Studies suggest the potential role of a sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} leak in cardiac contractile dysfunction in sepsis. However, direct supporting evidence is lacking, and the mechanisms underlying this SR leak are poorly understood. Here, we investigated the changes in cardiac Ca\textsuperscript{2+} handling and contraction in LPS-treated rat cardiomyocytes and a mouse model of polymicrobial sepsis produced by cecal ligation and puncture (CLP). LPS decreased the systolic Ca\textsuperscript{2+} transient and myocyte contraction as well as SR Ca\textsuperscript{2+} content. Meanwhile, LPS increased Ca\textsuperscript{2+}/spark–mediated SR Ca\textsuperscript{2+} leak. Preventing the SR leak with ryanodine receptor (RyR) blocker tetracaine restored SR load and increased myocyte contraction. Similar alterations in Ca\textsuperscript{2+} handling were observed in cardiomyocytes from CLP mice. Treatment with JTV-519, an anti-SR leak drug, restored Ca\textsuperscript{2+} handling and improved cardiac function. In the LPS-treated cardiomyocytes, mitochondrial reactive oxygen species and oxidative stress in RyR2 were increased, whereas the levels of the RyR2-associated FK506-binding protein 1B (FKBP12.6) were decreased. The Toll-like receptor 4 (TLR4)–specific inhibitor TLR4 activation-induced mitochondrial reactive oxygen species production and the resulting oxidative stress in RyR2 contribute to the SR Ca\textsuperscript{2+} leak.

Sepsis is the most common cause of mortality in intensive care units, and the incidence is increasing (1). Myocardial dysfunction is a recognized manifestation of sepsis, which occurs in 40% of patients diagnosed with sepsis and dramatically increases mortality from 20% to as high as 70–90% (2). The most common defect in cardiac performance during sepsis is impaired contractility of the ventricles (3). Evidence suggests that dysregulation of myocardial Ca\textsuperscript{2+} handling accounts for the reduced contractile force in septic cardiomyopathy (4, 5). The abnormalities in Ca\textsuperscript{2+} regulation have been suggested to occur at practically all main steps of Ca\textsuperscript{2+} handling, including decreased sarcolemmal Ca\textsuperscript{2+} entry through L-type Ca\textsuperscript{2+} channels, impaired sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} release and recycling, and reduced myofilibrillar Ca\textsuperscript{2+} sensitivity, although some results are conflicting (6, 7).

In the mammalian heart, the major source of Ca\textsuperscript{2+} required for contractile activation is the SR (8). During cardiac EC coupling, depolarization activates Ca\textsuperscript{2+} entry through L-type Ca\textsuperscript{2+} channels in the sarcolemmal membrane, triggering a large amount of Ca\textsuperscript{2+} release from SR via ryanodine receptors (RyRs) through a Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release mechanism (4, 9). The simultaneous systolic SR Ca\textsuperscript{2+} release gives rise to a global intracellular Ca\textsuperscript{2+} transient, which consequently initiates myofilament contraction. In septic cardiomyopathy, the systolic Ca\textsuperscript{2+} transient is decreased, which is associated with a decrease in the SR Ca\textsuperscript{2+} content (4, 10, 11). It is well established that the SR Ca\textsuperscript{2+} content is finely tuned by the SR Ca\textsuperscript{2+} recycling
through SR Ca2+-ATPase and diastolic SR Ca2+ release, also called SR Ca2+ leak, through the RyRs (9, 12). In a number of reports, the reduced SR Ca2+ content has been attributed to the depressed SR Ca2+-ATPase function (12–14). Another potential cause of the reduced SR Ca2+ content is increased diastolic SR Ca2+ leak. Zhu et al. (15) demonstrated that increased Ca2+ spark frequency and diminished SR Ca2+ content were simultaneously present in cardiomyocytes from septic rats. Although enhanced Ca2+ spark–mediated SR Ca2+ leak may explain the reduced SR Ca2+ content, direct experimental support for the causal relationship is lacking. Particularly, the underlying mechanism for the increased SR Ca2+ leak in septic cardiomyopathy remains poorly understood.

It is well established that excessive inflammatory response and intracellular oxidative stress play important roles in the development of septic cardiomyopathy (16). The cross-talk between inflammation and reactive oxidative species (ROS) further promotes intracellular oxidative stress. In heart failure and burn-generated cardiac dysfunction, the enhancement of intracellular oxidative stress has been suggested to cause SR Ca2+ leak by increasing diastolic RyR activity (17–19). In sepsis, the activation of the Toll-like receptor 4 (TLR4) signaling pathways stimulates inflammatory and oxidative responses, leading to the development of septic cardiomyopathy (20, 21). However, it remains unknown whether TLR4 mediates cardiac dysfunction through inducing SR Ca2+ leak in sepsis.

