Coxsackievirus B3 Induces Viral Myocarditis by Upregulating Toll-Like Receptor 4 Expression

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Abstract—In the present study, we investigated the potential pathogenesis of coxsackievirus B3 (CVB3)-induced viral myocarditis and the promising protective effect of silencing RNA (small interfering RNA, siRNA). One hundred and twenty mice were included in the study, and 30 mice were intraperitoneally inoculated with CVB3 to establish an acute viral myocarditis model. The survival rate was observed for the CVB3-infected mouse model (MOD), and myocardial injury was examined by HE (hematoxylin and eosin) staining assay. Real-time PCR (RT-PCR) and Western blot assay were selected to detect the toll-like receptor 4 (TLR4) expression in myocardial tissues. The TLR4 gene was silenced for the MOD mice, and the effects of this treatment were observed. The results indicate that the expression of TLR4 mRNA and the protein significantly and persistently increased during the progression of CVB3-induced myocarditis. The activities of cardiac enzymes including CK, LDH, AST, and CK-MB were also enhanced in CVB3-induced myocardial tissues. Interestingly, when the TLR4 gene was silenced, the CVB3-induced TLR4 production was significantly decreased and the severity of myocarditis was significantly lessened. In conclusion, CVB3 may induce viral myocarditis by upregulating toll-like receptor 4 expression. The viral myocarditis can be ameliorated by silencing the TLR4 gene in the CVB3 viral myocarditis model, which may be a feasible therapeutic method for treatment of viral myocarditis.

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In clinics, viral myocarditis is a leading reason for heart failure in young adults, and it often progresses to chronic myocarditis, dilated cardiomyopathy, and congestive heart failure [1, 2]. Coxsackievirus B3 (CVB3) is known to be the most common causative agent for human myocarditis [3]. Though many studies have been performed in recent years, the specific pathogenesis of viral myocarditis is still unclear. Thus, effective drug and therapeutic methods have not been found.

CVB3 can lead the serious inflammatory response, which then causes damage to myocytes [4, 5]. Some researchers have proposed that the inflammatory response can be related closely with signal-transduction activation, such as the toll-like receptor 4/nuclear factor κB signal pathway (TLR4/NF-κB) [6, 7]. However, the key factors of the TLR4/NF-κB pathway, especially for TLR4, were little studied in previous reports except for those of Frisancho-Kiss et al. [8, 9]. Some therapeutic methods have been reported to regulate the signal-transduction pathway [10]. Zou et al. [11] reported that the TLR4/NF-κB-dependent pathway is involved in the regulation of innate immune response in myocarditis. However, the specific mechanism of TLR4/NF-κB-mediated viral myocarditis is also elusive. So the search for specific drugs that can effectively target TLR4/NF-κB is critical for therapy of myocarditis.

In this study, CVB3 virus was employed to establish a mouse model of myocarditis. The TLR4 factor was studied in the CVB3-induced myocarditis mouse model to investigate the mechanism of viral myocarditis.

MATERIALS AND METHODS

Mice and virus. Six-week-old male BALB/C mice of 20-g weight were purchased from the Animal Center of
Xi’an Jiaotong University. All of the mice were specific pathogen free (SPF). CVB3 (Nancy strain) maintained by passage through HeLa cells was obtained from the Medical College of Xi’an Jiaotong University. The virus was routinely determined prior to infection by a 50% tissue culture infectious dose (TCID₅₀) assay of a HeLa cell monolayer. All animal procedures were performed according to the Guide for the Care and Use of Medical Laboratory Animals (Ministry of Health, P. R. China, 1998). The study was approved by the ethics committee of the First Hospital of Xi’an.

**Grouping and treatment.** The 120 mice employed in the study were divided into four groups including (i) a normal (control) mouse group (CN), (ii) a DMEM-treated group (DMEM), (iii) a CVB3-infected group (model mice, MOD), and (iv) a TLR-silencing group (model mice treated with siRNA targeting the TLR gene) or RNA-silenced control (model mice treated with control plasmid). The CVB3 model mice were infected by an intraperitoneal injection with 10⁵ TCID₅₀ CVB3 at day 0. The DMEM group was treated with the same volume of DMEM. No treatment was given to the mice in the CN group. The mice in TLR-silencing group and the RNA-silenced control group were infected by an intraperitoneal injection with 10⁵ TCID₅₀ CVB3 at day 0. Furthermore, the pRNAT-U6.1/Neo siRNA (0.2 μg) and RNA-silencing control plasmid (0.2 μg) were also transferred by intraperitoneal injection mediated by 0.2 μl Lipofectamine 2000 transfection reagent (Invitrogen, USA) at day 0 (but not at the same injection site with the CVB3 infection).

