SHOCK, Vol. 50, No. 4, pp. 483–492, 2018

TOLL-LIKE RECEPTOR 4 AND NLRP3 CASPASE 1- INTERLEUKIN-1β-AXIS ARE NOT INVOLVED IN COLON ASCENDENS STENT PERITONITIS-ASSOCIATED HEART DISEASE

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Received 25 Sep 2017; first review completed 9 Oct 2017; accepted in final form 6 Nov 2017

ABSTRACT—Hemodynamic collapse and myocardial dysfunction are among the major causes of death in severe sepsis. The purpose of this study was to assess the role played by toll-like receptor 4 and by the NLRP3 inflammasome in the cardiac dysfunction that occurs after high-grade polymicrobial sepsis. We performed the colon ascendens stent peritonitis (CASP) surgery in *Tlr4*$^{-/-}$, *Nlrp3*$^{-/-}$, and *caspase-1*$^{-/-}$ mice. We also assessed for the first time the electrical heart function in the colon ascendens stent peritonitis (CASP) model. The QJ interval was increased in wild-type C57BL/6J mice after CASP when compared with sham controls, a result paralleled by an increase in the cardiac action potential (AP) duration (APD). The decreases in ejection fraction (EF), left ventricle end diastolic volume, stroke volume, and cardiac output found after CASP were similar among all groups of mice. Similar heart response was found when *Nlrp3*$^{-/-}$ mice were submitted to high-grade cecal ligation and puncture. Despite developing cardiac dysfunction similar to wild types after CASP, *Nlrp3*$^{-/-}$ mice had reduced circulating levels of interleukin (IL)-1β, IL-6 and tumor necrosis factor-α. Our results demonstrate that the genetic ablation of *Tlr4*, *Nlrp3*, and *caspase-1* does not prevent the cardiac dysfunction, despite preventing the increase in pro-inflammatory cytokines, indicating that these are not feasible targets to therapy in high-grade sepsis.

KEYWORDS—Cardioimmunology, electrophysiology, interleukin-1β, innate immunology, sepsis

ABBREVIATIONS—CaMKII—Ca^{2+}/calmodulin-dependent protein kinase II; CASP—colon ascendens stent peritonitis; CLP—cecal ligation and puncture; EF—ejection fraction; IL-1β—interleukin 1-beta; IL-6—interleukin 6; LVDV—left ventricular diastolic volume; LVEDV—left ventricular end diastolic volume; LVESV—left ventricular end systolic volume; LVID(d)—left ventricular internal diameter in diastole; LVID(s)—left ventricular internal diameter in systole; LVSV—left ventricular systolic volume; MyD88—myeloid differentiation primary response 88; NLRP3—NOD-like receptor protein3; SF—shortening fraction; SIRS—systemic inflammatory response syndrome; TLR4—toll-like receptor 4; TNF-α—tumor necrosis factor alpha; TRIF—TIR-domain containing adapter inducing interferon-β

INTRODUCTION

Sepsis is life-threatening organ dysfunction caused by a dysregulated host response to infection (1). The severe tissue damage caused by sepsis can result from an initial infection that cannot be contained by the host and spreads to assume systemic proportions, but also from an exacerbated pro-inflammatory response that causes more tissue damage than the initial infective insult. Lungs, liver, kidney, and heart are deeply affected and contribute to a general imbalance that may result in death (1). Hemodynamic collapse and myocardial dysfunction with impaired contractility and diastolic function are among the major causes of death in severe sepsis. A role in cardiac dysfunction has been assigned to the exaggerated pro-inflammatory cytokine production found in sepsis (2).

The peritonitis caused by cecal ligation and puncture (CLP) is the most widely used model of low-grade polymicrobial sepsis. Pro-inflammatory cytokine production, such as tumor necrosis factor alpha (TNF-α) (3) and interleukin (IL)-6 (4), plays a pivotal role in the pathophysiology of CLP and is mainly related to the activation of different sensors of the innate immune system. Despite the role of toll-like receptor 4 (TLR4)

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Author contributions: EM and MLA designed the research; EM, MLA, JFR, FF, HAPN, MS, MVP, ABC, IR, HB, CP, analyzed and interpreted the data; EM, MLA, JFR, FF, MS, ABC, IR, HB performed the research; EM, MLA, MVP, CP wrote and revised the paper; and all authors approved the final version of the manuscript.

This work was supported by the Brazilian National Research Council (CNPq, grants: 308168/2012-7 and 475218/2012-4), the Carlos Chagas Filho Rio de Janeiro State Research Foundation (FAPERJ, grants no.: E-26/103.222/2011 and E-26/111.171/2011) and National Institutes of Science and Technology for Biology Structural and Bioimaging and National Institutes of Science and Technology for Regenerative Medicine (grant no.: 573767/2008-4 and 465656/2014-5), Brazil.

The authors declare no conflicts of interest.

Supplemental digital content is available for this article. Direct URL citation appears in the printed text and is provided in the HTML and PDF versions of this article on the journal's Web site (www.shockjournal.com).

DOI: 10.1097/SHK.0000000000001059

in mediating acute myocardial dysfunction in endotoxemia and *Escherichia coli* injection (5), previous works showed that the genetic ablation of TLR4 decreases survival and worsens cardiac function in CLP (6), an effect associated with the role of TLR4 in bacterial clearance and the compensatory increase in tissue-damage mediator IL-6. On the other hand, the genetic ablation of TLR2 (7) and also of myeloid differentiation primary response 88 (MyD88) and TIR-domain containing adapter inducing interferon-β (TRIF) (8) improves cardiac function in CLP, with a correspondent reduction in the amounts of systemic and cardiac inflammatory cytokines. Still, in low/medium-grade polymicrobial sepsis the genetic ablation of NLRP3 or IL-1β improves survival and increases bacterial clearance (9) while preserving cardiac function (10) and reducing the production of TNF-α and IL-6 by cardiomyocytes (11).

The need for a high model of polymicrobial sepsis emerged from the disparities between certain clinical conditions found in human sepsis and experimental models that attempt to mimic it.

