Involvement of NLRP3 inflammasome in CVB3-induced viral myocarditis

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Viral myocarditis, which is most prevalently caused by coxsackievirus B3 (CVB3) infection, is a serious clinical condition characterized by cardiac inflammation. Inflammasome plays an essential role in the regulation of diverse inflammatory responses by serving as a platform for caspase-1 activation and caspase-1-dependent proteolytic maturation and secretion of IL-1β. Although inflammasome has been reported to be crucial for the development of many inflammatory diseases, its role in the pathogenesis of viral myocarditis is still elusive. The present study aims to investigate whether CVB3 infection activates inflammasome and whether the activation of inflammasome contributes to CVB3-induced myocarditis. Our results showed that CVB3 infection induced inflammasome activation both in vitro and in vivo. With the inhibition of inflammasome activation, the severity of CVB3-induced myocarditis was significantly alleviated as evidenced by less weight loss, decreased serological indexes of creatine kinase and creatine kinase-MB activities, as well as less severe myocardial injury. Of importance, echocardiography results showed that inhibition of inflammasome activation also efficiently improved cardiac function as revealed by enhanced left ventricular ejection fraction and left ventricular fractional shortening. Despite that CVB3 infection significantly increased the expression of both retinoic acid-inducible gene 1 and NOD-like receptor family, pyrin domain containing 3 (NLRP3) in cardiac myocytes, CVB3-induced inflammasome activation was NLRP3-, but not retinoic acid-inducible gene 1, dependent. Further study showed that reactive oxygen species production and K⁺ efflux were critical for the activation of NLRP3 inflammasome upon CVB3 infection. Collectively, our study demonstrated a crucial role of the NLRP3 inflammasome in the pathogenesis of CVB3-induced myocarditis, and modulation of inflammasome activation might represent a promising therapeutic strategy for viral myocarditis.

coxsackievirus B3; viral myocarditis; inflammasome; interleukin-1β; NLRP3

VIRAL MYOCARDITIS (VMC) is a principal cause of sudden death in young adults, which may progress to dilated cardiomyopathy and even congestive heart failure (33). Coxsackievirus group B type 3 (CVB3), an enterovirus of the picornaviridae family, is believed to be the most common causative agent in VMC (1, 39). Despite that extensive efforts have been made in the past several decades, the pathogenesis of CVB3-induced myocarditis is still unclear. It is reported that CVB3 can directly damage myocardium (4, 11, 28); however, accumulating evidence demonstrates that the excessive host immune responses may play a more critical pathogenic role in the course of CVB3-induced myocarditis (11, 33, 39). It has been reported that the VMC mice had elevated levels of the proinflammatory cytokines, such as TNF-α and IL-1β (9, 29). Treatment of mice model with the neutralization antibody against IL-1β has been proven to lead to sustained reduction in systemic inflammation and ameliorate symptoms of VMC. Blockade of MyD88 signaling pathway in CVB3-infected mice is also revealed to decrease proinflammatory response in cardiac tissue and thus improve the survival rate significantly (17, 51). Of importance, a variety of clinical studies have demonstrated the elevation of proinflammatory cytokines (e.g., TNF-α, IL-1β, and IL-6) in patients with myocarditis (10, 12). Given the importance of proinflammatory cytokines in VMC, appropriate modulation of the production is important for homeostasis of host.

Inflammasome, an intracellular multiprotein complex containing a sensor protein, the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC), and the inflammatory protease caspase-1, is responsible for the activation of the inflammatory cytokines IL-1β and IL-18 (15, 32). Studies have demonstrated that inflammasome activation contributes to host immune responses to a wide range of microbial pathogens and endogenous danger signals through regulating caspase-1-induced inflammatory responses (15, 21, 49); however, in some circumstances, inflammasome activation may lead to the development of inflammatory diseases (6, 23, 25, 48). Evidence has also shown that the dysregulation of inflammasome activity is associated with numerous proinflammatory diseases including arthritis, neurodegenerative disorders, metabolic disorders, and inflammatory bowel disease (6, 8, 23, 27, 30). However, whether CVB3 infection could induce inflammasome activation and the role of inflammasome activation in CVB3-induced myocarditis is still unclear.