Therefore, in this study, we studied the possible contribution of TLR4 signaling to the abnormal SR function and cardiac dysfunction in sepsis by using an LPS-induced cell model of sepsis and a cecal ligation and puncture (CLP)–induced mouse model of polymicrobial sepsis. Furthermore, we explored the potential therapeutic effect of JTV-519, a newly developed drug with the activity of preventing SR Ca2+ leak, possibly by stabilizing RyR channels, on treatment of septic cardiomyopathy.

Results

**LPS decreases intracellular Ca2+ transient and cardiac contractility by reducing SR Ca2+ content**

We first examined the effect of LPS on intracellular Ca2+ transient and cell shortening in cardiomyocytes paced with 1-Hz field stimulation. The results show that LPS treatment for 30 min significantly decreased Ca2+ transient and cell shortening in a dose-dependent manner. The amplitude of Ca2+ transient was decreased by 10.8, 26.9, and 34.2% by 250, 500, and 1000 ng/ml LPS, respectively (Fig. 1, A and B). Consistently, the cardiac contractility was remarkably decreased by LPS treatment in a dose-dependent manner, where the cell shortening was decreased by 8.1, 17.8, and 36.7% by 250, 500, and 1000 ng/ml LPS, respectively (Fig. 1C). Furthermore, LPS slowed the kinetics of Ca2+ transient, where the half-time of decay of the Ca2+ transient (T50) was increased by LPS treatment (Fig. 1D).

It is known that SR is the major source for intracellular Ca2+ transient, and the reduction in SR load results in a decrease of the Ca2+ transient (9). We thus explored whether the reduction in systolic Ca2+ transient is related to the alteration of SR Ca2+ content. In parallel to the decrease of Ca2+ transient, LPS reduced SR Ca2+ content in a dose-dependent manner (Fig. 1, E and F). The amplitude of caffeine-induced Ca2+ transient, which reflects SR load, was decreased by 22.1, 36.2, and 45.5% by 250, 500, and 1000 ng/ml LPS, respectively (Fig. 1F). The results suggest that LPS decreases systolic Ca2+ transient and cardiac contractility through reducing SR Ca2+ content. The half-time of decay (T50) of the Ca2+ transient was not significantly changed (Fig. 1G), suggesting that LPS had no effect on the activity of Na+-Ca2+ exchanger.

**Increased Ca2+ spark–mediated SR leak contributes to the reduction of SR load in LPS-treated cardiomyocytes**

We next explored the possible contribution of SR Ca2+ leak to the reduction of SR Ca2+ content. Observing diastolic Ca2+ spark provides a window visualizing the resting RyR Ca2+ release (or Ca2+ leak) and SR function. We found that LPS dose-dependently increased the occurrence of Ca2+ spark. The frequency of Ca2+ sparks was increased by 51.1, 81.8, and 120% by 250, 500, and 1000 ng/ml LPS, respectively (Fig. 2, A and B). Meanwhile, the amplitude of Ca2+ sparks was decreased (Fig. 2C), which is consistent to the reduction of SR Ca2+ content. LPS had no significant effect on the size (full width of half-maximum, FWHM) (Fig. 2D) and kinetics (full duration of half-maximum, FWHM) of the Ca2+ sparks (Fig. 2E).

To probe the causal relationship between Ca2+ spark–mediated SR Ca2+ leak and the reduction of SR Ca2+ content upon LPS stimulation, we used RyR blocker, tetracaine, to inhibit Ca2+ spark–mediated SR Ca2+ leak and investigated the effect on the SR Ca2+ load. Previous studies have demonstrated that tetracaine dose-dependently regulates Ca2+ handling and myocyte contractility. The study by Venetucci et al. (22) showed that tetracaine at lower concentration (20–50 μM) had no significant effect on the amplitude of Ca2+ transient in isoproterenol-treated cardiomyocytes with no diastolic release but increased Ca2+ transient amplitude in the cells preceded by diastolic release (22). Our previous study had also shown that tetracaine at 50 μM prevented the increased diastolic SR Ca2+ leak without affecting normal Ca2+ handling (20). We thus pre-treated the cells with 50 μM tetracaine for 30 min before LPS stimulation. Tetracaine significantly decreased Ca2+ spark frequency (Fig. 3A) and largely restored the reduced SR load (Fig. 3B) in LPS-treated cells, indicating that SR leak is an important reason for the diminished SR load. Furthermore, the peak systolic Ca2+ transient and cell shortening were significantly increased with the restoration of SR load (Fig. 3, C and D), confirming the notion that LPS decreased myocyte contractility by partially depleting SR Ca2+ content.