Data collection in the low-grade CLP model depicts an attempt to wall-off an infection. Tissue injury with intra-abdominal abscess formation and necrotic bowels is largely documented, and bacteremia is observed only at the late stages. On the other hand, the colon ascendens stent peritonitis (CASP) surgery has been shown to reproduce the effects of severe, high-grade sepsis (12), and appears to mimic the clinical course of diffuse peritonitis, with increasing infection due to persisting intestinal leakage and development of a systemic inflammatory response syndrome (SIRS), which plays a major role in the rate and the cause of death. The mortality in CASP depends on TLR2 (13), TLR4 (14), TLR9 (15), MyD88 (16), while antibacterial defenses and survival are associated with inducible nitric oxide synthase, IL-12 (17), complement (18), and calcium diastolic leakage (19). The role of TLR4-NLRP3-IL-1β axis in causing cardiac alterations during a high-grade polymicrobial sepsis remains unknown.

We have recently demonstrated that the progress of type I diabetes to associated heart arrhythmias is mediated by NLRP3 inflammasome activation and IL-1β production, promoting CAMKII oxidation and an increase in calcium sparks (20). We have also shown that the heart contractile dysfunction in CASP is mediated by CAMKII (19). Here, we studied the role played by TLR4-NLRP3-IL-1β axis in the electrical and mechanical cardiac dysfunction that follows CASP in mice. The results show that despite reducing the level of key inflammatory cytokines IL-1β, TNF-α, and IL-6, the genetic ablation of innate immunity sensors TLR4 and NLRP3 is not capable of preventing cardiac dysfunction, indicating that neither these sensors nor the cytokine storm evoked by this axis is involved in cardiac depression associated with high-grade sepsis.

MATERIALS AND METHODS

Animals and experimental protocol

This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Brazilian National Council of Animal Experimentation (http://www.cobea.org.br/) and Federal Law 11.794 (October 8, 2008).

The protocols of the present study were approved by the Committees for Animal Care and Use at the Federal University of Rio de Janeiro (under no.: 137/16). Wild-type (WT) C57BL/6J and TLR4 ($Tlr4^{-/-}$) were kindly provided by Prof. Sergio Costa Oliveira at Federal University of Minas Gerais (Belo Horizonte, Brazil), *Knockout* to NLRP3 ($Nlpr3^{-/-}$)(Genentech, San Francisco, Calif) (no. OM-214220) (21), and CASP-1 ($Caspase1^{-/-}$) (22) (MTO #14865) male mice were generated in the C57BL/6J background. Animals, studied at the age of 8 to 10 weeks, were kept at constant temperature (23°C) in a standard light/dark cycle (12 h/12 h) with free access to standard chow and water. Mice, were bred and maintained under specific pathogen-free conditions at the animal facilities of the Federal University of Rio de Janeiro, and sacrificed by cervical dislocation. The animals were divided in two groups: (a) Sham mice (Sham) and (b) operated mice (CASP). CASP surgery procedures were carried out as previously described by Traeger et al., using a specific 14-gauge catheter (22). Briefly the procedure to induce CASP is characterized by the fixed insertion of a stent (catheter) into the colon ascendens after abdominal incision. Fecal content is milked from the cecum into the stent and finally may leak from the stent into the peritoneal cavity, which leads to polymicrobial peritonitis and subsequently sepsis. The severity of the disease is defined by the diameter of the stent, thus here we used a 14-gauge, which induce 100% lethality.

CLP surgery was carried out as previously described with slight modifications (23). Briefly, mice were anesthetized with isofluorane and a small incision was made in the abdomen through which the cecum was externalized. Cecum was ligated at 75% of its length and nine punctures were made using a 16-G needle. In pilot experiments, we determined that this procedure resulted in similar mortality between CLP and CASP models (100% within 48 h). Cecum was then returned to the peritoneal cavity and wound was closed. Animals received 1 mL subcutaneous saline for fluid reposition and analgesics.

Cardiac mechanical/structural and electrical function

Left ventricular structural and functional assessment using echocardiography—In vivo transthoracic echocardiogram was performed using a high resolution imaging system (VEVO 770; VisualSonics, Toronto, Ontario, Canada) equipped with a 30-MHz scanhead (VisualSonics). Mice were placed in an induction chamber with constant inflow of 2% isoflurane mixed with 100% oxygen. Once the mouse was asleep, it was removed from the induction chamber, trichotomized in precordial region and placed on a heating platform with electrocardiogram contact pads and maintained anesthetized by a nose cone with 1% to 2% isoflurane in 100% oxygen. Excess gases were evacuated passively using an activated charcoal absorption filter (VaporGuard, VetEquip, Livermore, Calif). A rectal probe was lubricated with gel, placed in the rectum and taped to the platform. The temperature was maintained at 36.5°C to 37.5°C. B-Mode and M-mode at the level of the papillary muscle images were obtained. Quantitative measurements were made offline using analytic software (VisualSonics). The percent of shortening fraction (SF) was calculated from M-mode measurements using the leading edge to leading edge method via the formula: % SF = left ventricular internal diameter (diastole) [LVID (d)] − left ventricular internal diameter (systole) [LVID (s)]/LVID (d) × 100. Ejection fraction (% EF) as follow: left ventricular diastolic volume (LVDV) − left ventricular systolic volume (LVSV)/left ventricular diastolic volume (LVDV) × 100. Cardiac stroke = left ventricular end diastolic volume (LVEDV) − left ventricular end systolic volume (LVESV). Cardiac output: Bpm × stroke volume.

ECG and cardiac AP recording—In order to assess the cardiac electrical activity *in vivo*, an electrocardiogram recording was carried out in conscious animals by noninvasive method. Electrodes were positioned in DI derivation and connected by flexible cables to a differential AC amplifier (model 1700; A-M Systems, Sequim, Wash), with signal low-pass filtered at 500 Hz and digitized at 1 kHz by a 16-bit A/D converter (Minidigi 1-D; Axon Instruments, Union City, Calif) using Axoscope 9.0 software (Axon Instruments). Data were stored in a PC for offline processing. The following parameters were analyzed using LabChart 7.3 software (AD Instruments, Bella Vista, New South Wales, Australia). RR interval and QJ interval as a measurement of early repolarization were also analyzed.