In present investigation, we demonstrated an essential role of inflammasome in mediating the pathological response to CVB3 infection. We found that NOD-like receptor family, pyrin domain containing 3 (NLRP3) but not retinoic acid-inducible gene 1 (RIG-I) was involved in CVB3-induced inflammasome activation and the subsequent IL-1β secretion. Furthermore, the production of reactive oxygen species (ROS) and the potassium efflux, but not the lysosomal destabilization, were revealed to be crucial for inflammasome activation in response to CVB3 infection.

MATERIALS AND METHODS

Mice. Specific pathogen-free male BALB/c mice (H-2b), 6 wk of age, were purchased from Shanghai Experimental Animal Centre of Chinese Academy of Sciences. All animal experiments were performed according to the Guide for the Care and Use of Medical Laboratory Animals (Ministry of Health, P.R. China, 1998) and with
the ethical approval of the Shanghai Medical Laboratory Animal Care and Use Committee (permit number, SYXX 2009-0036) as well as the Ethical Committee of Fudan University (permit number, 20090106).

**Virus infection and virus titration.** The original stock of CVB3 (Nancy strain) was maintained by passage through Hela cells (ATCC number: CCL-2). Mice were infected by an intraperitoneal injection with $10^5$ 50% tissue culture infectious dose (TCID$_{50}$) of CVB3, and hearts from individual mice were aseptically harvested, weighted, and homogenized in complete basal Eagle’s medium (DMEM) containing 2% FBS. Cellular debris was removed by centrifugation, and viral titers were determined on Hela cell monolayers using TCID$_{50}$ assay as previously described (19).

**Inhibition of caspase-1.** In vivo inhibition of caspase-1 was achieved using a highly specific, cell-permeable, and competitive inhibitor of caspase-1 as previously described (14, 34, 50). Briefly, 6 h after CVB3 infection, mice received the first intraperitoneal injection of 0.5 mg Ac-Tyr-Val-Ala-Asp-2,6-dimethylbenzoxoylmethyl ketone (Ac-YVAD-CHO, Bachem Biochemica, Heidelberg, Germany) dissolved in 0.1 ml sterile phosphate-buffered saline (pH 7.4). This was repeated after 12 h and continued at 12-h intervals until surviving mice were euthanized at the end of the observation period. Infected mice receiving phosphate-buffered saline treatment were used as control.

For caspase-1 inhibition in vitro, cardiac myocytes were incubated with caspase-1 inhibitor Ac-YVAD-CHO (10 $\mu$M) for 30 min before CVB3 infection.

**Neutralization of IL-1β in vivo.** In vivo blockade of IL-1β was achieved using the neutralizing antibody against IL-1β as previously described (41). Briefly, mice were injected intraperitoneally with 100 $\mu$g/mouse of either anti-IL-1β antibody (R&D Systems; Minneapolis, MN) or an isotype control antibody (R&D Systems) 24 h before CVB3 infection.

**Preparation of neonatal murine cardiac myocytes.** Cardiac myocytes from neonatal mice within 72 h of birth were prepared as previously reported (43). Briefly, the hearts were finely minced and subjected to stepwise enzymatic digestion with 0.25% trypsin. The dissociated cells were washed with DMEM and depleted of endothelial cells and fibroblasts by two sequential 1-h adsorptions to plastic flasks at 37°C. After the noadherent myocytes were removed, the stuck myocytes were washed once, resuspended in complete basal medium, and dispensed into tissue culture wells. After a period of 48 h, the myocytes were firmly attached to the plastic. According to observations on the shape and beating activity of the cells obtained, more than 95% cells were identified as cardiac myocytes.

**Histopathology and myocarditis grading.** Seven days following CVB3 infection, the hearts were cut longitudinally, fixed in 10% phosphate-buffered formalin, paraffin embedded, sectioned, and stained with hematoxylin and eosin. Sections were examined by two independent investigators in a blinded manner, and the severity of myocarditis was assessed by previously described 0–4 scale (20), in which 0 = no inflammation; 1 = one to five distinct mononuclear inflammatory foci with involvement of 5% or less of the cross-sectional area; 2 = more than five distinct mononuclear inflammatory foci, or involvement of over 5% but not over 20% of the cross-sectional area; 3 = diffuse mononuclear inflammation involving over 20% of the area, without necrosis; and 4 = diffuse inflammation with necrosis.