**Tissue histopathology and myocarditis grading.** On days 7, 14, and 21 after CVB3-infection, heart tissues were collected, sectioned, and stained with hematoxylin and eosin (HE). The sections were examined by two independent investigators in blind manner, and the severity of myocarditis was assessed following a previously described report [12]. Grading of myocarditis was defined on a 0 to 4 scale: 0 – no inflammation or injury; 1 – one to five distinct mononuclear inflammatory foci with involvement of ≤5% of the cross-sectional area; 2 – more than five distinct mononuclear inflammatory foci, or involvement of 5–20% of the cross-sectional area; 3 – diffuse mononuclear inflammation (or injury) involving over 20% of the area; 4 – diffuse inflammation with necrosis or injury.

**Semiquantitative RT-PCR.** The forward primer for detecting TLR4 was 5′-GAATCACCTGGGCACGAC-3′, and the reverse primer was 5′-GCTCGGCC-CACGTACATACT-3′. The individual GAPDH was used as the internal control (forward primer: 5′-AAGGC-CTACCATCTTTCCAG-3′, reverse primer: 5′-TGAGCCTTTCCACTGGC-3′). The RNA samples were isolated and separated by 15% SDS-PAGE and electrophoresed on 1.5% agarose gel, the gel images of each PCR product were digitally captured with a CCD camera and analyzed with NIH Imager beta version 2. Relative transcriptional values of each factor in semiquantitative RT-PCR are presented as the ratio of the signal value of the specific PCR product and that of the individual GAPDH. The procedures and the statistical analysis were performed as previously described [13].

**Examination of cardiac enzymes.** Cardiac enzymes can reflect heart function to some degree. Thus, levels of aspartate transaminase (AST), creatine kinase (CK), creatine kinase isoenzyme (CK-MB), and lactate dehydrogenase (LDH) in supernatants of heart homogenates were detected using a fully automatic biochemical analyzer following the manufacturer’s instructions (Thermo, USA).

**Western blot.** Mouse myocardial tissue lysates were isolated and separated by 15% SDS-PAGE and electrotransferred onto nitrocellulose (NC) membranes. The NC membranes were blocked with 5% defatted milk that was dissolved in PBST (phosphate buffered saline, pH 7.6, containing 0.05% Tween-20) overnight at 4°C. Then the NC membranes were incubated with 1 : 500 TLR4-specific monoclonal antibodies (mAb) (Santa Cruz, USA), 1 : 2000 GADPH-specific mAb for 1 h at 37°C and then incubated with 1 : 3000 horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Santa Cruz). The specific procedures were performed according to Xu et al. [14].

**TLR4-siRNA design and transfection.** Three target sequences of the TLR4 gene were selected. The oligonucleotides containing sequences specific for TLR4 (5′-GATAGGAACCACTCTTT-3′, 5′-CCTTCT-3′, 5′-GTTCCATTGCTTGCGAA-3′) were synthesized and annealed. A pair of annealed DNA oligonucleotides was inserted into a pRNAT-U6.1/Neo siRNA expression vector that had been digested with BamHI and HindIII (GenScript, USA). The pRNAT-U6.1/Neo siRNA vector (0.2 μg) was also injected (transfection) into the mice together with the CVB3 virus, but not at the same site.

**Statistical analysis.** The image of the immunoblot was scanned with Typhoon software (Pharmacia, USA) and then digitized and saved in TIF format. All data are presented as mean ± SD. Statistical analysis was performed using SPSS 19.0 software, and the t-test was employed. The value of overall survival rate was calculated using Cheson’s criteria. Differences with p < 0.05 were considered statistically significant.