To assess cardiac electrical activity an AP recording was performed. Thus, a muscle strips were obtained and placed to the bottom of a tissue bath in order to expose the endocardial side. The preparations were superfused with Tyrode's solution containing (in mM): 150.8 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 11.0 D-glucose, 10.0 HEPES (pH 7.4 adjusted with NaOH at 37.0 ± 0.5°C) saturated with carbogen mixture (95% O_2/5% CO_2) at a flow of 5 mL/min (Miniplus3; Gilson, Middleton, Wis). The tissue was stimulated at three different basic cycle lengths: 200, 300 and 500 ms. Transmembrane potential was recorded using glass microelectrodes (10–40 MΩ DC resistance) filled with 2.7 M KCl connected to a high input impedance microelectrode amplifier (Electro 705; WPI,

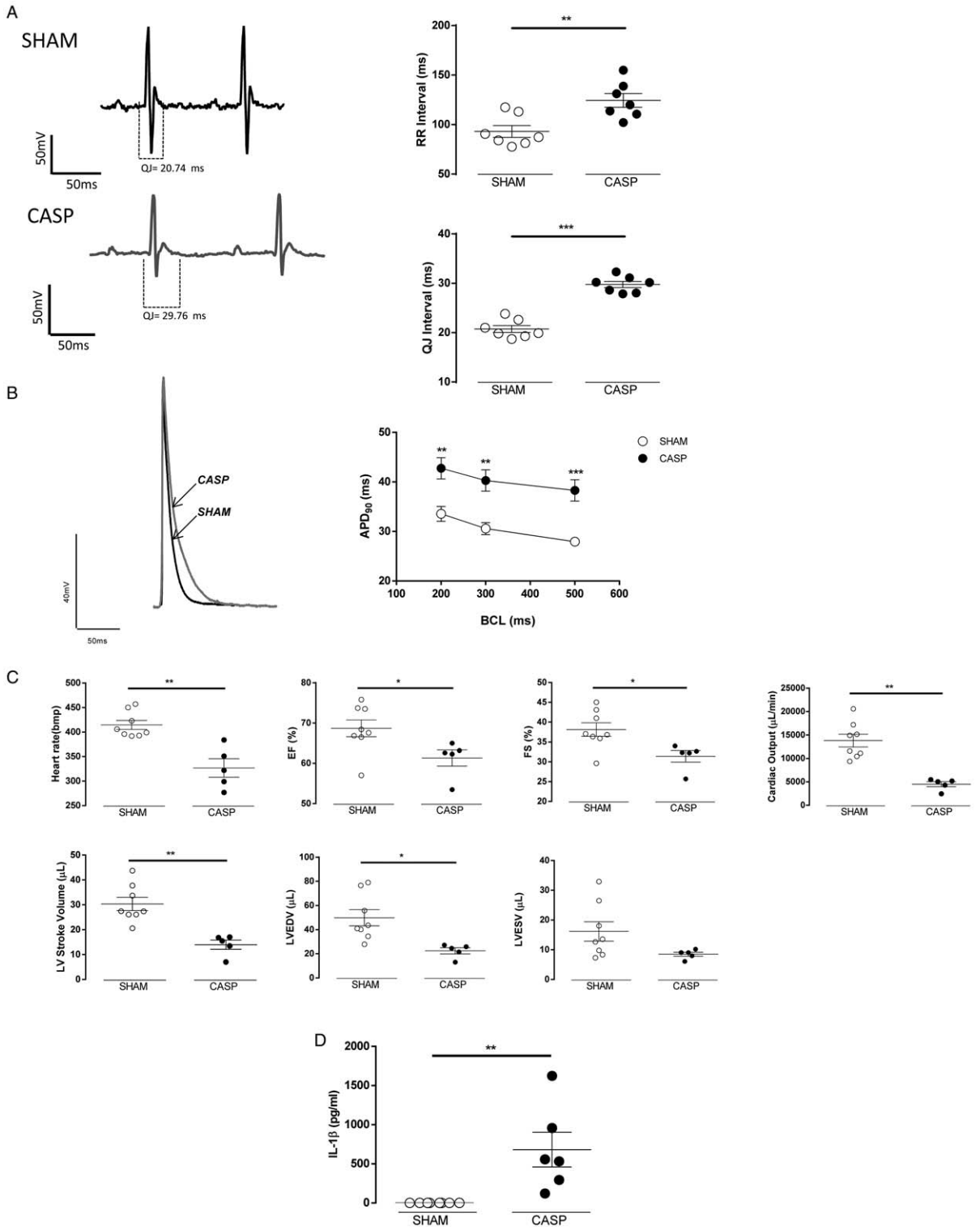

FIG. 1. **Impairment of electrical and mechanical cardiac function after colon ascendens stent peritonitis (CASP).** (A) Representative ECG traces of C57BL/6 WT mice highlighting QJ interval prolongation in CASP compared with sham mice (left). RR and QJ interval duration (right). For all groups, n = 7. (B) Representative action potential (AP) traces from the endocardial layer of left ventricle strips paced at 200 ms basic cycle length (BCL) in sham and CASP WT mice (left). AP duration at 90% of repolarization (APD90) at different BCL (200, 300, and 500 ms) in both groups (right). For all groups, n = 7. (C) Echocardiogram parameters of sham and CASP wild type (WT) mice. WT sham n = 8 and CASP n = 5. (D) Interleukin (IL)-1β serum levels. All parameters were analyzed 24 hour post-CASP induction. Results were expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. EF (%) indicates ejection fraction; LVEDV, left ventricular end diastolic volume; LVESV, left ventricular end systolic volume; SF (%), shortening fraction.

Sarasota, Fla). Amplified signals were digitized (1440 Digidata A/D Interface; Axon Instrument Inc) and stored in a computer for later analysis using software LabChart 7.3 (AD Instruments). The following parameters were analyzed: resting membrane potential, AP amplitude, AP duration at 90% (APD90), and 30% (APD30) repolarization. The Maximum rate of depolarization (Vmax) was calculated using five-point linear regression centered on the sample. The maximum negative slope (Max. Neg. Slope) was calculated by the steepest downhill slope starting 5 ms after the peak using a linear regression during a window of 4 ms. The AP triangulation was calculated by subtracting APD40 from APD90.

Inflammatory plasma profile—Blood was harvested from mice and kept for 30 minutes at room temperature. Blood was then centrifuged (800g × 15 min) and serum was collected and stored at -80 until use. A Bioplex Assay Kit (Bio-Rad, Hercules, Calif) was used to measure serum cytokine levels of IL-1β in Sham and Septic WT and *Nlrp3*$^{-/-}$ mice. To measure IL-6, IL-4 and TNF-α a CBA kit (BD, Franklin Lakes, NJ) was used.