**Echocardiography.** At day 7 after CVB3 infection, echocardiography was performed on isoflurane-anesthetized mice with high-resolution ultrasound imaging system (Vevo2100, Visual Sonics) equipped with a 30-MHz microscan transducer. The echocardiographic measurements of left ventricular ejection fraction and left ventricular fractional shortening were performed according to the operator’s manual.

**Real-time PCR analysis.** Total RNA was extracted from cardiac myocytes or mouse hearts with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The cDNA was synthesized with PrimeScript RT reagent kit (Takara, Dalian, China). The expression of the genes encoding caspase-1, NLRP3, and ASC was quantified by real-time PCR using a Lightcycler 480 and SYBR Green system (Roche Diagnostic Systems, Mannheim, Germany) following the manufacturer’s protocol using oligonucleotide primer specific for mouse caspase-1 (forward, 5’-TGGTCTTTGACTTTTGAGGAC-3’ and reverse, 5’-GGTCAACCTTACGAGTGG-3’), mouse NLRP3 (forward, 5’-CGAGACTCTTGGAAGAGGT-3’ and reverse, 5’-GCATACATTGAGAAGTGATGC-3’), and mouse ASC (forward, 5’-CAGAGTCAGCCAGAAGAGTC-3’ and reverse, 5’-GGTGGTCCTGACGAAAAGCC-3’).

**Gene silence.** To block NLRP3 or RIG-I expression, cardiac myocytes were transfected with specific sets of small interfering RNA (siRNA) for mouse NLRP3 (target, 5’-GGUGAAAUUGACCUAUAUAC-3’ (26), as well as siRNA for mouse RIG-I (target, 5’-CCACACACUUGGAGUGUC-3’) (2) and nonsense sequence used as scrambled siRNA were obtained from GenePharma (Shanghai, China). siRNA (10 nM) was used for transfection using HiPerFect Transfection Reagent (Qiagen) according to the manufacturer’s protocol. Briefly, primary cardiac myocytes were washed twice with PBS before transfection, and siRNA was added to wells in DMEM supplemented with 10% fetal bovine serum for 72 h at 37°C and 5% CO$_2$. Thereafter, cells were treated with CVB3 (multiplicity of infection = 10) for the indicated time points. The mRNA and protein expression levels for NLRP3 or RIG-I were determined by real-time PCR and Western blot analysis, respectively.

**Caspase-1 assay.** Caspase-1 activation assay was performed using caspase-1 assay kit (Beyotime Institute of Biotechnology), which is based on the ability of caspase-1 to cleave acetyl-Tyr-Val-Ala-Asp-p-nitroanilide (Ac-YVAD-pNA) into the yellow formazan product p-nitroaniline (pNA). Cell lysates were centrifuged at 12,000 g for 10 min, and the protein concentrations in the supernatants were determined by the Bradford protein assay. Protein (30 µg) from each sample was used for the assay according to the supplier’s instructions. Briefly, cellular extracts were incubated in a 96-well microtiter plate with 1 mM of Ac-YVAD-pNA overnight at 37°C. The absorbance values of pNA at 405 nm (OD$_{405}$) were measured using a 96-well plate reader (Multiskan MK3, Thermo Scientific), and the increase in OD$_{405}$ indicated activation of caspase-1 and shown as fold change compared with control.

