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TLR4 AND NLRP3 CASPASE 1- IL-1B- AXIS ARE NOT INVOLVED IN COLON ASCENDENS STENT PERITONITIS (CASP)-ASSOCIATED HEART DISEASE

Maria Micaela Lopez Alarcón[a], Julieta Fernández Ruocco[a], Fabiano Ferreira[b], Heitor A. Paula-Neto[c], Marisa Sepúlveda[d], Martín Vila Petroff[d], Adriana Bastos Carvalho[a], Isalira Peroba Ramos[e], Hugo Justino Branda[a], Claudia Neto Paiva[b], Emiliano Medei[a,e*]

[a]Carlos Chagas Filho Biophysics Institute, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil; [b]Institute of Microbiology, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil; [c]Laboratory of Molecular Targets, Department of Pharmaceutical Biotecnology, Faculty of Pharmacy, , Federal University of Rio de Janeiro, Rio de Janeiro, Brazil, [d]Cardiovascular Research Center, Conicet, National University of La Plata, La Plata, Buenos Aires, Argentina; and [e]National Center for Structural Biology and Bioimaging – CENABIO/UFRJ, Rio de Janeiro, Brazil.

** Corresponding author at: Prof. Emiliano Medei. Carlos Chagas Filho Biophysics Institute – UFRJ, Avenida Carlos Chagas Filho, 373 –CCS- Bloco G, Rio de Janeiro, RJ 21941-902, Brasil. Tel./Fax: 55 21 39386555.*

E-mail address: emedei70@biof.ufrj.br

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NLRP3 axis do not prevent cardiac dysfunction in CASP

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 TLR4 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 CASP model. The QJ interval was increased in wild-type C57BL/6J mice after CASP when compared to sham controls, a result paralleled by an increase in the cardiac action potential duration (APD). The decreases in ejection fraction (EF), left-ventricle end diastolic volume (LVEDV), 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 CLP. Despite developing cardiac dysfunction similar to wild-types after CASP, *Nlrp3*$^{-/-}$ mice had reduced circulating levels of IL-1β, IL-6 and TNF-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.

Key words: Cardioimmunology, Sepsis, Innate immunology, electrophysiology, IL-1β

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 TNF-α [3] and 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) in mediating acute myocardial dysfunction in endotoxemia and *E. 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 toll like receptor 2 (TLR2) [7] and also of MyD88 and 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 surgery colon ascendens stent peritonitis (CASP) 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 iNOS[17], 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 n°: 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, USA) (# 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 8–10 weeks, were kept at constant temperature (23 ^{o}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 2 groups: a) Sham mice (Sham) and b) operated mice (CASP). CASP surgery procedures were carried out as previously described by Traeger T.et al.

(2010), using a specific 14 G catheter [21]. 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 [22]. 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 9 punctures were made using a 16G gauge needle. In pilot experiments, we determined that this procedure resulted in similar mortality between CLP and CASP models (100% within 48h). 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, Toronto, Ontario, Canada). 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 1–2% isoflurane in 100% oxygen. Excess gases were evacuated passively using an activated charcoal absorption filter (VaporGuard, VetEquip, Livermore, California, USA). A rectal probe was lubricated with gel, placed in the rectum and taped to the platform. The temperature was maintained at 36.5–37.5 °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, Toronto, Ontario, Canada). The percent of shortening fraction was calculated from M-mode measurements using the leading edge to leading edge method via the formula: % Shortening Fraction (%SF) = left ventricular internal diameter (diastole) [LVID(d)] − left ventricular internal diameter (systole) [LVID(s)]/LVID(d) x 100. Ejection fraction (%EF) as follow: left ventricular diastolic volume (LVDV) - left ventricular systolic volume (LVSV) / left ventricular diastolic volume (LVDV) x100. Cardiac stroke = left ventricular end diastolic volume (LVEDV) – left ventricular end systolic volume (LVESV). Cardiac output: Bpm x stroke volume.

ECG and cardiac action potential 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, Washington USA), 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, California, USA) using Axoscope 9.0 software (Axon Instruments, Union City, California, USA). 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). R-R interval and Q-J interval as a measurement of early repolarization were also analyzed.