Statistical analysis—Data are presented as mean ± SEM. Comparison between two groups was analyzed by t test. Multiple comparisons were performed using analysis of two-way variance (ANOVA), followed by Sidak's post-test to selected pairs. Values of $P < 0.05$ were considered statistically

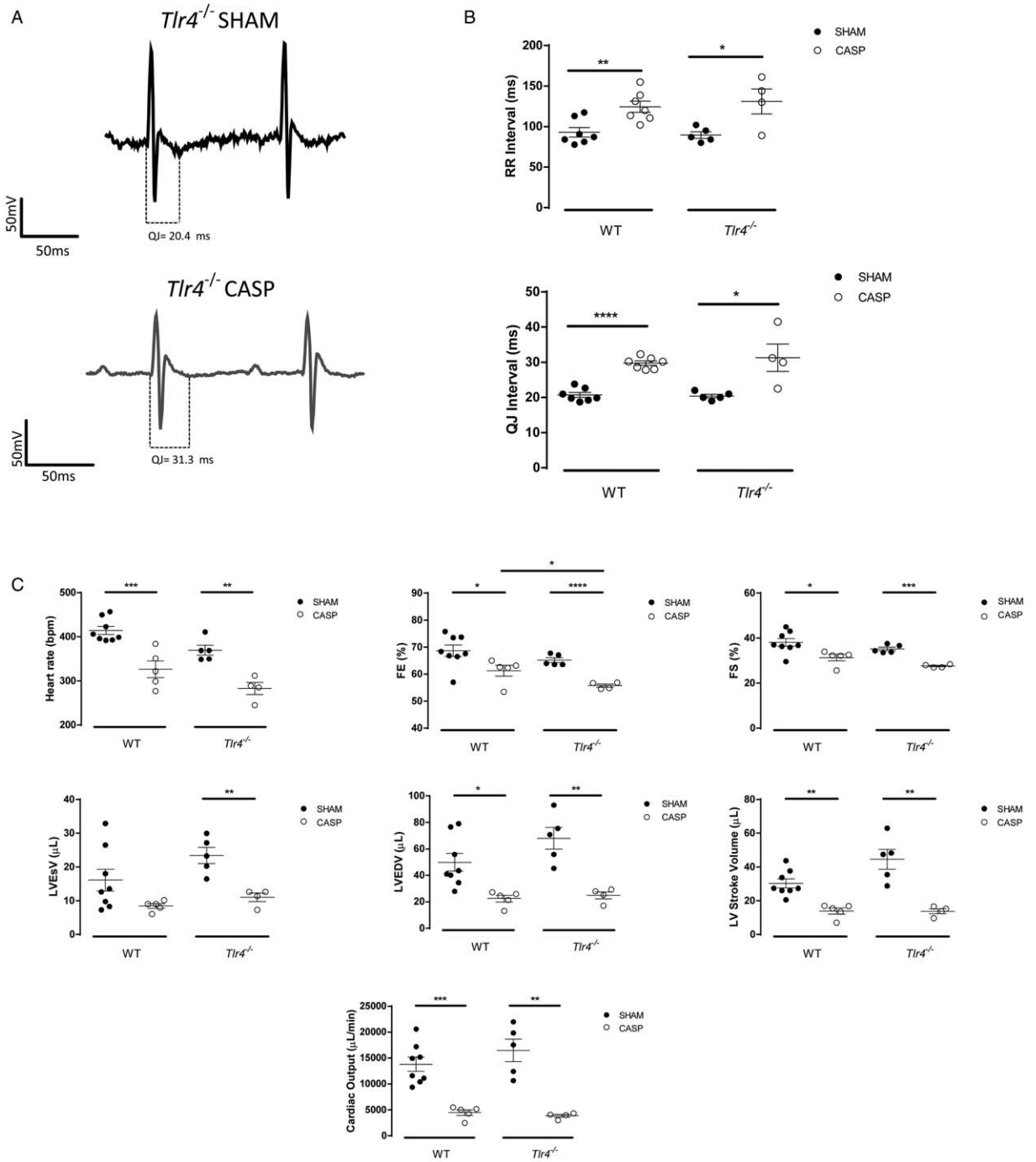

FIG. 2. **The genetic ablation of toll-like receptor 4 (TLR4) does not prevent colon ascendens stent peritonitis (CASP)-induced cardiac dysfunction.** (A) Representative ECG traces of *Tlr4*$^{-/-}$ sham and CASP mice showing QJ interval prolongation after CASP (left). RR and QJ interval duration of wild type (WT) and *Tlr4*$^{-/-}$ sham and CASP groups (right). WT sham n = 7, *Tlr4*$^{-/-}$ sham n = 5, WT CASP n = 7, *Tlr4*$^{-/-}$ CASP n = 4. (B) Echocardiogram parameters for WT and *Tlr4*$^{-/-}$ sham and CASP mice. WT sham n = 8, WT CASP n = 5, *Tlr4*$^{-/-}$ sham n = 5, *Tlr4*$^{-/-}$ CASP n = 4. Results were expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

significant. All analysis were made using GraphPad Prism 6.0 (GraphPad Software, La Jolla, Calif).

RESULTS

Sepsis by CASP impairs both cardiac electrical and mechanical activity

In order to assess the cardiac electrical function, ECG was recorded after CASP induction in C57BL/6J mice. Prolonged RR and QJ intervals were observed 24 hours after CASP induction as compared to the sham group (Fig. 1A). Mice also presented prolonged APD at 90% of repolarization after CASP induction (Fig. 1B and Supplemental Table 1, http://links.lww.com/SHK/A674). We observed a more depressed left ventricular function after CASP surgery than in sham-operated controls (Fig. 1C), as we previously described (19). Since sepsis

