB3 on day 0 and inflammatory (A) or alternative activation/regulatory (B) cell markers assessed at day 10 postinfection using qRT-PCR and shown as relative gene expression normalized to hypoxanthine phosphoribosyltransferase 1 (Hprt). Data show the means $\pm$ SE of 6 or 7 mice/group. B6.129 and TLR3$^--$ groups were evaluated using the Mann-Whitney rank sum test; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. 

$P = 0.03$ (Fig. 1A) and in the heart at day 2 ($P = 0.0001$) and day 10 ($P = 0.0002$) (Fig. 1B) postinfection compared with PBS-inoculated controls.

$P = 0.03$ (Fig. 1A) and in the heart at day 2 ($P = 0.0001$) and day 10 ($P = 0.0002$) (Fig. 1B) postinfection compared with PBS-inoculated controls.
cludes monocyte/macrophages, neutrophils, some dendritic cells, and mast cells (P = 0.0001), CD3+ cells (T cells) (P = 0.04), GR1+ cells (neutrophils) (P = 0.002), and F4/80+ cells (mature macrophages) (P = 0.04) were significantly increased in the heart of TLR3-deficient mice compared with WT controls by qRT-PCR (Fig. 3A). We report for the first time that chronic inflammation, necrosis, and fibrosis were significantly increased in TLR3-deficient mice at day 35 postinfection compared with WT mice (P = 6.0 × 10−7) (Fig. 2, D and E). Necrosis and fibrosis were not present in WT or TLR3-deficient mice at day 10 after infection (data not shown). Thus, TLR3 deficiency overcame resistance to chronic myocarditis in B6.129-resistant background mice.

*Increased Th2-associated cytokines and a shift to an immunoregulatory phenotype.* TLR3 signaling is important for the production of IFNs and a Th1 response (37). Previously, TLR3-deficient mice were shown to have significantly reduced IFN-γ levels in the heart at days 3 and 10 postinfection (1, 46). Although IFN-γ was significantly decreased in the heart of TLR3-deficient mice at day 10 postinfection during acute CVB3 myocarditis (P = 0.03) (1), IFN-α (P = 0.20), and IFN-β (P = 0.50) levels were unaltered (Fig. 4A). No significant difference was observed in cardiac IL-17A levels between WT and TLR3-deficient mice (P = 0.36) (Fig. 4A). However, cytokines associated with a Th2 response were significantly increased in the heart of TLR3-deficient mice compared with WT controls at day 10 postinfection, including IL-4 (P = 0.03) (1), IL-10 (P = 0.008), IL-13 (P = 0.002), and TGF-β1 (P = 0.005) (Fig. 4B). To confirm that TLR3-deficient mice developed a Th2-type immune response during acute myocarditis we looked for markers of alternative activation of macrophages. CD11b, GR1, F4/80, and T-cell immunoglobulin mucin-3 (Tim-3) during acute CVB3 myocarditis (21, 26, 27). Here, we found that TLR3-deficient mice had significantly increased expression of Arg1 (P = 0.0001) (1), Ym1 (P = 0.0002) (1), IL-4 receptor (P = 0.0001) (1), and Tim-3 (P = 0.0001) in their hearts compared with WT mice during acute myocarditis (Fig. 3B). Thus, TLR3 deficiency causes a switch from a Th1- to a Th2-type regulatory phenotype during acute CVB3 myocarditis.

**TLR3 deficiency increases viral replication during acute myocarditis.** Previously, TLR3 deficiency was reported to be associated with increased CVB3 levels in the spleen at day 3 postinfection and the heart at days 3, 6, 8, and 10 postinfection (1, 46, 58). To determine the effect of TLR3 deficiency on viral replication in our autoimmune model of CVB3 myocarditis, we assessed infectious virus levels in the pancreas (main target organ for CVB3) and the heart over time (Fig. 5). We found that there was no significant difference between WT and TLR3-deficient mice in viral replication in the pancreas for any timepoint examined except for day 7 postinfection, when viral replication was increased in TLR3-deficient mice (P = 0.0009) (Fig. 5A). Infectious CVB3 was cleared from the pancreas by day 10 postinfection (Fig. 5A). In contrast, TLR3 deficiency was associated with significantly increased viral replication in the heart in our CVB3 model at day 3 (P = 0.02), day 5 (P = 0.002), day 7 (P = 0.0009), day 10 (P = 0.02), and day 12 (P = 0.01) postinfection, but it could not be detected in the heart at day 18 or 35 postinfection (Fig. 5B). These data indicate that the peak of viral replication in the heart in WT and knockout mice occurs around day 7 postinfection and that increased viral replication due to TLR3 deficiency is fairly specific to the heart.