To assess cardiac electrical activity an action potential 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 CaCl2, 1.0 MgCl2, 11.0 D-glucose, 10.0 HEPES (pH 7.4 adjusted with NaOH at 37.0 ± 0.5 °C) saturated with carbogen mixture (95% O2/5% CO2) at a flowof 5 ml/min (Miniplus3 Gilson, Middleton, Wisconsin, USA). 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, Sarasota, Florida, USA). Amplified signals were digitized (1440 Digidata A/D interface, Axon Instrument Inc., Union City, California, USA) and stored in a computer for later analysis using software LabChart 7.3 (ADInstruments, Bella Vista, New South Wales, Australia). The following parameters were analyzed: resting membrane potential (RMP), action potential amplitude (APA), action potential 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 x 15 min) and serum was collected and stored at -80 until use. A Bioplex Assay Kit (Bio-Rad, Hercules, California, USA) 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, New Jersey, USA) 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 significant. All analysis were made using GraphPad Prism 6.0 (GraphPad Software, La Jolla, California, USA).

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 h after CASP induction as compared to the sham group (Figure 1A). Mice also presented prolonged action potential duration at 90% of repolarization after CASP induction (Figure 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 (Figure 1C), as we previously described [19]. Since sepsis 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 (Figure 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 wild-type mice and assessed their cardiac function by electro and echocardiography.The genetic ablation of TLR4 (*Tlr4*$^{-/-}$ mice) did not prevent either electrical (Figure 2A, B) or mechanical (Figure 2C) cardiac reactions to CASP, and *Tlr4*$^{-/-}$ mice presented cardiac alterations similar to wild type-septic mice, such as increased RR and QJ intervals, decreased EF and FS. The similar behavior of *Tlr4*$^{-/-}$ and wild-type hearts after CASP show TLR4 is not a key molecule regulating the deleterious cardiac alterations.

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

Nlrp3$^{-/-}$ mice behaved similarly to wild-type mice concerning electrical (Figure 3A) and mechanical (Figure 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 wild-type 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 [23]. Therefore, since the results presented above (Figure 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, 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 wild-type mice after CASP as compared to sham-operated mice (Figure 5A). Also, different from wild-type mice, *Nlrp3*$^{-/-}$ mice reacted to CASP with a blunted increase in IL-6 and TNF-α plasma levels (Figure 5B, 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 wild-type and *Nlrp3*$^{-/-}$ mice and the blunted production of pro-inflammatory cytokines found in *Nlrp3*$^{-/-}$ as compared to wild-type 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 (Figure 6A) and electrical (Figure 6B) heart functions were similarly affected after WT or *Nlrp3*$^{-/-}$ mice were submitted to either CLP or CASP surgeries. The

production of IL-1β, TNF, and IL-6 was blunted in *Nlrp3*$^{-/-}$ mice submitted to CASP when compared to the response of WT mice, but after CLP, similar levels of inflammatory cytokines were found in *Nlrp3*$^{-/-}$ versus WT mice (Figure 6A-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 follows CASP in mice. We found that the genetic deficiency of TLR4, NLRP3, or CASP1 does not interfere with cardiacdysfunction 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 [24].

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 (LPS) kills the host by inducing a severe cytokine storm [25], 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 (29). 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 action potential duration, while it also promotes CaMKII

oxidation and increases calcium sparks in cardiomyocytes [26]. 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 MIP-1α, IFN-γ, and IL-12 [27-29]. 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.

AUTHOR CONTRIBUTIONS

E. Medei and M. Lopez Alarcón designed the research; E. Medei, M. Lopez Alarcón, J.FernándezRuocco, F. Ferreira, M. Sepúlveda, M. Vila Petroff, A. Bastos Carvalho, I. Ramos, H. Branda, C. Paiva, analyzed and interpreted the data; E. Medei, M. Lopez Alarcón, J. FernándezRuocco, F. Ferreira, M. Sepúlveda, A. Bastos Carvalho, I. Ramos and H. Branda performed the research; E. Medei, M. Lopez Alarcón, M. Vila Petroff and C. Paiva wrote and revised the paper; and all authors approved the final version of the manuscript.

REFERENCES

1. Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, Bellomo R, Bernard GR, Chiche JD, Coopersmith CM, et al.: The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). JAMA 315(8):801-10, 2016.

2. Drosatos K, Lymperopoulos A, Kennel PJ, Pollak N, Schulze PC, Goldberg IJ: Pathophysiology of Sepsis-Related Cardiac Dysfunction: Driven by Inflammation, Energy Mismanagement, or Both?. *Curr. Heart Fail Rep* 12(2):130-40, 2015.