**Prevention of SR Ca2+ leak with JTV-519 improves cardiac function in septic mice**

A mouse model of polymicrobial sepsis was produced by CLP. Cardiac function was monitored with echocardiography 6 h after the surgery (23–25). Consistent with a previous report, the cardiac function in septic mice was impaired as compared with control, where the left ventricular (LV) functions indexed by the fractional shortening (FS) and ejection fraction (EF) were remarkably decreased (Fig. 4, A–C). The Ca2+ handling and myocyte contraction were examined in cardiomyocytes isolated from the hearts of control (sham) or septic mice. The
Figure 1. LPS decreased intracellular Ca\(^{2+}\) transient and myocyte contraction and decreased SR Ca\(^{2+}\) content in cardiomyocytes. A, representative confocal line-scan images of Ca\(^{2+}\) transient along with time courses of Ca\(^{2+}\) transient and cell shortening in control and LPS (1000 ng/ml)-treated cardiac myocyte paced at 1 Hz. B and C, average of the amplitude of Ca\(^{2+}\) transient (\(\Delta F/F_0\); B) and percentage (%) of maximum cell shortening (C) in control and different doses (250–1000 ng/ml) of LPS-treated cells. D, average of the rise time (bottom) and half-time of decay (\(T_{50}\); top) of Ca\(^{2+}\) transient. n = 125–140 cells in each group. E, representative images of caffeine-elicited Ca\(^{2+}\) transient in control and LPS (1000 ng/ml)-treated cardiac myocytes. F, statistics of the amplitude of caffeine-elicited Ca\(^{2+}\) transient (SR Ca\(^{2+}\) content) in control and different doses of LPS-treated cells. n = 15–25 cells in each group. G, half-time of decay (\(T_{50}\)) of caffeine-elicited Ca\(^{2+}\) transient (n = 15–25 cells in each group). *, \(p < 0.05\); **, \(p < 0.01\) versus control. Error bars, S.D.
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**Figure 2.** LPS increased \( \text{Ca}^{2+} \) sparks in cardiomyocytes. A, representative \( \text{Ca}^{2+} \) spark images in control and LPS (1000 ng/ml)-treated cells. B, average of the frequency of \( \text{Ca}^{2+} \) sparks in control and 250–1000 ng/ml LPS-treated cells. C–E, statistics of the amplitude \( (F/F_0) \), full width of half-maximum \( (\text{FWHM}; D) \), and full duration at half-maximum \( (\text{FDHM}; E) \) of \( \text{Ca}^{2+} \) sparks in control and LPS groups \( (n = 53–75 \text{ cells/group}) \). *, \( p < 0.05 \); **, \( p < 0.01 \) versus control. Error bars, S.D.

**Figure 3.** Effects of tetracaine on LPS modulation of \( \text{Ca}^{2+} \) handling and myocyte contraction in cardiomyocytes. A–D, statistics of the frequency of \( \text{Ca}^{2+} \) sparks \( (n = 54–79 \text{ in each group}) \); the amplitude of caffeine-elicited \( \text{Ca}^{2+} \) transient \( (\text{SR Ca}^{2+} \text{ content}, n = 13–31) \); the amplitude of action potential-elicited \( \text{Ca}^{2+} \) transient \( (n = 39–58) \); and maximum of cell shortening \( (n = 39–58) \) in control and LPS (1000 ng/ml)-treated cells with or without tetracaine (50 \( \mu \text{M} \)) pretreatment. *, \( p < 0.05 \); **, \( p < 0.01 \). Error bars, S.D. Error bars, S.D.
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frequency of Ca\(^{2+}\) sparks was dramatically increased (Fig. 4, D and E), and the SR Ca\(^{2+}\) content (Fig. 4, G and H) and systolic intracellular Ca\(^{2+}\) transient and cell shortening (Fig. 4, I–K) were remarkably decreased. The results are consistent with the findings in LPS-treated cardiomyocytes.

JTV-519 is a newly developed 1,4-benzothiazepine drug with antiarrhythmic and cardioprotective properties relating to the role of preventing increased Ca\(^{2+}\) leak from the SR (26, 27). We thus explored the therapeutic effect of JTV-519 on the impaired contractility in septic cardiomyopathy by incubation of JTV-519 (1 \(\mu\)M) with cardiomyocytes from sham or CLP mice for 1 h (28, 29). Fig. 4 (D and E) demonstrates that JTV-519 significantly decreased the rate of Ca\(^{2+}\) sparks in cardiomyocytes isolated from septic mouse hearts. Concomitantly, JTV-519 restored the reduced SR Ca\(^{2+}\) content (Fig. 4, G and H) and increased the systolic Ca\(^{2+}\) transient and cell shortening (Fig. 4, I–K). JTV-519 applied in vivo (0.5 mg/kg/h, i.v., 2 h before the surgery) (30) improved cardiac function in CLP mice, where the EF and FS were significantly increased as compared with CLP mice without JTV-519 treatment (Fig. 4, A–C). Comparing the Ca\(^{2+}\) handling and myocyte contraction in cardiomyocytes isolated from CLP mice with or without JTV-519 treatment shows that the abnormal Ca\(^{2+}\) handling and the impaired myocyte contraction were largely corrected by JTV-519 treatment in vivo (Fig. S1). No matter whether it was applied in vitro or in vivo, JTV-519 had no significant effects on the production of pro-inflammatory cytokines, including IL-6, IL-1\(\beta\), and TNF-\(\alpha\), which were increased in CLP mouse serum or in the cultural medium of septic cardiomyocytes (Fig. S2). The results confirm the central role of SR Ca\(^{2+}\) leak in impaired cardiac contractility in sepsis and highlight the therapeutic potential of JTV-519 in the treatment of septic cardiomyopathy.