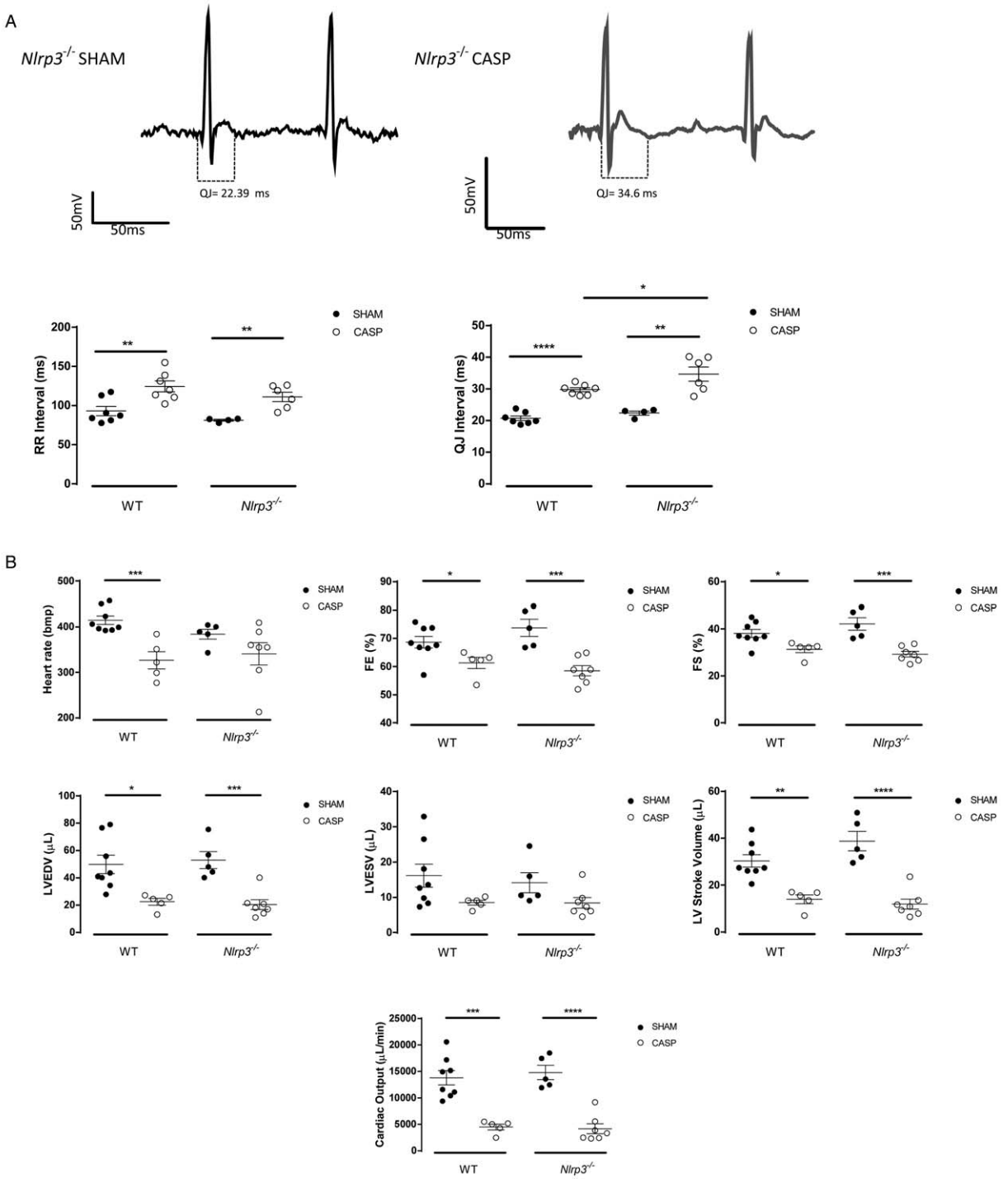

FIG. 3. **The genetic ablation of *Nlrp3* does not prevent colon ascendens stent peritonitis (CASP)-induced cardiac dysfunction.** (A) Representative ECG traces of *Nlrp3*$^{-/-}$ sham and CASP mice highlighting QJ interval prolongation in *Tlr4*$^{-/-}$ after CASP (left). RR and QJ interval duration of wild type (WT) and *Nlrp3*$^{-/-}$ sham and CASP groups (right). WT sham n = 7, *Nlrp3*$^{-/-}$ sham n = 4, WT CASP n = 7, *Nlrp3*$^{-/-}$ CASP n = 6. (B) Echocardiogram parameters for WT and *Nlrp3*$^{-/-}$ sham and CASP mice. WT sham n = 8, *Nlrp3*$^{-/-}$ sham n = 5, WT CASP n = 5, *Nlrp3*$^{-/-}$ CASP n = 7. Results were expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

is associated with a strong innate immune response and with cardiac dysfunction, we evaluated the production of the arrhythmogenic cytokine IL-1β. Much greater amounts of circulating IL-1β were found in septic compared to sham-operated mice (Fig. 1D).

TLR4 is not associated with contractile or electrical cardiac dysfunction in CASP

To investigate the role of TLR4 in the cardiac function during high-grade sepsis, we performed CASP in TLR4-deficient mice (*Tlr4*$^{-/-}$) and C57BL/6J WT mice and