**Baseline hemodynamics of uninfected WT and TLR3-deficient mice.** In general, uninfected WT B6.129 and TLR3-deficient mice displayed normal ventricular function (Table 1, day 0). Maximum ventricular power (PMX) was significantly lower in TLR3-deficient mice than WT controls (P = 0.001) (Table 1). Overall, indices of diastolic or systolic function were not significantly different between WT and TLR3-deficient mice.

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**Fig. 4. Cytokines in the heart of TLR3-deficient (TLR3−/−) mice during acute CVB3 myocarditis.** Wild-type (WT) B6.129 and TLR3−/− mice were infected with CVB3 on day 0 and Th1/Th17-type (A) and Th2 or regulatory-type (B) cytokines assessed in the heart during acute myocarditis at day 10 postinfection by ELISA. Data show the means ± SE from three separate experiments that were collapsed together; n = 17–25 mice/group. WT and TLR3−/− groups were evaluated using the Mann-Whitney rank sum test; *P < 0.05; **P < 0.01.
TLR3 DEFICIENCY INDUCES CHRONIC INFLAMMATORY DCM

Fig. 5. TLR3 deficiency increases viral replication in the heart. Wild-type (WT) B6.129 and TLR3-deficient (TLR3−/−) (KO) mice were infected with CVB3 on day 0, and viral replication was assessed by plaque assay at days 0, 3, 5, 7, 10, 12, 18, and 35 postinfection in the pancreas (A) and heart (B). Data show the median and 25th to 75th percentiles for 7–10 mice/group and are representative of three separate experiments for day 7, 10, 12, and 35 postinfection. Differences between B6.129 and TLR3−/− were assessed using a permutation procedure (See Methods). PFU, plaque-forming units. Permutation-corrected P values: *P < 0.05; **P < 0.01; ***P < 0.001.

significantly different between uninfected WT and TLR3-deficient mice at day 0.

TLR3-deficient mice with myocarditis develop progressively worse heart function. Unlike a previous report of CVB3 myocarditis, in which only around 50% of TLR3-deficient mice survive to day 14 postinfection (46), nearly 100% of WT and TLR3-deficient mice survive out to day 35 postinfection in our autoimmune model of CVB3 myocarditis (data not shown) (1). Recently, we showed that TLR3-deficient mice develop worse cardiac function compared with WT mice during acute CVB3 myocarditis at day 10 postinfection (1). To assess cardiac function as mice progressed from myocarditis to iDCM, we compared baseline features in uninfected mice (day 0) to mice with acute (day 10 postinfection) and chronic (day 35 postinfection) myocarditis by echocardiography in the same individual mice over time (Fig. 6). By echocardiography (Fig. 6, A and D) and pressure-volume relationships (Table 1), cardiac function became significantly worse over time. LV end-systolic dimension (LVESD) was significantly increased over the time course (P = 0.03), LV end-diastolic dimension was unaltered (P = 0.43), while % fractional shortening and ejection fraction (EF) significantly decreased over time (P = 0.009 and P = 0.01, respectively) (Fig. 6, A and D). At day 10 postinfection, there was no significant difference between WT or TLR3-deficient mice in heart weight (HW)-to-tibia length (TL) ratio (P = 0.62) (Fig. 6B), a measure of hypertrophy, but by day 35 postinfection, there was a significant reduction in HW to TL in TLR3-deficient mice (P = 5.31 × 10−3) (Fig. 6C).