**Oxidative stress in RyR2 underlies the SR Ca\(^{2+}\) leak in LPS-treated cardiomyocytes**

The occurrence of high-frequency Ca\(^{2+}\) spark under a low level of SR Ca\(^{2+}\) content indicates that the activity of RyR2 is increased by LPS. A series of studies demonstrates that oxidative modification of RyR2 leads to conformational change of RyR2 and thus alteration of RyR2 gating and open probability (28, 31, 32). Enhanced oxidative stress in RyR2 has been shown to be a major reason for SR Ca\(^{2+}\) leak in heart failure (28, 31–33). Therefore, we examined whether oxidative stress contributes to the increased RyR Ca\(^{2+}\) release in septic cardiomyopathy. As illustrated in Fig. 5 (A and B), the free thiol groups in RyR2 indicated by monobromobimane (mBB) fluorescence were significantly decreased in LPS-treated cells, indicating enhancement of oxidative stress in RyR2.

FK506-binding protein 1B (FKBP12.6) is an accessory protein of RyR2, and oxidative stress in RyR2 has been suggested to induce FKBP12.6 dissociation from RyR2, leading to hyperactive RyR2 in heart failure (34–36). We examined the content of FKBP12.6 associating with RyR2 in LPS-treated cardiomyocytes. The RyR2 complex was pulled down by co-immunoprecipitation with anti-RyR2 antibody. The protein levels of RyR2 and FKBP12.6 in the precipitations were quantified by Western blotting, and the ratio of FKBP12.6 to RyR2 was calculated to estimate the combination of FKBP12.6 with RyR2 (37–39). LPS treatment remarkably decreased the ratio of FKBP12.6 to RyR2 (Fig. 5, C and D). In contrast, LPS had no significant effect on the expression of FKBP12.6, where the total protein level of FKBP12.6 in the cell lysates indicated as the input remains unaltered by LPS treatment (Fig. 5C, bottom). The results indicate FKBP12.6 dissociation from RyR2 in response to LPS stimulation.

In cardiac myocytes, mitochondria occupy 30–40% of the cellular volume and constitute the major source of intracellular reactive oxygen species (ROS) production (40). It has been suggested that sepsis induces mitochondrial dysfunction, and mitochondria-derived ROS plays an important role in the development of septic cardiomyopathy (16, 41). To explore whether this accounts for the enhanced oxidative stress in RyR2 in LPS-treated cells, we examined the mitochondrial ROS (mitoROS) level indicated by the fluorescence intensity of 5-(and-6)-chloromethyl 2’,7’- dichlorodihydrofluorescein diacetate (MitoSOX) in control and LPS-stimulated cells. Fig. 5 (E and F) illustrates that LPS stimulation remarkably increased MitoSOX fluorescence. Using mito-TEMPO (25 \(\mu\)M, applied 60 min before LPS treatment) to scavenge mitoROS (Fig. 5, E and F) increased the free thiol groups in RyR2 (mBB fluorescence; Fig. 5, A and B) and the association of FKBP12.6 with RyR2 (Fig. 5, C and D) in LPS-stimulated cardiomyocytes. The LPS-induced high-frequency Ca\(^{2+}\) sparks were concomitantly suppressed (Fig. 5G). The results collectively indicate that mitoROS accumulation causes oxidative stress in RyR2 and FKBP12.6 dissociation from the channel, resulting in SR Ca\(^{2+}\) leak.

**TLR4 mediates intracellular oxidative stress and SR leak**

TLR4 is the receptor of LPS and plays a critical role in cardiac dysfunction in sepsis. TLR4-activated signaling pathways can induce mitochondrial dysfunction and excessive intracellular ROS accumulation (42). To explore the contribution of TLR4 activation in intracellular oxidative stress and consequent SR Ca\(^{2+}\) leak, we pretreated the cells with TLR4-specific inhibitor TAK-242 (1 \(\mu\)M) for 30 min before LPS stimulation. TAK-242 suppressed the occurrence of high-frequency Ca\(^{2+}\) sparks (Fig. 4, D and E), and the SR Ca\(^{2+}\) content (Fig. 4, G and H) and systolic intracellular Ca\(^{2+}\) transient and cell shortening (Fig. 4, I–K) were remarkably decreased. These results are consistent with the findings in LPS-treated cardiomyocytes.
largely inhibited LPS-induced increase of MitoSOX fluorescence (Fig. 6, A and B). The relative content of free thiol groups in RyR2 was significantly increased (Fig. 6, C and D). Furthermore, TAK-242 significantly reduced the high-frequency Ca2+ sparks stimulated by LPS (Fig. 6E). The results indicate that TLR4 mediates mitoROS production and oxidative stress in RyR2, resulting in enhancement of SR Ca2+ leak. With the correction of SR Ca2+ leak, TAK-242 increased SR Ca2+ content and intracellular Ca2+ transient and cell shortening in LPS-treated cells (Fig. 6, F and G).