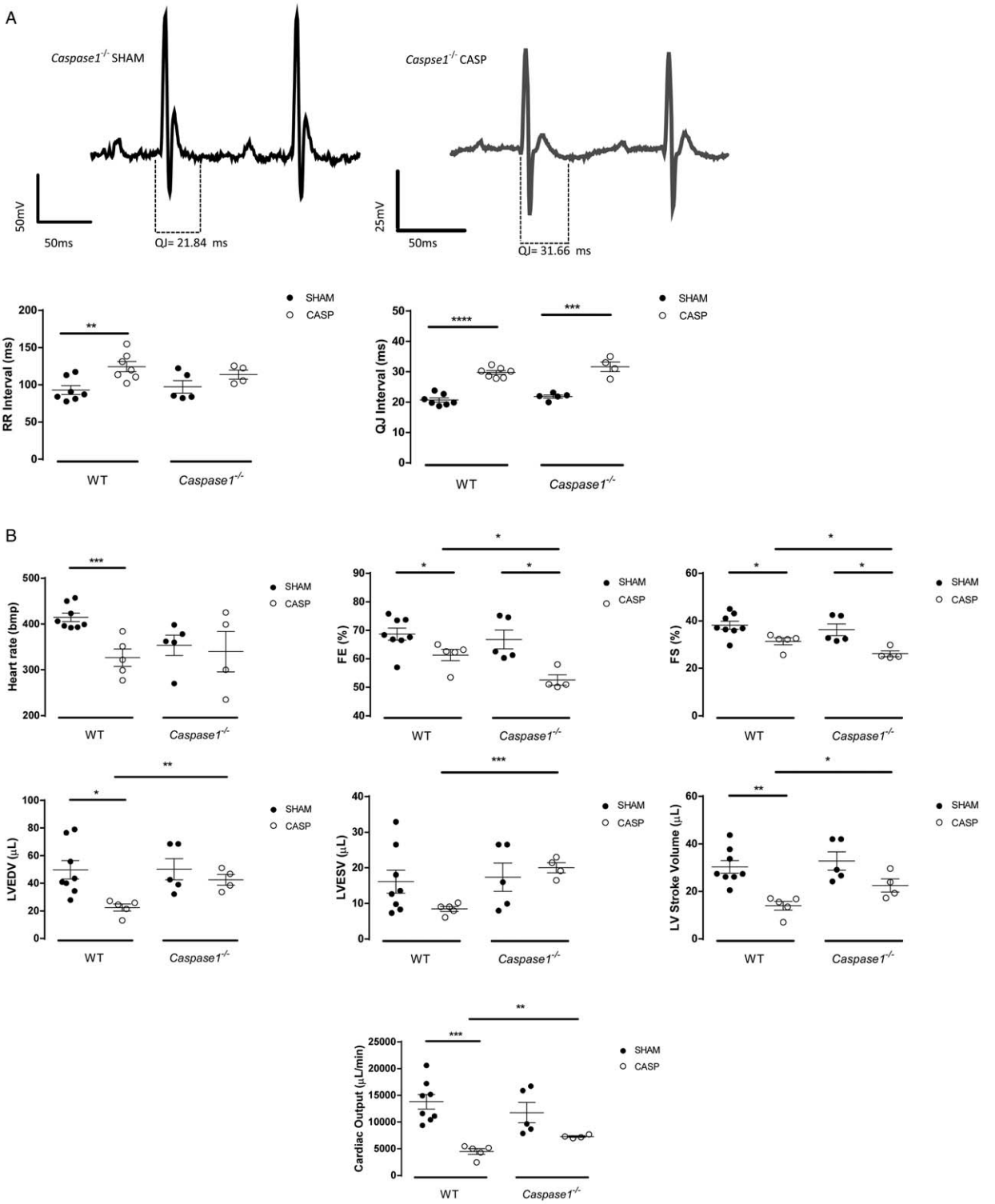

FIG. 4. **The genetic ablation of *Caspase-1* does not prevent colon ascendens stent peritonitis (CASP)-induced cardiac dysfunction.** (A) Representative ECG traces of *caspase-1*$^{-/-}$ sham and CASP mice highlighting QJ interval prolongation in *caspase-1*$^{-/-}$ after CASP (left). RR and QJ interval duration of wild type (WT) and *caspase1*$^{-/-}$ sham and CASP groups (right). WT sham n = 7, WT CASP n = 7, *caspase-1*$^{-/-}$ sham n = 5, *caspase-1*$^{-/-}$ CASP n = 4. (B) Echocardiogram parameters of WT and *caspase-1*$^{-/-}$ sham and CASP mice. WT sham n = 8, CASP n = 5 and *caspase-1*$^{-/-}$ sham n = 5, *caspase-1*$^{-/-}$ CASP n = 4. Results were expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

assessed their cardiac function by electro- and echocardiography. The genetic ablation of TLR4 (*Tlr4*$^{-/-}$ mice) did not prevent either electrical (Fig. 2, A and B) or mechanical (Fig. 2C) cardiac reactions to CASP, and *Tlr4*$^{-/-}$ mice presented cardiac alterations similar to WT-septic mice, such as increased RR and QJ intervals, decreased EF and FS. The similar behavior of *Tlr4*$^{-/-}$ and WT hearts after CASP show TLR4 is not a key molecule regulating the deleterious cardiac alterations.

Activation of NLRP3 or Caspase 1 is not involved in cardiac deterioration after CASP

Nlrp3$^{-/-}$mice behaved similarly to WT mice concerning electrical (Fig. 3A) and mechanical (Fig. 3B) cardiac responses to CASP, undergoing increases in RR and QJ intervals and decreases in EF and FS as compared to sham controls, which were similar in magnitude to that found in WT mice. These data indicate that NLRP3 does not participate in the cardiac deterioration that follows induction of high-grade sepsis.

The maturation of IL-1β critically depends on caspase-1 activation. This phenomenon is a consequence of inflammasome activation (24). Therefore, since the results presented above (Fig. 3) strongly suggested that the NLRP3 inflammasome is not involved in the CASP-induced cardiac damage, we also tested whether these cardiac alterations depend on caspase-1 activation using mice deficient in caspase-1 (*Caspase1*$^{-/-}$). As shown in Figure 4 A and B, neither electrical nor mechanical cardiac function was preserved in the *Caspase1*$^{-/-}$ mice after CASP surgery. Collectively, these data suggest that the suppression of this unique pathway does not interfere with the cardiac dysfunction induced by CASP.

NLRP3 controls pro-inflammatory cytokine production in CASP

In order to understand whether the lack of inflammasome activation could interfere with CASP-induced cytokine storm, we measured the level of key circulating cytokines. As expected, in *Nlrp3*$^{-/-}$ mice, the circulating IL-1β did not reach levels as high as in WT mice after CASP as compared to sham-operated mice (Fig. 5A). Also, different from WT mice, *Nlrp3*$^{-/-}$ mice reacted to CASP with a blunted increase in IL-6 and TNF-α plasma levels (Fig. 5, B and C). Together, these data indicate that NLRP3 activation participates in the production of key inflammatory cytokines during CASP-induced high-grade sepsis. The sharp contrast between the similar cardiac deterioration found in WT and *Nlrp3*$^{-/-}$ mice and the blunted production of pro-inflammatory cytokines found in *Nlrp3*$^{-/-}$ as compared to WT mice in response to CASP indicates that these pro-inflammatory cytokines are not pivotal in the cardiac deterioration induced by CASP.