**RESULTS**

**CVB3 infection triggers significant myocardial injury.** The mice for establishing the acute viral myocarditis model were administered intraperitoneal injection with 2·10² TCID₅₀ CVB3 on day 0, and the model was observed for 21 days. Figure 1a indicates that the CVB3 virus took
on higher efficacy of virus delivery. We detected the survival rate of the mice in each group. Only two mice died in the CN group and three mice in the DMEM group by the 21st day. The survival of the mice in the MOD group was significantly decreased, decreasing by six on the 7th day, nine on the 14th day, and the survival diminished to 70% on the 21st day (Fig. 1a). We also examined the virus titer in each group. The results of virus titer examination indicated that the virus titer peaked on the 14th day, and then decreased gradually in the following days to day 21 (Fig. 1b). However, no virus titer could be detected in the CN and DMEM groups, which showed that none of these mice was infected by the CVB3 virus.

The myocarditis was evaluated to determine the histopathological status. The histopathology results showed that myocardial injury was observed on the 7th day and was increasingly severe on the following 14th and 21st days ($p < 0.05$) (Fig. 1c and d).

**TLR4 levels are upregulated in the MOD group.** To explore the mechanism of the pathology of CVB3-triggered viral myocarditis, the TLR4/NF-κB pathway factor TLR4 was examined by real-time PCR (RT-PCR) and Western blot assay, respectively. The results illustrated that the TLR4 mRNA expression in the MOD group was significantly upregulated compared with the CN group on the 21th day (Fig. 2a, $p < 0.01$). The results of Western blot assay indicated the same changes of TLR4 protein (Fig. 2b).

**CVB3 infection triggers enhanced level of cardiac enzymes.** In this study, cardiac enzymes including AST, LDH, CK, and CK-MB were also detected. The results indicated that all of the above four cardiac enzymes were significantly increased compared with the CN group, and the peak value appeared on the 14th day after infection (Fig. 3).

**Gene silencing of TLR4 decreases cardiac injury.** To investigate the role of TLR4 in the pathogenesis of CVB3-induced viral myocarditis, the TLR4 gene in the CVB3-infected mice was silenced. According to our preliminary results, the transfection of TLR4 silencing has no effects on
the CVB3 infection and delivery. The results indicated that the TLR4 mRNA expression (Fig. 4a) and TLR4 protein expression (Fig. 4b) decreased significantly in the TLR4-silenced group compared with the MOD group ($p < 0.05$).

Histopathological studies with HE staining indicated that when the $TLR4$ gene was silenced, the myocardial injury was significantly lessened starting from the 7th day to the 21th day compared with the MOD group (Fig. 4c,
Especially on the 21th day, even complete lack of cardiac inflammation could be found in the TLR4-silenced group. Gene silencing of TLR4 alleviates cardiac enzyme upregulation. We also monitored the cardiac enzymes when TLR4 was silenced. The results showed that the levels of AST, LDH, CK, and CK-MB were significantly decreased in the TLR4-silenced group compared with the MOD group (Fig. 5). Thus, the TLR4 gene silencing blocked the cardiac enzyme upregulation of the mice with viral myocardial infection.

Control siRNA silencing does not affect TLR4 status and does not alleviate cardiac injury. To give convincing data for cardiac improvement by TLR4 gene silencing and to demonstrate the specificity of the particular siRNA effects, control siRNA was employed to treat mice. The results indicated that the control siRNA could not affect the TLR4 mRNA (Fig. 6a) and protein expression (Fig. 6b) and could not alleviate the cardiac injury (Fig. 6c). Furthermore, the cardiac enzymes including AST, LDH, CK, and CK-MB in the control siRNA group did not change compared with the MOD group (Fig. 6d). These results confirmed that the cardiac improvements of gene silencing of TLR4 were specific.

DISCUSSION

Viral myocarditis is mainly characterized by excessive inflammation of the myocardium. Previous reports indicated that cytokines, inflammatory factors, and chemokines play important roles in the pathogenesis of viral myocarditis [15]. In clinics, many therapeutic methods have targeted the expression of cytokines for treatment of viral myocarditis patients. In this study, the TLR4/NF-κB inflammatory response signal-transduction pathway
was explored. Our data show that the CVB3 virus can trigger myocardial injury even from day 1 in our mouse model. The virus titer peaked on day 14 in the cardiac tissues, and then gradually decreased in the following days until day 21. However, the most serious myocardial injury was found on day 21 post-infection, which may be caused by the cumulative effects of the virus. Also, the lowest survival rate of the mice in the MOD group appeared on day 14, which remained to day 21 post virus infection.