**Deletion of TLR4 improves cardiac function by preventing SR Ca2+ leak in septic mice**

We further investigated the critical role of SR Ca2+ leak in TLR4-induced cardiac dysfunction in CLP septic mice with or without TLR4 gene knockout. Deletion of TLR4 (TLR4−/−; Fig. 7, A and B) significantly increased cardiac function in the CLP mice, where EF and FS were increased by 32.5 and 42.1%, respectively, compared with WT CLP mice (Fig. 7C). The survival rate was significantly increased in TLR4−/− CLP mice (Fig. 7D).

**Figure 5. Effects of LPS on RyR2 oxidation, FKBP12.6 association with RyR2, and mitochondrial ROS generation.** A, representative images of mBB fluorescence intensity and Coomassie-stained gels in parallel in control and LPS-treated cells with or without mito-TEMPO pretreatment. Mito-TEMPO (25 μM) was incubated with the cardiomyocytes for 60 min before LPS treatment to scavenge mitochondrial ROS. B, relative free thiol content (%) of RyR2 measured by normalizing mBB fluorescence to RyR2 level (n = 3 in each group). C and D, co-immunoprecipitation analysis of the relative amount of FKBP12.6 associated with RyR2. RyR2 complex was pulled down with anti-RyR2 antibody. Total cytosolic FKBP12.6 protein level indicated as input is shown in the bottom panel. The relative amount of FKBP12.6 associated with RyR2 was calculated as the ratio of the protein content of FKBP12.6 to RyR2 in the precipitation (n = 3–6 in each group). E, MitoSOX red fluorescence recorded from control, LPS, LPS + mito-TEMPO, and mito-TEMPO groups. F, averages of MitoSOX fluorescence in four groups (n = 26–50 cells in each group). G, averages of the frequency of Ca2+ sparks in four groups (n = 30–67 in each group). **, p < 0.01. Error bars, S.D. IP, immunoprecipitation; IB, immunoblotting.
The frequency of Ca\(^{2+}\) sparks in cardiomyocytes from TLR4\(^{-/-}\) septic mice was much lower than that in cardiomyocytes from WT septic mice (Fig. 8A). Mirroring the decrease in Ca\(^{2+}\) spark–mediated SR leak, the SR Ca\(^{2+}\) content was remarkably increased in TLR4\(^{-/-}\) septic mice compared with WT septic mice (Fig. 8B). Consistently, the systolic Ca\(^{2+}\) tran-
**TLR4 mediates septic cardiomyopathy by increasing RyR leak**

Figure 7. TLR4 deficiency attenuated cardiac dysfunction and increased survival rate in septic mice.  

**A**, representative images of Western blots of TLR4 and GAPDH proteins and statistics of TLR4 abundance in WT and TLR4−/− groups (n = 6 in each group).  
**B**, representative images generated by echocardiography and quantification of LV FS and LV EF (n = 10–13 in each group).  
**C**, TLR4 deficiency increases survival outcome in CLP-induced septic mice. TLR4−/− and age-matched WT mice (20 in each group) were subjected to CLP, and the survival was carefully monitored for half a day. ***, p < 0.01. Error bars, S.D.**

Figure 8. Deletion of TLR4 restores Ca2+ handling and myocyte contraction in septic mice.  
**A**, average of the frequency of Ca2+ sparks in WT-sham, WT-CLP, TLR4−/−-sham, and TLR4−/−-CLP groups (n = 43–74 in each group).  
**B**, statistics of the amplitude of SR Ca2+ content in four groups (n = 16–28 in each group).  
**C** and **D**, action potential–elicited Ca2+ transient and average of the peak Ca2+ transient (C) and maximum cell shortening (D; n = 51–90 in each group). *, p < 0.05; ***, p < 0.01. Error bars, S.D.
sient and myocyte contractility were significantly increased (Fig. 8, C and D). The data indicate that TLR4 activation contributes to septic cardiac dysfunction through inducing SR Ca\(^{2+}\) leak.