**Western blot analysis.** To examine caspase-1 activation, culture supernatants of cardiac myocytes treated with indicated stimuli were subjected to trichloroacetic acid precipitation. After being washed with ice-cold ethanol, the precipitates were dissolved in $\times$1 SDS-PAGE loading buffer and analyzed by Western blot analysis to detect processed mature caspase-1 p10 fragment; cell lysates were blotted with pro-caspase-1 and GAPDH antibodies to show the levels of pro-caspase-1 in cell lysates and protein loading, respectively. Anti-caspase-1 antibody used here was obtained from Santa Cruz Biotechnology. ASC protein expression in the heart of VMC mice was evaluated by Western blot analysis using ASC antibody (Sigma-Aldrich). NLRP3 or RIG-I expression in cardiac myocytes was assessed by Western blot analysis with NLRP3 antibody (Santa Cruz Biotechnology) or RIG-I antibody (Cell Signaling Technology), respectively, and quantified using ImageJ software.

**ELISA for IL-1β.** Levels of IL-1β of cell culture supernatants and heart homogenates were determined by enzyme-linked immunosorbent assay (ELISA) (eBioscience) following the manufacturers’ instructions.

**Measurement of ROS.** Intracellular ROS generation in control and treated cells was assessed using the Reactive Oxygen Species Assay Kit (Beyotime Institute of Biotechnology) as previously described (45). To examine the role of ROS formation in the assembly of inflammasome, ROS inhibitor 5 mM N-acetyl cysteine (NAC) (Beyotime Institute of Biotechnology) was used. Stock solution of NAC was neutralized before use to ensure that it would not change the PH of the culture media. Briefly, cardiac myocytes were treated for 2 h with 5
mM NAC, followed by mock or CVB3 infection for 4 h. After being washed, the cells were incubated with 10 mM 2′,7′-dichlorofluorescein diacetate for 30 min at 37°C in the dark, followed by the detection of fluorescence intensity using FACS calibur flow cytometer (BD Biosciences; excitation, 488 nm; and emission, 530 nm).

Statistical analysis. Data were shown as means ± SE. Statistical analysis of the data was performed using the GraphPad Prism (Version 5.0) statistical program. The unpaired Student’s t-test was used to compare differences between two groups, whereas comparison of multiple groups was performed using ANOVA with post hoc tests to compare differences between individual groups. Pearson’s coefficient was used to determine the correlation between groups. The survival rates of CVB3 infected mice were compared and analyzed with Kaplan-Meier plot. \( P < 0.05 \) was considered statistically significant.

RESULTS

CVB3 infection induced production of IL-1β in cardiac tissues of VMC mice, positively correlated with the severity of myocarditis. To explore the role of IL-1β in the pathogenesis of VMC, we generated VMC murine model by intraperitoneal injection in male BALB/c mice with \( 10^5 \) TCID\(_{50} \) CVB3 as previously described (43). As shown in Fig. 1A, the body weight changes exhibited significant and continuous decrease since day 2 postinfection. The hematoxylin and eosin-stained muscle sections also showed the apparent myocardial injury in CVB3-infected mice (Fig. 1B). We then examined the IL-1β levels in VMC mice by ELISA. Results showed that the IL-1β production was significantly increased in mouse sera since day 4 (Fig. 1C) and in cardiac tissue since day 2 (Fig. 1D). Of note, correlation analysis revealed that the myocardial IL-1β levels were positively correlated with the heart pathological score (Fig. 1E) and body weight loss (Fig. 1F). Together, this result suggested that the levels of IL-1β were increased in response to CVB3 infection and highly correlated with the pathological process of VMC.

CVB3 infection induced inflammasome activation both in vivo and in vitro. To investigate whether inflammasome was involved in CVB3-induced myocarditis, we determined the expression of the inflammasome components, caspase-1, and the adapter protein ASC by real-time PCR analysis and Western blot analysis, respectively. Results showed that CVB3 infection significantly upregulated the expression of ASC, caspase-1, and caspase-1 p10 subunit in the heart tissue at both mRNA and protein levels (Fig. 2, A and B). Further immunohistochemical analysis also showed significant upregulation in both caspase-1 and ASC expression (Fig. 2C). Moreover, CVB3 infection was found to significantly enhance caspase-1 enzymatic activity after CVB3 infection (Fig. 2D).