During acute myocarditis at day 10 postinfection, TLR3-deficient mice displayed more severe functional impairment compared with WT mice as assessed by pressure-volume relationships (Table 1). End-systolic pressure (ESP) in TLR3-deficient mice (96 ± 4.7 mmHg) was significantly decreased compared with WT controls (108 ± 2.6 mmHg, P = 0.03). EF dropped to 48% in TLR3-deficient mice vs. 66% in WT controls (P = 0.03). For TLR3-deficient mice, this was a 27% decrease in EF from baseline, whereas EF in WT controls was only 11% lower (Table 1). Peak flow rate (PFR) normalized to end-diastolic volume (EDV) (i.e., PFR/EDV) remained at 37 ±

Table 1. Invasive hemodynamics of TLR3-deficient mice based on pressure-volume analysis

<table>
<thead>
<tr>
<th>Day 0 (Baseline)</th>
<th>Day 10 pi</th>
<th>Day 35 pi</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6.129</td>
<td>TLR3−/−</td>
<td>B6.129</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TLR3−/−</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>596 ± 3.7</td>
<td>598 ± 7.6</td>
</tr>
<tr>
<td>ESP, mmHg</td>
<td>111 ± 3.8</td>
<td>110 ± 1.9</td>
</tr>
<tr>
<td>EDP, mmHg</td>
<td>6.9 ± 0.8</td>
<td>6.8 ± 0.6</td>
</tr>
<tr>
<td>dP/dt max</td>
<td>10766 ± 250</td>
<td>9892 ± 404</td>
</tr>
<tr>
<td>dP/dt min</td>
<td>-10022 ± 309</td>
<td>-8808 ± 635</td>
</tr>
<tr>
<td>EF, %</td>
<td>74 ± 2.2</td>
<td>69 ± 2.2</td>
</tr>
<tr>
<td>ESV, μl</td>
<td>4 ± 0.4</td>
<td>6 ± 0.9</td>
</tr>
<tr>
<td>EDV, μl</td>
<td>16 ± 0.5</td>
<td>19 ± 1.3</td>
</tr>
<tr>
<td>SV, μl</td>
<td>12 ± 0.5</td>
<td>12 ± 0.9</td>
</tr>
<tr>
<td>PFR/EDV, s−1</td>
<td>39 ± 3</td>
<td>39 ± 3</td>
</tr>
<tr>
<td>CO, μl/min</td>
<td>6940 ± 318</td>
<td>7396 ± 317</td>
</tr>
<tr>
<td>SW, mmHg × μl</td>
<td>969 ± 40</td>
<td>1087 ± 105</td>
</tr>
<tr>
<td>PRSW, mmHg</td>
<td>68 ± 5.4</td>
<td>86 ± 7.4</td>
</tr>
<tr>
<td>PMX, mW</td>
<td>13.0 ± 0.5</td>
<td>11.6 ± 0.6*</td>
</tr>
<tr>
<td>PMX/EDV2</td>
<td>5.6 ± 0.4</td>
<td>3.3 ± 0.4</td>
</tr>
<tr>
<td>Ea/Ees</td>
<td>1.3 ± 0.1</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>Vo, μl</td>
<td>-14.4 ± 1.2</td>
<td>-8.3 ± 0.8</td>
</tr>
<tr>
<td>Tau, Weiss, ms</td>
<td>5.1 ± 0.3</td>
<td>5.7 ± 0.3</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE for 10 mice/group per timepoint. CO, cardiac output; dP/dt max, peak rate of pressure rise (mmHg/s); dP/dt min, peak rate of pressure decline (mmHg/s); Ea/Ees, arterial elastance normalized to Ees; EDP, end-diastolic pressure; EDV, end-diastolic volume; Ees, LV end-systolic elastance (stiffness); EF, ejection fraction; ESP, end-systolic pressure; ESV, end-systolic volume; PFR/EDV, peak flow rate normalized to EDV; PMX, maximum ventricular power (mW); PRSW, preload recruitable stroke work; SV, stroke volume; SW, stroke work; Tau, time constant of diastolic relaxation; Vo, intercept of the ESP-volume relationship. *P < 0.05 comparing B6.129 to TLR3−/− by ANOVA for all timepoints corrected for multiple testing (see Methods). **P < 0.01, and ***P < 0.001 compare B6.129 to TLR3−/− at each timepoint by Mann-Whitney rank sum test for parameters that were significant by ANOVA corrected for multiple testing. ¥P < 0.05 over time by ANOVA not corrected for multiple testing.
4.0 in WT mice but diminished to 25 ± 2.0 s−1 in TLR3−/− mice (P = 0.02), a 36% decline from uninfected controls. Stroke work (SW) dropped from 1,069 ± 100 mmHg × μl in WT to 717 ± 83 in TLR3-deficient mice (P = 0.02). Maximum ventricular power (PMX) in WT mice was 13 ± 0.7 mW, while power had declined to 9 ± 1.2 mW in TLR3-deficient mice (P = 0.03). The parameters described above reveal a decline in systolic function in TLR3-deficient mice; however, diastolic heart function was also impaired in knockout mice. dP/dt minimum (dP/dt min) in TLR3-deficient mice was 30% lower at −6,795 ± 615 mmHg/s than WT mice at −9,549 ± 454 mmHg/s (P = 0.002).