**Discussion**

**Enhancement of SR Ca\(^{2+}\) leak contributes to the contractile defect in septic cardiomyopathy**

One major finding in this study is strong evidence indicating that enhancement of Ca\(^{2+}\) spark–mediated SR Ca\(^{2+}\) leak plays a critical role in the contractile dysfunction in septic cardiomyopathy. There are three types of supporting evidence. First, LPS, the major cause of sepsis, increased Ca\(^{2+}\) spark–mediated SR Ca\(^{2+}\) leak but decreased SR Ca\(^{2+}\) content, systolic Ca\(^{2+}\) transient, and cell shortening in cardiomyocytes. Preventing the SR leak with RyR blocker, tetracaine restored SR Ca\(^{2+}\) content and significantly increased systolic Ca\(^{2+}\) transient and cell shortening. The results indicate the causal relationship between the increased SR Ca\(^{2+}\) leak and the decreased SR Ca\(^{2+}\) content and the resulting contractile dysfunction in LPS-treated cardiomyocytes. Second, preventing the hyperactive Ca\(^{2+}\) spark–mediated SR leak with JTV-519 restored the SR Ca\(^{2+}\) content and increased systolic Ca\(^{2+}\) transient and myocyte contraction in mouse septic cardiomyopathy. Third, blocking or deletion of TLR4, the critical molecule mediating myocyte contraction in mouse septic cardiomyopathy. Activation of TLR4 stimulates mitochondrial ROS generation and enhances oxidative stress in RyR2, leading to hyperactive RyR2 and subsequent SR Ca\(^{2+}\) leak. Despite the direct effect of ROS on increasing RyR2 activity, it may also act through activating Ca\(^{2+}\)/calmodulin-dependent kinase II, which increases RyR activity by phosphorylation of RyR2 (45).

**Possible role of FKBP12.6 dissociation in the oxidative stress-induced SR Ca\(^{2+}\) leak**

FKBP12.6 is an accessory protein of RyR2, and the dissociation of FKBP12.6 from RyR2 has been shown to cause SR Ca\(^{2+}\) leak in heart failure in previous studies (34, 46). In septic cardiomyocytes, we found that oxidative stress in RyR2 induces FKBP12.6 dissociation from RyR2. Relieving oxidative stress in RyR2 restored the interaction of FKBP12.6 with RyR2, suggesting that FKBP12.6 dissociation may be an important link for oxidative stress-induced SR Ca\(^{2+}\) leak in septic cardiomyopathy.

Of note, the reports on the role of FKBP12.6 in the gating of RyR2 and the regulation of SR Ca\(^{2+}\) release are extremely controversial because RyRs are intracellular channels inaccessible to direct electrophysiological measurements. Lipid bilayers have been widely used to study the role of FKBP12.6 in RyR gating at the single-channel level. However, the experimental setting has the shortcoming of not being able to reconstruct the native environment, such as the interacting proteins (calsequestrin, junctin, triadin, etc.) and the exact intracellular ionic composition. Thus, the results vary greatly among laboratories. Single RyRs from FKBP12.6-knockout mice or treated with rapamycin/FK506 to dissociate FKBP12.6 were found to have increased open probability and partial opening/subcon-duction in some studies but not in others (47–50). In intact cardiomyocytes, the role of FKBP12.6 in the regulation of SR Ca\(^{2+}\) release is studied by observing Ca\(^{2+}\) sparks; however, it is still highly controversial. Xin et al. (51) demonstrated that FKBP12.6 knockout increases the amplitude and duration but not the frequency of Ca\(^{2+}\) sparks. By contrast, some reports have shown that FK506 treatment or FKBP12.6 dissociation increases the spontaneous Ca\(^{2+}\) spark frequency (52). FKBP12.6 knockout mice develop lethal arrhythmia during exercise (53). A recent study by Zhao et al. (54) demonstrated that FKBP12.6 dissociation increases the frequency but not the amplitude and kinetics of Ca\(^{2+}\) sparks by using a combination of FKBP12.6-knockout mice and FK506/rapamycin pharmacology. As reported by a previous study, only <20% of

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**TLR4 mediates septic cardiomyopathy by increasing RyR leak**

The TLR4–mediated signaling pathways play a critical role in sepsis-induced cardiac dysfunction through inducing inflammation and ROS production (43). Recent studies have linked TLR4 signaling to mitochondrial dysfunction, where the activation of TLR4 induces mitochondrial ROS generation by interfering with mitochondrial respiratory chain (44). In septic cardiomyocytes, we found that inhibition or deletion of TLR4 decreased mitochondrial ROS production, prevented Ca\(^{2+}\) spark–mediated SR Ca\(^{2+}\) leak, and improved cardiac function in septic cardiomyopathy. Taken together, we conclude that TLR4 plays a critical role in inducing SR Ca\(^{2+}\) leak in septic cardiomyocytes. Activation of TLR4 stimulates mitochondrial ROS generation and enhances oxidative stress in RyR2, leading to hyperactive RyR2 and subsequent SR Ca\(^{2+}\) leak. Despite the direct effect of ROS on increasing RyR2 activity, it may also act through activating Ca\(^{2+}\)/calmodulin-dependent kinase II, which increases RyR activity by phosphorylation of RyR2 (45).
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FKBP12.6 binding sites on RyRs are occupied by FKBP12.6 (55), which calls into question the critical role of FKBP12.6 in the regulation of normal RyR2. Recent structural analysis has indicated that FKBP is inserted into the gap between the JSol (handle) domain and SPRY triangle of RyR (56–59). Based on these findings, Zhao et al. (54) proposed a model in which a single-subunit occupation of FKBP12.6 stabilizes two adjacent subunits of an RyR (which has four FKBP12.6-binding sites on its four subunits), and a ~20% occupation of FKBP12.6-binding sites stabilizes ~59% of RyRs to explain the robust effect of FKBP12.6 knockout on RyR2 activity. Nevertheless, we may not exclude the possibility that oxidative stress in the RyR2 channel itself mediates the SR Ca$^{2+}$ leak in septic cardiomyopathy, and the role of FKBP12.6 is minor in view of the extremely controversial reports on the role of FKBP12.6 in regulating RyR gating and SR Ca$^{2+}$ release.