We further isolated primary cardiac myocytes from neonatal BALB/c mice and infected these cells with CVB3 for 24 h at varying multiplicity of infection. As shown in Fig. 3A, IL-1β secretion exhibited a dose-dependent increase in CVB3-infected cardiac myocytes. Caspase-1 activation was also evident following CVB3 infection as demonstrated by the increase of caspase-1 enzymatic activity (Fig. 3B) and induction of caspase-1 subunit p10 (Fig. 3C). Time course analysis also showed that CVB3 infection enhanced IL-1β production,
caspase-1 activity as well as caspase-1 subunit p10 induction in a time-dependent manner (Fig. 3, D-F). Taken together, these data demonstrated that CVB3 infection could induce inflammasome activation both in vivo and in vitro.

Inflammasome activation was crucial for the development of CVB3-induced myocarditis. To examine the role of inflammasome activation in the pathogenesis of CVB3-induced myocarditis, we treated CVB3-infected mice with caspase-1 inhibitor Ac-YVAD-CHO to block inflammasome activation and then examined parameters of the severity of myocarditis. Result showed that inhibition of caspase-1 activity significantly alleviated the symptoms of VMC as evidenced by less weight loss (Fig. 4A), higher survival rate (>80%) (Fig. 4B), decreased serological indexes of creatine kinase (CK), CK-MB activities (Fig. 4, C and D), and less severe myocardial injury (Fig. 4E). Of note, M-mode echocardiography showed that inhibition of inflammasome activation also improved cardiac function as revealed by enhanced left ventricular ejection fraction and left ventricular fractional shortening (Fig. 4, F and G). However, the levels of viral titer in the heart exhibited no significant decrease after Ac-YVAD-CHO treatment (Fig. 4H).

Furthermore, our data demonstrated that inhibiting caspase-1 activity as well as caspase-1 subunit p10 induction in a time-dependent manner (Fig. 3, D-F). Taken together, these data demonstrated that CVB3 infection could induce inflammasome activation both in vivo and in vitro.
activity reduced the levels of IL-1β both in vivo (Fig. 4I) and in vitro (Fig. 4J).

To determine whether the contribution of inflammasome for CVB3-induced myocarditis was mediated by IL-1β, we used neutralizing antibody against IL-1β by intraperitoneal injection 24 h before CVB3 infection. The result showed that blockade of IL-1β ameliorated the symptoms of VMC as demonstrated by less fluctuation in body weight (Fig. 5A), higher survival...
rate (Fig. 5B), decreased serum CK and CK-MB levels (Fig. 5, C and D), and less severe myocardial injury (Fig. 5E) compared with those of the control antibody treatment group. All these data indicated that CVB3-induced inflammasome activation played a critical role in CVB3-induced myocarditis through regulating IL-1β production.

**NLRP3 inflammasome was responsible for CVB3-induced IL-1β secretion in cardiac myocytes.** Various sensor proteins, including NLRP1, NLRP3, NLRC4, NLRP6, AIM2 and RIG-I, have been revealed to initiate the activation of inflammasome platforms. To identify the receptor involved in inflammasome activation in response to CVB3 infection, we determined the expression levels of these receptors in CVB3-infected cardiac myocytes by real-time PCR analysis. As shown in Fig. 6A, only the expression levels of NLRP3 and RIG-I were upregulated in cardiac myocytes infected with CVB3.

To evaluate the role of RIG-I and NLRP3 in caspase-1 activation in response to CVB3 infection, we knocked down the expression of RIG-I or NLRP3 by siRNA technique. Results from real-time PCR and Western blot analysis showed that transfection of RIG-I or NLRP3 siRNA in cardiac myocytes significantly inhibited the expression of their corresponding gene (Fig. 6, B–E). Further results showed that the knockdown of RIG-I had no effect on IL-1β secretion upon CVB3 infection (Fig. 6F); however, NLRP3 downregulation decreased IL-1β production by about 50% after CVB3 infection (Fig. 6G). Moreover, NLRP3 siRNA treatment also led to marked reduction in caspase-1 activity (Fig. 6H) and caspase-1 p10 production (Fig. 6I) in response to CVB3 infection. These results indicated that NLRP3 but not RIG-I was implicated in CVB3-induced inflammasome activation.