In WT mice, heart function returned to normal after the acute phase of myocarditis abated, but several important parameters of heart function assessed using pressure-volume relationships indicated that TLR3-deficient hearts remained significantly impaired out to day 35 postinfection (Table 1). ESP in WT mice at day 35 postinfection was improved (116 ± 3.4 mmHg) compared with uninfected WT mice (111 ± 3.8 mmHg), while ESP in TLR3-deficient mice continued to decline (90 ± 6.1 mmHg, P = 0.001). PFR/EDV recovered in WT mice by day 35 postinfection (45 ± 3 s−1), while TLR3-deficient mice did not (29 ± 2 s−1, P = 5 × 10−4). Preload recruitable stroke work also failed to recover in TLR3-deficient mice by day 35 postinfection (9 ± 1.7 mW) compared with WT controls at day 35 postinfection (14 ± 1.0 mW, P = 0.01). While PMX in uninfected TLR3-deficient mice was 18% lower than WT controls, this gap widened to 26% at day 10 and 38% by day 35 postinfection. Diastolic measures of heart function remained impaired at day 35 postinfection in TLR3-deficient mice. dP/dt min was −7,757 ± 904 mmHg/s in TLR3-deficient mice and −10,852 ± 432 mmHg/s for WT controls (P = 0.005). Overall, on the basis of pressure volume analysis, TLR3-deficient mice developed progressively impaired cardiac function, while WT mice recovered following acute myocarditis.

**IL-4 deficiency reduces cardiac dysfunction.** To determine whether increased IL-4 in TLR3-deficient mice could impair cardiac function, we assessed heart function using echocardiography and pressure-volume relationships in IL-4-deficient mice during acute CVB3 myocarditis at day 10 postinfection (Table 2). Although indicators of poor heart function such as SW, maximum ventricular power (PMX), and ejection fraction continued to decline in TLR3-deficient mice by day 35 postinfection (60 ± 10 mmHg) but did in WT mice (115 ± 6.4 mmHg, P = 6 × 10−4), PMX continued to decline in TLR3-deficient mice (9 ± 1.7 mW) compared with WT controls at day 35 postinfection (14 ± 1.0 mW, P = 0.01). While PMX in uninfected TLR3-deficient mice was 18% lower than WT controls, this gap widened to 26% at day 10 and 38% by day 35 postinfection. Diastolic measures of heart function remained impaired at day 35 postinfection in TLR3-deficient mice. dP/dt min was −7,757 ± 904 mmHg/s in TLR3-deficient mice and −10,852 ± 432 mmHg/s for WT controls (P = 0.005). Overall, on the basis of pressure volume analysis, TLR3-deficient mice developed progressively impaired cardiac function, while WT mice recovered following acute myocarditis.