In summary, this study provides direct evidence indicating the critical role of Ca$^{2+}$ spark-mediated SR Ca$^{2+}$ leak in the development of septic cardiomyopathy. Mechanistically, the activation of the signal axis, TLR4–mitoROS accumulation–enhancement of RyR2 oxidative stress, induces hyperactive RyR2 and the increased SR Ca$^{2+}$ leak. The dissociation of FKBP12.6 from RyR2 may participate in but not be essential to oxidative stress-induced RyR2 hyperactivity. Furthermore, this study demonstrates for the first time the therapeutic potential of JTV-519 in the treatment of septic cardiomyopathy by preventing SR Ca$^{2+}$ leak.

Materials and methods

Animals and CLP model

Adult Sprague-Dawley rats of either sex, weighing 200–220 g, and wild-type male C57BL/6 mice weighing 18–22 g were purchased from the Animal Center of Southern Medical University, and TLR4$^{-/-}$ mice were purchased from the Model Animal Research Center of Nanjing University. All animal experiments were handled in accordance with a protocol approved by the institutional care and use committee of Shenzhen University, which conforms to the ethical standards formulated in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication 85-23, revised 1996). CLP was performed to induce sepsis published by the National Institutes of Health (NIH Publication 85-23, revised 1996). CLP was performed to induce sepsis.

Adult rat ventricular myocytes

Adult rat ventricular myocytes were isolated from adult SD rats as described previously (19, 61). Briefly, after deep anesthesia with trichloroacetaldehyde monohydrate (0.5 g/kg, i.p.), the heart was quickly removed from the rat chest; cleaned and flushed with nominally Ca$^{2+}$-free Tyrode solution consisting of 137 mM NaCl, 5.4 mM KCl, 1.2 mM MgCl$_2$, 1.2 mM Na$_2$HPO$_4$, 10 mM glucose, and 20 mM HEPES (pH 7.3, adjusted with NaOH); and perfused using a Langendorff apparatus at 37 °C. After 5 min, the solution was switched to the enzyme solution with 0.5 mg/ml collagenase (Worthington; Type II) and 0.06 mg/ml protease (Sigma; Type XIV) for 15 min. All solutions were equilibrated with 100% O$_2$. Then the heart was minced into small chunks, and single cells were shaken loose from the heart tissue and stored in HEPES-buffered external solution containing 137 mM NaCl, 5.4 mM KCl, 1.2 mM MgCl$_2$, 1.2 mM Na$_2$HPO$_4$, 20 mM glucose, and 20 mM HEPES (pH 7.4).

Isolation of adult mouse ventricular myocytes

Adult mouse ventricular myocytes were isolated from anesthetized C57BL/6 mice as described previously (62). Briefly, the heart was quickly removed and cleaned and flushed with a Ca$^{2+}$-free buffer containing 120 mM NaCl, 5.4 mM KCl, 1.2 mM MgSO$_4$, 1.2 mM Na$_2$HPO$_4$, 5.6 mM glucose, 20 mM NaHCO$_3$, 10 mM 2,3-butanedione monoxime (BDM; Sigma), 5 mM taurine, 10 mM HEPES (pH 7.4) and perfused using a Langendorff apparatus. All solutions were bubbled with 100% O$_2$. The enzymatic digestion was initiated by adding collagenase type B (0.75 mg/ml; Worthington) and protease type XIV (0.02 mg/ml; Sigma) to the perfusion solution. When the heart became swollen and hard after 3 min of digestion, 50 μM Ca$^{2+}$ was added to the enzyme solution and perfused for about 30 min. Following the perfusion procedure, the heart was minced into small chunks, and single cells were shaken loose from the heart tissue and stored in HEPES-buffered solution containing 1 mM CaCl$_2$, 137 mM NaCl, 5.4 mM KCl, 15 mM dextrose, 1.3 mM MgSO$_4$, 1.2 mM Na$_2$HPO$_4$, and 20 mM HEPES, adjusted to pH 7.4 with NaOH. Cells were used for the following experiments within 4 h after isolation.