**ROS and potassium efflux were crucial for NLRP3 inflammasome activation in response to CVB3 infection.** Activation of the NLRP3 inflammasome can be mediated by different mechanisms, including production of ROS, stimulation of K⁺ efflux, and induction of lysosomal destabilization (47). To elucidate the cellular mechanism by which CVB3 triggered the NLRP3 inflammasome, we used NAC to inhibit ROS, K⁺-rich medium, or an ATP-sensitive potassium channel inhibitor glibenclamide to prevent K⁺ efflux, and bafilomycin A1 to...
blocklysosome destabilization, respectively. The results showed that inhibition of ROS generation (Fig. 7A) by NAC impaired caspase-1 activity and IL-1β production during CVB3 infection (Fig. 7, B–D). Further results revealed that CVB3-induced caspase-1 activation and IL-1β secretion were markedly suppressed when K+ efflux was prevented by culturing cells with K+-rich medium or glibenclamide (Fig. 7, E–G), whereas the pretreatment of cardiac myocytes with bafilomycin A1 had no effect on blocking CVB3-induced caspase-1 activation and IL-1β secretion (Fig. 7, H and I). Collectively, these data demonstrated that generation of ROS and K+ efflux during CVB3 infection might act as the stress signals for NLRP3 inflammasome activation, which in turn were crucial for CVB3-induced IL-1β secretion, whereas lysosomal destabilization appeared not to be involved in the activation of the NLRP3 inflammasome in response to CVB3 infection.

**DISCUSSION**

VMC is an inflammation of the myocardium induced by virus infection (31). Although direct injury induced by CVB3
has been found in CVB3-induced myocarditis (4, 11, 28), the inflammation is concerned as the more prominent reason for the injury of CVB3-induced myocarditis (9, 17, 29, 51). A growing body of evidence has suggested that proinflammatory cytokines are critical for the development of CVB3-induced myocarditis (9). Among the cytokines, increased IL-1β levels in the heart directly correlate with increased acute CVB3-induced myocarditis in murine model (9, 29). Furthermore, a broad spectrum of inflammatory cytokines is induced by CVB3 replication via a pathway that requires IL-1 signaling (18, 38). Inflammasome is a key modulator of inflammatory responses via regulating IL-1β release and has been demonstrated to be involved in a wide variety of inflammatory and autoimmune diseases; however, its role in CVB3-induced myocarditis is still unknown.

In present study, we investigated whether inflammasome was activated by CVB3 infection and the role of inflammasome activation in CVB3-induced myocarditis. During the prepara-

Fig. 7. Treatment with N-acetyl-cysteine (NAC) or high concentration of K+ inhibited the inflammasome activation and subsequent IL-1β secretion. A–D: cardiac myocytes were infected with CVB3 in the absence or presence of NAC (5 mM for 2 h) to inhibit reactive oxygen species (ROS) generation. Representative FACS plot showed intracellular ROS production after 4 h of CVB3 infection and was represented with the mean fluorescent intensities in mock-infected control, CVB3 alone, and CVB3 plus NAC treatment (A). Activation of caspase-1 was measured after 12 h of CVB3 infection (10 MOI for 12 h) in presence or absence of NAC (B). Active caspase-1 p10 and pro-caspase-1 were analyzed by Western blot analysis (C). IL-1β in the culture supernatants was assessed by ELISA (D). E–G: cardiac myocytes were infected with CVB3 (10 MOI for 12 h) in presence or absence of NaCl (150 mM), KCl (150 mM), or glibenclamide (Glib; 100 μM). Caspase-1 activity was assessed using an enzymatic assay (E). Active caspase-1 p10 and pro-caspase-1 were analyzed by Western blot analysis (F). IL-1β in the culture supernatants was assessed by ELISA (G). H and I: cardiac myocytes were infected with CVB3 (10 MOI for 12 h) in presence or absence of bafilomycin A1 (100 nM). Activation of caspase-1 was measured (H), and IL-1β in the culture supernatants was assessed (I). Data were presented as means ± SE of 3 separate experiments. *P < 0.05.
tion of our paper, a report by Toldo et al. (46) was published in which the presence of inflammasome in samples directly obtained from acute myocarditis patients was described. The authors identified the formation of the inflammasome in heart biopsy samples of all 11 cases of acute myocarditis patients (100%) and in heart samples of 10 of 11 postmortem cases (91%) and in none of the controls. Our data and conclusion in vitro and in murine model studies correlate well with the data from the sample directly obtained from patients reported (46) and corroborated and strengthen the conclusion that CVB3 activates in inflammasome and is a direct cause of VMC. Further results showed that inhibition of inflammasome activation significantly alleviated the symptoms of CVB3-induced myocarditis. Collectively, these data illustrated that CVB3 infection induced inflammasome activation both in vitro and in vivo and suggested the significant role of the inflammasome in the pathogenesis of CVB3-induced myocarditis.