**Table 2. In vivo hemodynamics of IL-4-deficient mice during acute CVB3 myocarditis based on pressure-volume analysis and echocardiography**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT</th>
<th>IL-4−/−</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, bpm</td>
<td>568 ± 6.7</td>
<td>552 ± 11.9</td>
<td>0.27</td>
</tr>
<tr>
<td>ESP, mmHg</td>
<td>92 ± 3.1</td>
<td>106 ± 3.7</td>
<td>0.006</td>
</tr>
<tr>
<td>ED, mmHg</td>
<td>5.1 ± 0.5</td>
<td>6.4 ± 0.8</td>
<td>0.19</td>
</tr>
<tr>
<td>dP/dt max, mmHg/s</td>
<td>9627 ± 428</td>
<td>10942 ± 443</td>
<td>0.04</td>
</tr>
<tr>
<td>dP/dt min, mmHg/s</td>
<td>−8994 ± 333</td>
<td>−10024 ± 528</td>
<td>0.11</td>
</tr>
<tr>
<td>EF, %</td>
<td>46 ± 1.6</td>
<td>43 ± 2.2</td>
<td>0.42</td>
</tr>
<tr>
<td>SW, μl</td>
<td>10.4 ± 0.6</td>
<td>11.8 ± 0.6</td>
<td>0.14</td>
</tr>
<tr>
<td>PFR/EDV, s−1</td>
<td>25.1 ± 1.4</td>
<td>22.5 ± 1.5</td>
<td>0.23</td>
</tr>
<tr>
<td>CO, ml/min</td>
<td>5.5 ± 0.3</td>
<td>6.5 ± 0.3</td>
<td>0.02</td>
</tr>
<tr>
<td>Vt, μl</td>
<td>3.1 ± 1.6</td>
<td>2.5 ± 1.0</td>
<td>0.01</td>
</tr>
<tr>
<td>Ees, mm</td>
<td>13.7 ± 2.4</td>
<td>7.4 ± 0.8</td>
<td>0.02</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>2.1 ± 0.2</td>
<td>2.2 ± 0.2</td>
<td>0.60</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>3.6 ± 0.1</td>
<td>3.4 ± 0.1</td>
<td>0.22</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE for 11 to 12 mice/group. CO, cardiac output; dP/dt Max, peak rate of pressure rise (mmHg/s); dP/dt min, peak rate of pressure decline (mmHg/s); LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension. P values compare WT BALB/c to IL-4−/− mice by Mann-Whitney rank sum test at day 10 postinfection. †P < 0.05 after correction for multiple testing.
TLR3-deficiency induces chronic inflammatory DCM (dysfunctional cardiomyopathy) following acute myocarditis (46, 52). However, in contrast to other models of virus-induced myocarditis, we observe no reduction in survival in IL-4-deficient mice with experimental autoimmune myocarditis at days 6 and 8 postinfection compared with controls, but not in heart mice with experimental autoimmune myocarditis at days 3 and 5 postinfection (46, 48). That TLR3 levels are elevated in the heart during the peak of experimental autoimmune myocarditis but not during the peak of experimental autoimmune myocarditis (46, 48) suggests that TLR3 signaling is a key factor in the pathophysiology of chronic inflammatory DCM. Therefore, IL-4 deficiency increases regulatory T-cell and macrophage populations within the heart without altering susceptibility to viral infection. IL-4 deficiency increases regulatory T-cell and macrophage populations within the heart without altering susceptibility to viral infection.

The only other study to examine the role of TLR3 deficiency in chronic inflammatory DCM (46, 52) reported that IL-4 deficiency had no effect on acute CVB3 myocarditis reported that IL-10 deficiency increases regulatory T-cell and macrophage populations within the heart without altering susceptibility to viral infection. IL-4 deficiency increases regulatory T-cell and macrophage populations within the heart without altering susceptibility to viral infection.

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postinfection (46) but did not examine cytokine levels at other
timepoints during acute myocarditis (e.g., at day 10 postinfec-
tion), as we did in this study.

We are the first to report that TLR3 deficiency switches the
protective antiviral Th1 response induced during acute CVB3
myocarditis to a Th2-type immune response with increased
IL-4, IL-10, IL-13 and TGF-β1 in the heart. Here, we describe
that TLR3 deficiency progressively worsens heart function
following CVB3 infection and that increased IL-4 levels in
TLR3-deficient mice may impair cardiac function. IL-10 and
IL-13 have been shown previously to protect against myocar-
ditis (11, 21, 42), while TGF-β released by alternatively
activated macrophages and/or regulatory T cells (Treg) can
reduce inflammation but increase fibrosis (18, 21, 55). Previous
studies found that treatment of CVB3-infected mice with
recombinant (r)IL-4 or an IL-4-expressing plasmid signifi-
cantly reduces acute myocarditis while improving heart func-
tion and survival (34, 41). However, we previously reported
that IL-4-associated responses in male mice promote myocar-
ditis and iDCM in autoimmune CVB3 and experimental autoim-
une myocarditis models (4, 5, 8, 18, 19). Although Tim-3 is
found on Th1 cells where it induces apoptosis (14, 24), in this
study and previous publications, we have found that Tim-3 is
present on regulatory macrophages regardless of whether mice are
skewed to a Th1 (i.e., IL-4-deficient mice) or Th2 (i.e., TLR3-
deficient mice) response (24, 26, 27). Elevated IL-4 and Th2
responses in A/J and BALB/c mice have been found to result in
increased chronic myocarditis (1–3, 8, 17, 18). The fact that only
Th2-type-responding mouse strains like BALB/c and A/J mice are
susceptible to chronic myocarditis and iDCM suggests that Th2
responses contribute to the cardiac dysfunction that leads to
chronic cardiomyopathy (20, 38).