Since our results in CASP differed from a previous work which showed attenuation of the cardiac deterioration in *Nlrp3*$^{-/-}$ mice submitted to medium-grade CLP (10), we performed high-grade CLP in order to test whether severe polymicrobial sepsis could bypass the need for Nlrp3-dependent inflammation to affect cardiac function. Systolic (Fig. 6A) and electrical (Fig. 6B) heart functions were similarly affected after WT or *Nlrp3*$^{-/-}$ mice were submitted to either CLP or

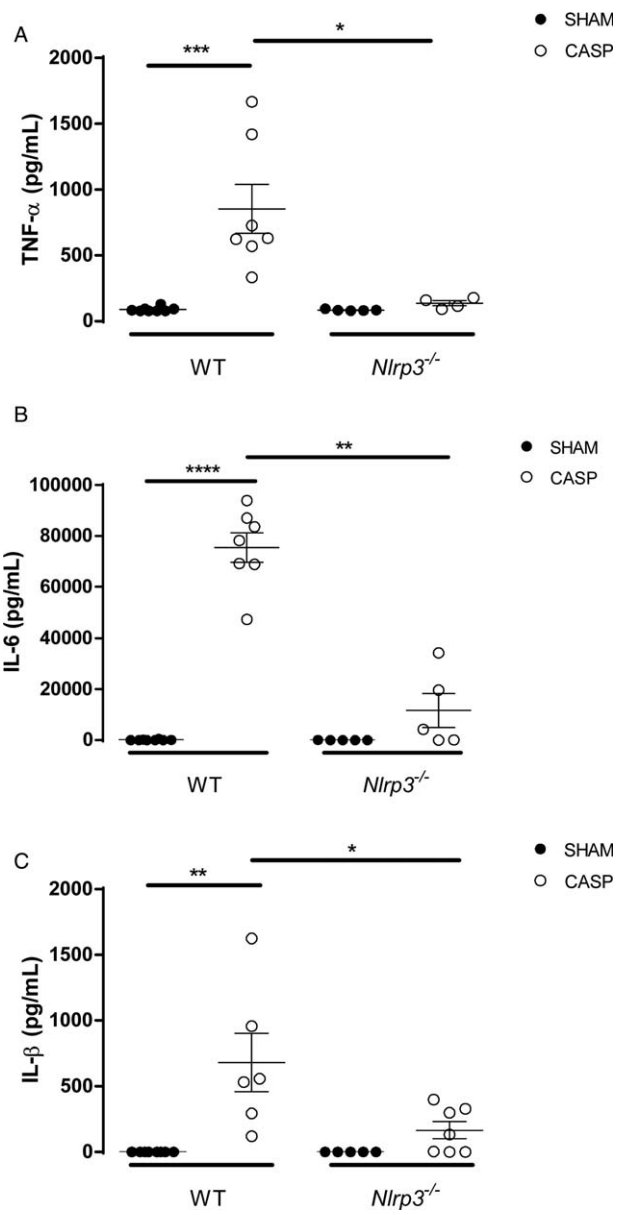

FIG. 5. **Circulating levels of pro-inflammatory cytokines after colon ascendens stent peritonitis (CASP) depend on *Nlrp3*.** Serum levels of cytokines for wild type (WT) sham, WT CASP, *Nlrp3*$^{-/-}$ sham and *Nlrp3*$^{-/-}$ CASP: (A) tumor necrosis factor alpha (TNF-α), (B) interleukin (IL)-6, and (C) IL-1β. Results were expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

CASP surgeries. The production of IL-1β, TNF, and IL-6 was blunted in *Nlrp3*$^{-/-}$ mice submitted to CASP when compared with the response of WT mice, but after CLP, similar levels of inflammatory cytokines were found in *Nlrp3*$^{-/-}$ versus WT mice (Fig. 6, A–C). Thus, while the production of pro-inflammatory cytokines depended on the chosen polymicrobial sepsis model, our results indicate that when the severity of sepsis is high, NLPR3-dependent inflammation is not relevant to the cardiac function.

DISCUSSION

Herein, we studied the role played by TLR4-NLRP3-IL-1β axis in the electrical and mechanical cardiac dysfunction that