Based on the sensor protein involved, at least six types of inflammasomes have been identified, namely, NLRP1, NLRP3, NLRC4, NLRP6, AIM2, and RIG-I inflammasome (12). Many of these sensors are promoted by specific stimuli. For example, NLRP1 inflammasome activation is stimulated by muramyl dipeptide (MDP) and lethal toxin (5, 13); AIM2 inflammasome is activated in response to cytoplasmic DNA (16, 37); RIG-I inflammasome is promoted by the 5′-triphosphate RNA generated by virus (36). However, NLRP3 inflammasome responds to numerous physically and chemically diverse stimuli (3, 22, 35, 40, 42). In our study, both RIG-I and NLRP3 in cardiac myocytes were upregulated upon CVB3 infection. Whereas knockdown of RIG-I had no effect on caspase-1 activation and IL-1β releasing in response to CVB3 infection, downregulation of NLRP3 in cardiac myocytes significantly decreased the levels of IL-1β upon CVB3 infection. These results indicated the important role of NLRP3 but not RIG-I in CVB3-induced inflammasome activation.

Although NLRP3 inflammasome responds to physically and chemically diverse stimuli, the actual triggering of NLRP3 is controlled by integration of a comparatively small number of signals such as K+ efflux, elevated levels of ROS, and lysosomal destabilization (27). Corresponding to previous reports that CVB3 infection could induce ROS generation (44), our result revealed that exposure to CVB3 resulted in an increased ROS production in cardiac myocytes. Inhibition of ROS with NAC led to significant reduction of caspase-1 activity as well as the levels of IL-1β production in response to CVB3 infection, verifying the essential role of ROS in the activation of NLRP3 inflammasome. Furthermore, our results showed that blockage of K+ efflux with K+-rich medium significantly decreased IL-1β release and caspase-1 activation upon CVB3 infection. To exclude the possibility that K+-rich medium may change the cardiac myocytes functions through not only potassium channels but also some voltage-gated sodium channels, we used an ATP-sensitive potassium channel inhibitor glibenclamide and found that blockage of K+ efflux by glibenclamide also markedly reduced IL-1β release in response to CVB3 infection. This data confirmed the importance of K+ efflux in CVB3-induced NLRP3 inflammasome activation. It was also reported that the phagocytosis of particulate or agonists or live pathogens could be followed by disruption of the lysosomal membrane, causing release of putative NLRP3-activating lysosomal contents into the cytosol (17, 24, 48).

However, in our experiment, lysosome inhibitor had no effect on CVB3-induced caspase-1 activation and IL-1β release. These data indicated that CVB3 infection induced the inflammasome activation mainly through ROS and K+ efflux but not lysosomal destabilization signals.

In conclusion, the results of the present study clearly indicated that CVB3-induced inflammasome activation had an important role in the pathogenesis of VMC. Furthermore, NLRP3 but not RIG-I activation was involved in CVB3-induced inflammasome activation, and the activation was mediated by ROS production and K+ efflux. These data suggest that the modulation of the inflammasome might represent a promising therapeutic strategy for VMC.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s). No conflict of interest, financial or otherwise.

AUTHOR CONTRIBUTIONS

Y.W. performed experiments; Y.W. analyzed data; Y.W. interpreted results of experiments; Y.W. prepared figures; Y.W. drafted manuscript; B.G. and S.X. edited and revised manuscript; B.G. and S.X. approved final version of manuscript.

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