Ca$^{2+}$ spark and Ca$^{2+}$ transient detection and contraction measurement

Isolated ventricular myocytes loaded with Ca$^{2+}$ indicator Fluo-4 AM (5 μM/liter at room temperature for 8 min) (Invitrogen) were placed in a recording chamber. Ca$^{2+}$ sparks and transients were recorded as reported previously (19). For Ca$^{2+}$ spark recording, confocal line-scan imaging was carried out in resting cells at 488-nm excitation and 505-nm collection with a Zeiss 710 inverted confocal microscope (Carl Zeiss, Oberkochen, Germany) with a ×40 oil immersion lens (numerical aperture 1.3). Line-scan images were acquired at a sampling rate of 3.84 ms/line, along the longitudinal axis of the cell. For
the detection of systolic Ca\(^{2+}\) transient, after the cells were stimulated with field stimulation (1 Hz) to reach a steady state, confocal line-scan imaging was performed with the same confocal parameters used for Ca\(^{2+}\) spark recording under field stimulation (1 Hz). Myocyte contraction was measured by detecting the length of two edges of the cell along with the time of stimulation. Myocytes were superfused with HEPES-buffered external solution during the experiment.

**Measurement of SR Ca\(^{2+}\) load**

Short puffs of caffeine (20 mmol/liter) were applied to completely empty the SR, following a train of 1-Hz field stimulation to achieve steady-state SR Ca\(^{2+}\) loading in ventricular myocytes. SR Ca\(^{2+}\) content was assessed by detecting the amplitude of a caffeine-elicited Ca\(^{2+}\) transient. Cells were superfused with HEPES-buffered external solution.

**Measurement of ROS in mitochondria**

Isolated cardiomyocytes were loaded with 5 μM MitoSOX (Invitrogen) for 15 min at room temperature (63, 64). Frame fluorescence images (excitation at 488 nm and emission at 505–530 nm, laser intensity 4%, 6.6 s/frame) were acquired with a Zeiss 710 inverted confocal microscope with ×40 lens. Because MitoSOX is light-sensitive and oxidized progressively, we used the same scanning parameters for all of the related experiments.

**Oxidative stress level in RyR2**

The content of the free thiols (i.e. the number of reduced cysteines) in RyR2 in cardiomyocytes was determined with the mBB (Calbiochem) fluorescence technique (17, 28). Heavy SR vesicles were prepared from different groups of cells under non-reducing conditions. Samples were incubated with 400 μmol/liter mBB for 1 h in the dark at room temperature. Then proteins were acetone-precipitated and subjected to SDS-PAGE (in a 6% polyacrylamide gel). The mBB fluorescence was measured using BIO-PEOFL (Vilber Lourmat Biotechnology (Marne-la-Vallé, France); excitation 365 nm and emission 400–600 nm). Images were acquired and analyzed using Biscopt software. After that, the same gel was stained with Coomassie Blue. The mBB fluorescence in the RyR2 (~560 kDa) was normalized by protein abundance of RyR2 determined by Coomassie Blue staining of the same gel, which was defined as the relative content of free thiols in the RyR2.

**Co-immunoprecipitation and Western blotting to detect the relative amount of FKBP12.6 associated with RyR2**

We examined FKBP12.6 association with RyR2 as described previously (37, 38). Briefly, cardiomyocytes were lysed in modified radioimmune precipitation lysis buffer, shaking on ice for 20 min. The lysates were centrifuged at 12,000 × g for 15 min at 4 °C. The supernatants were collected, and the protein concentrations were determined with a bichinonic acid (BCA) protein assay kit (Thermo Fisher Scientific). The 100 μg of supernatant protein was incubated with 2 μg of anti-RyR2 antibody (Abcam) in 0.1 ml of modified radioimmune precipitation buffer and shaken slowly overnight at 4 °C. The samples were incubated with 40 μl of protein A/G-agarose beads at 4 °C for 3 h. The resins were washed three times with radioimmune precipitation buffer, and the eluted immunoprecipitated proteins were boiled for 5 min at 95 °C and loaded into wells in the 10% SDS-PAGE before being transferred to PVDF membranes and then probed with primary antibody: anti-RyR2 (1:1000; Abcam), FKBP12.6 (1:2000; Elabscience). Bound antibodies were visualized using the enhanced chemiluminescence (ECL) detection kit (Beyotime). The FKBP12.6 associated with RyR2 was calculated as the ratio of FKBP12.6 to RyR2 protein content in RyR2 immunoprecipitates. Total FKBP12.6 protein level in cell lysates was detected as input to indicate the expression level of FKBP12.6.

**Data analysis**

All values were expressed as means ± S.D. Statistical analyses were performed by unpaired two-tailed t test or one-way analysis of variance when appropriate, using SPSS Statistics version 20.0 software (IBM Corp., Armonk, NY). Values of p < 0.05 were considered statistically significant.

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