In the present study, the results indicate that the infection with CVB3 can induce overexpression of TLR4/NF-κB signal-transduction factor, TLR4, in cardiac tissues on day 1, 7, 14, and 21. This result is consistent with previous reports [16, 17], which also indicated that the CVB3 can induce upregulation of TLR4. However, Roberts et al. [18] found that TLR signaling in males correlated with increased myocarditis susceptibility, but not in females. This conclusion was different from ours, thus further study should investigate the sex differences in TLR4-related viral myocarditis. Also, the cardiac CK, CK-MB, LDH, and AST activities significantly increased from day 1, and achieved the peak at day 14. Chen et al. [19] also found that the cardiac enzymes CK, LDH, AST, and CK-MB were enhanced in CVB3-induced myocardial tissues, but the peak appeared on day 10 post-infection. This difference may be caused by the different strain or titer of the CVB3 virus.

To investigate the further function of TLR4 in CVB3-caused viral myocardial injury, small interfering RNA (siRNA) of TLR4 was employed to silence the TLR4 expression. From the results, we found that TLR4 mRNA and protein expression was significantly decreased compared with the MOD group at every time point from day 1 to day 21. These data prove that the transfection system of RNA silencing is effective and stable.

The inflammatory response and injury were clearly triggered by viral infection. The TLR4/NF-κB signal-transduction pathway was considered as a prototypical proinflammatory signaling pathway. TLR4/NF-κB could mediate the activation of some proinflammatory genes including cytokines, chemokines, and adhesion molecules and toll-like receptor 4 molecules [16]. Thus, in this study we detected TLR4 expression and blocked the TLR4 over-expression by silencing the TLR4 gene. TLR4 is a toll-like receptor protein that plays a key role in the innate immune system as well as in the digestive system [17, 18]. TLR4 can also activate the TLR4/NF-κB pathway, which plays a critical role in proinflammatory cytokine-mediated signaling pathways [19]. However, not much is known about the role of TLR4 in viral myocarditis. Our results indicate
that the TLR4-mediated TLR4/NF-κB pathway is significantly inhibited in CVB3-infected mice transfected with silencing RNA, including lessening of myocardial injury and cardiac enzyme level enhancement. The preliminary experiment data of our team also show that blocking the expression of TLR4 can result in lower expression levels of proinflammatory cytokines, which is consistent with the results in the present study. Richer et al. [17] also showed that TLR4 could induce inflammatory cytokine production in CVB3-infected mice. Thus, the TLR4/NF-κB signal-transduction pathway might be a novel target for the therapy of viral myocarditis.

The function and the molecular mechanism responsible for TLR4/NF-κB inhibition of silencing RNA have never been clarified [20-23]. In this study, silencing RNA for the TLR4 gene was used for the first time to treat CVB3-induced viral myocarditis and to investigate the inhibitory function of silencing RNA. The results indicate that endogenous TLR4 is ubiquitylated in CVB3-infected cardiac myocytes. They suggest that silencing RNA can de-ubiquinate TLR4 to block the CVB3-activated TLR4/NF-κB pathway. To our knowledge, this is the first report illustrating that gene silencing is necessary to block the CVB3-activated TLR4/NF-κB pathway by restricting endogenous TLR4 expression. Other reagents have been found for TLR4 inhibition. Ding et al. [24] found that cinnamaldehyde could directly reduce the inflammation and injury in viral myocarditis mice by inhibiting the TLR4/NF-κB signal-transduction pathway. Chen et al. [19] also reported that Shenqi Fuzheng Injection could also reduce CVB3-induced TRAF6 production, which is a factor of the TLR4/NF-κB pathway. Therefore, factors in the TLR4/NF-κB pathway can act as the targeting molecules for viral myocarditis therapy. Of course, there may also be some other targeting molecules involved in CVB3-activated TLR4/NF-κB signaling.

Fig. 6. Effects of control siRNA silencing on TLR4 status and cardiac injury. a) TLR4 gene expression in MOD and control siRNA group; b) TLR4 protein expression in MOD and siRNA group; c) statistical analysis of HE staining images of myocarditis tissues were performed; d) activity of CK, CK-MB, LDH, and AST was monitored daily until day 21 post-infection.
In conclusion, our study shows for the first time that TLR4 participates in the pathogenesis of CVB3 virus by activating the TLR4/NF-κB signaling pathway. Gene silencing of TLR4 can decrease cardiac inflammation and lessen the severity of myocarditis. Thus, TLR4 gene silencing can control CVB3-induced viral myocarditis, which could be a feasible therapeutic treatment for viral myocarditis.

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