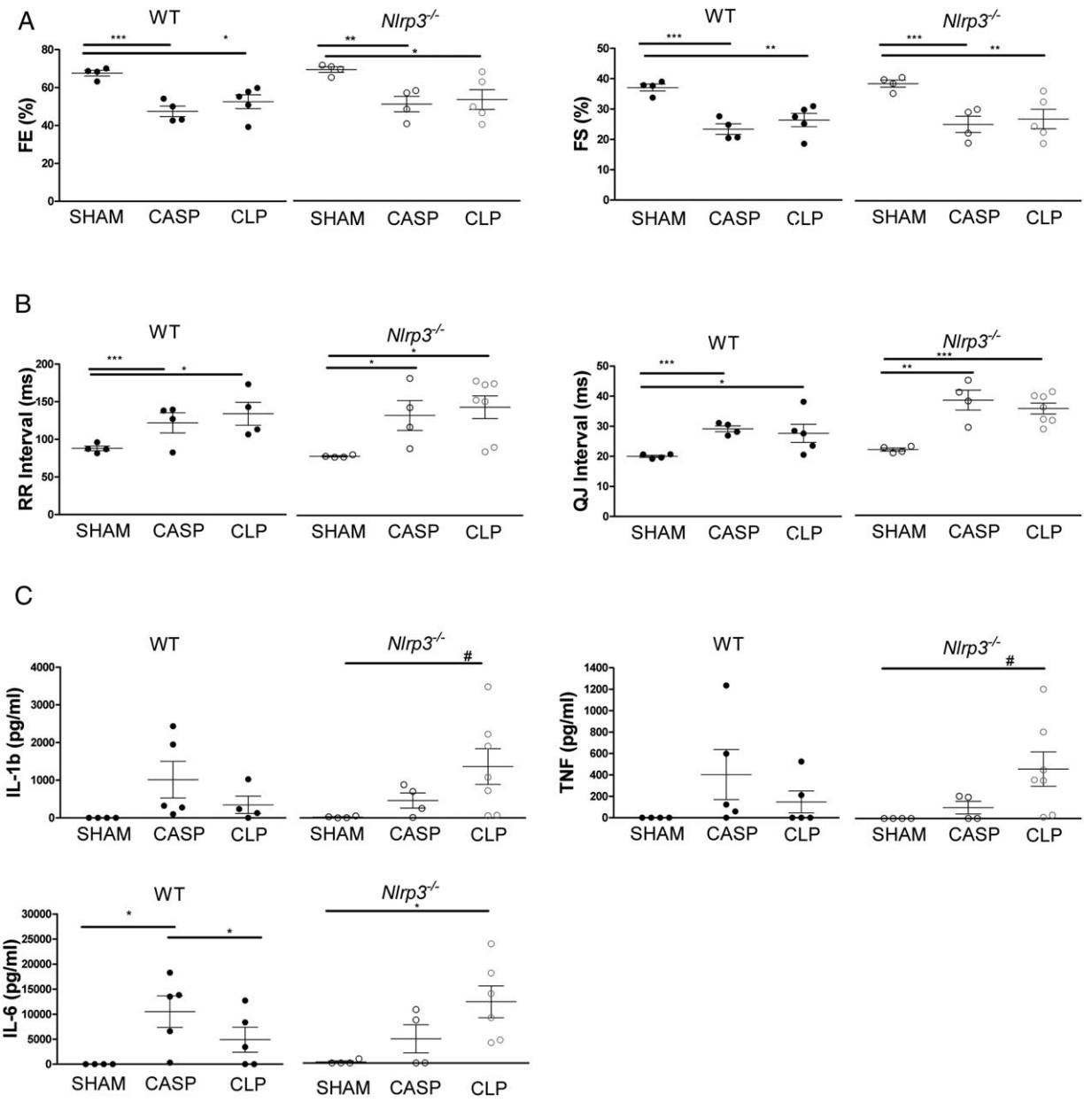

Fig. 6. **Cardiac function of *Nlrp3*$^{-/-}$ mice respond similarly to high-grade models of sepsis cecal ligation and puncture (CLP) and colon ascendens stent peritonitis (CASP).** (A) Left ventricle ejection fraction (EF) and fractional shortening (FS); (B) RR and QJ interval duration, and (C) IL-1β, tumor necrosis factor (TNF) and interleukin (IL)-6 plasma levels of wild type (WT) and *Nlrp3*$^{-/-}$ 18 hour after sham, CASP, or CLP. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{\#}P = 0.06$.

follows CASP in mice. We found that the genetic deficiency of TLR4, NLRP3, or CASP1 does not interfere with cardiac dysfunction in general, despite the profound inhibition of IL-1β, IL-6, and TNF-α production in *Nlrp3*$^{-/-}$ mice after CASP surgery. Our results indicate that IL-1β is unlikely to be a feasible therapeutic target in cases of sepsis in which diffuse peritonitis and SIRS predominate.

The genetic deficiency of NLRP3 prevented the decrease in heart rate, stroke volume, and LV end diastolic volume that occurred after medium-grade CLP surgery (10). On the other hand, we found here similar cardiac responses in WT and *Nlrp3*$^{-/-}$ after high-grade CLP or CASP surgeries, indicating that when the severity of sepsis is high, Nlrp3-dependent inflammation is not relevant to the cardiac function. We speculate that the reasons for the different results obtained with high-grade CLP and CASP performed by us and the previous medium-grade CLP performed by others (10) are likely due to the widespread availability of TLR ligands in high-grade sepsis, which can dispense with the inflammatory effects of cytokines and act on heart function through modulation of autonomic nervous function (25).

A previous work on the CLP model of medium-grade sepsis revealed that genetic deficiency of NLRP3 was capable of preventing the secretion of the inflammasome-dependent cytokine IL-1β and also of the non-NLRP3 related cytokine IL-6 (10). Here we found IL-1β, IL-6, and TNF-α production was

drastically decreased in *Nlrp3*$^{-/-}$ mice after high-grade CASP surgery, but not after high-grade CLP. The reasons for the discrepancy between medium- and high-grade CLP and also between high-grade CLP and CASP concerning NLRP3-dependent pro-inflammatory cytokine secretion remain unknown, as well as its relevance to the outcome of polymicrobial sepsis.

Toll-like receptor 4 (TLR4) activates the innate immune system in response to microbial products. The result is an inflammatory reaction that has both the benefit of clearing a potential infection, but can also result in collateral damage to host tissues. In endotoxemia, the TLR4 ligand lipopolysaccharide kills the host by inducing a severe cytokine storm (26), but in CLP, the genetic ablation of TLR4 worsens cardiac function (6), an effect that could stem from the capacity of TLR4 stimulation to increase the levels of IL-6 (27). Here we have shown that deficiency of TLR4 does not interfere with cardiac dysfunction in the CASP model of sepsis. Also, conditions that drastically reduce the levels of IL-6 do not interfere with cardiac dysfunction after CASP, further indicating that in high-grade sepsis there is no involvement of IL-6 production in cardiac dysfunction.

We have recently demonstrated that IL-1β is an arrhythmogenic agent per se, causing prolongation of the APD, while it also promotes CaMKII oxidation and increases calcium sparks in cardiomyocytes (20). We have also shown that heart contractile dysfunction in CASP is mediated by CAMKII oxidation and calcium diastolic leakage (19). Therefore, we raised the hypothesis that IL-1β could be responsible for the prolonged QJ, the CaMKII oxidation, the calcium diastolic leakage and the consequent contractile dysfunction after CASP. Nevertheless, genetic deficiency of either NLRP3 or caspase-1 did not interfere with cardiac dysfunction after sepsis, even though they abrogated the secretion of IL-1β. The redundancy in the effects of pro-inflammatory cytokines upon cardiomyocytes does not easily fit to explain these results, since IL-1β, IL-6, and TNF-α were all greatly reduced in *Nlrp3*$^{-/-}$ mice. However, we can not rule out the cardiac effects of non-studied cytokines which are known to interfere with heart physiology and are present in high levels after CASP, such as macrophage inflammatory protein 1 alpha, interferon gamma, and IL-12 (26, 28, 29). Another possibility is that pathogen associated molecular patterns other than TLR4 ligands or NLRP3 activators are directly sensed by cardiomyocytes, triggering alterations in heart physiology. Still, it is possible that mitochondrial ROS produced in response to a secondary metabolic dysfunction in sepsis play a role in cardiac dysfunction.

Our results show that the absence of either TLR4, NLRP3, or caspase-1 does not prevent cardiac dysfunction after CASP, but still, the genetic ablation of NLRP3 abrogates the production of IL-1β, TNF-α, and IL-6. Taken together, these results indicate that targeting TLR4, NLRP3, caspase-1, or IL-1β is not likely to ameliorate the cardiac dysfunction that accompanies the cases of sepsis in which diffuse peritonitis and SIRS predominate. These results in the CASP model contrast sharply with the great prevention of cardiac dysfunction found in *Nlrp3*$^{-/-}$ mice submitted to medium-grade CLP surgery, but are similar to our cardiac findings in high-grade sepsis, indicating the need to distinguish between low, medium and high-grade sepsis in humans in order to propose candidate therapies to clinical trials.

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