Extracellular matrix remodeling and fibrosis are critical for
the progression from CVB3 myocarditis to DCM (13, 36, 43).
Fibroblast proliferation and collagen deposition can be in-
creased by TNF, IL-1β, IL-4, IL-13, IL-17A, and/or TGF-β1
(7, 13, 18, 40). We observed significantly increased IL-4,
IL-13, and TGF-β1 in the heart of TLR3-deficient mice during
acute myocarditis in this study and progressively worse chronic
inflammation, fibrosis, and cardiomyopathy. Additionally, we
show here that IL-4 deficiency improves cardiac function,
establishing that IL-4 can induce negative effects on heart
function during acute CVB3 myocarditis by reducing regula-

Fig. 7. Flow cytometric analysis of IL-4 deficient (IL-4−/−) mice during acute CVB3 myocarditis. WT and IL-4−/− mice were infected with CVB3 on day 0, and immune cells were assessed by histology (A, left) or flow cytometry (A, right) at day 10 postinfection. Viral replication was not significantly altered in IL-4-deficient mice during acute CVB3 myocarditis (C). Percentages of Th1 and Th17 cells (D), Tim-3− macrophages (E), and regulatory T cells (F) are shown. Data are expressed as means ± SE of two separate experiments collapsed together; *P < 0.05; **P < 0.01; ***P < 0.001 by Mann-Whitney rank sum test.
tory macrophage and Treg cell populations in the heart. Previously, reduced cardiac Treg populations were associated with the progression to iDCM following acute myocarditis (5). Similar to the findings of other researchers using IL-4-deficient mice (54), we found that elevated Th1 responses in IL-4-deficient mice were associated with elevated IL-10. TBF-1, and IL-10 levels may be increased in TLR3-deficient hearts due to increased numbers of alternatively activated/regulatory macrophages and Treg (1, 21, 55).

Our findings in IL-4-deficient mice contrast with two previous studies that found that treatment of CVB3-infected mice with recombinant (r)IL-4 or an IL-4-expressing plasmid improved survival and reduced myocarditis (34, 41). Li et al. (41) found that rIL-4 treatment of mice significantly reduced CVB3 viral replication and inflammation at day 10 postinfection, while improving acute heart function. However, this effect of rIL-4 to reduce CVB3 replication is unusual because most investigators, including us, have found that IFN-β and/or IFN-γ are needed to reduce viral replication while IL-4/Th2-type responses allow increased viral replication (1, 19, 22, 39, 57). Jiang et al. (34) found that overexpression of IL-4 using a plasmid improved survival during acute CVB3 myocarditis, but ~75% of WT mice had died by day 8 postinfection. The results from that experiment are not very comparable to this study because in our CVB3 model of myocarditis, nearly 100% of WT mice survive to at least day 90 postinfection (1, 13, 18, 20). Overall, our findings indicate that IL-4 can contribute to cardiac dysfunction in an autoimmune model of CVB3 myocarditis. Our findings in the mouse model are supported by two clinical studies reporting that an elevated Th2 response was present in the heart of iDCM patients with an autoimmune etiology (28, 38), suggesting that an IL-4-driven Th2 response could play a role in the progression of myocarditis to iDCM. Thus, we have shown that TLR3 protects against the progression to chronic inflammatory heart disease following CVB3 infection, not only by reducing viral replication in the heart but also by inhibiting an IL-4 response.

**Perspectives and Significance**

IL-4-driven Th2-type immune responses are usually considered to protect against Th1-associated autoimmune diseases like myocarditis. However, mounting evidence indicates that elevated Th2 responses during acute myocarditis facilitate remodeling that leads to chronic iDCM, at least in male mice. In this study, we demonstrate that elevated IL-4 levels during acute myocarditis in male TLR3-deficient mice lead to progressively worse cardiac function. These findings indicate that TLR3 polymorphisms that reduce TLR3 function may predispose individuals to develop iDCM following infection with common enteroviruses like CVB3.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


47. Shi Y, Fukuoka M, Li G, Liu Y, Chen M, Konviser M, Chen X, Opavsky MA, Liu PP. Regulatory T cells protect mice against...