3. Lang CH, Pruznak AM, Frost RA: TNF-alpha Mediates Sepsis-Induced Impairment of Basal and Leucine-Stimulated Signaling via S6K1 and eIF4E in Cardiac Muscle. *J. Cell Biochem.* 94(2):419-31, 2005.

4. Riedemann NC, Neff TA, Guo RF, Bernacki KD, Laudes IJ, Sarma JV, Lambris JD, Ward PA: Protective effects of IL-6 blockade in sepsis are linked to reduced C5a receptor expression. *J. Immunol.* 170(1):503-7, 2003.

5. Avlas O, Fallach R, Shainberg A, Porat E, Hochhauser E: Toll-like receptor 4 stimulation initiates an inflammatory response that decreases cardiomyocyte contractility. *Antioxid. Redox Signal* 15(7):1895-909, 2011.

6. Zhang M,Zou L,Feng Y, Chen YJ, Zhou Q, Ichinose F, Chao W: Toll-like receptor 4 is essential to preserving cardiac function and survival in low-grade polymicrobial sepsis. *Anesthesiology* 121(6):1270-80, 2014.

7. Zou L, Feng Y, Chen YJ, Si R, Shen S, Zhou Q, Ichinose F, Scherrer-Crosbie M, Chao W: Toll-like receptor 2 plays a critical role in cardiac dysfunction during polymicrobial sepsis. *Crit. Care Med.* 38(5): 1335-42, 2010.

8. Feng Y, Zou L, Zhang M, Li Y, Chen C, Chao W: MyD88 and Trif signaling play distinct roles in cardiac dysfunction and mortality during endotoxin shock and polymicrobial sepsis. *Anesthesiology* 115(3):555-67, 2011.

9. Jin L, Batra S, Jeyaseelan S: Deletion of *Nlrp3* Augments Survival during Polymicrobial Sepsis by Decreasing Autophagy and Enhancing Phagocytosis. *J. Immunol*. 198(3):1253-62, 2016.

10. Kalbitz M, Fattahi F, Grailer JJ, Jajou L, Malan EA, Zetoune FS, Huber-Lang M, Russell MW, Ward PA: Complement-induced activation of the cardiac NLRP3 inflammasome in sepsis. *FASEB J.* 30(12): 3997-4006, 2016.

11. Atefi G, Zetoune FS, Herron TJ, Jalife J, Bosmann M, Al-Aref R, Sarma JV, Ward PA: Complement dependency of cardiomyocyte release of mediators during sepsis. *FASEB J.* 25(7): 2500-8, 2011.

12. Schabbauer G: Polymicrobial sepsis models: CLP versus CASP. *Drug Discovery Today: Disease Models* 9(1): e17-e21, 2012.

13. Weighardt H, Kaiser-Moore S, Ramunas M, Vabulas RM, Kirschning CJ, Wagner H, Holzmann B: Cutting Edge: Myeloid Differentiation Factor 88 Deficiency Improves Resistance Against Sepsis Caused by Polymicrobial Infection. *J. Immunol.*169(6):2823-27, 2002.

14. Daubeuf B, Mathison J, Spiller S, Hugues S, Herren S, Ferlin W, Kosco-Vilbois M, Wagner H, Kirschning CJ, Ulevitch R et al.: TLR4/MD-2 Monoclonal Antibody Therapy Affords Protection in Experimental Models of Septic Shock. *J. Immunol.*179(9):6107-14, 2007.

15. Lohner R, Schwederski M, Narath C, Klein J, Duerr GD, Torno A, Knuefermann P,Hoeft A, Baumgarten G, Meyer R. et al.: Toll-like receptor 9 promotes cardiac inflammation and heart failure during polymicrobial sepsis.*Mediators Inflamm*, 2013.

16. Reim D, Rossmann-Bloeck T, Jusek G, Prazeres da Costa O, Holzmann B: Improved host defense against septic peritonitis in mice lacking MyD88 and TRIF is linked to a normal interferon response. *J. Leukoc. Biol.* 90(3):613-20, 2011.

17. Entleutner M, Traeger T, Westerholt A, Holzmann B, Stier A, Pfeffer K, Maier S, Heidecke CD: Impact of interleukin-12, oxidative burst,andiNOS on the survival fecal peritonitis. *Int. J. Colorectal Dis.*21(1):64-70, 2005.

18.Yuan Y, Ren J, Cao S, Zhang W, Li J: Exogenous C3 protein enhances the adaptive immune response to polymicrobial sepsis through down-regulation of regulatory T cells. *Int. Immunopharmacol.*12(1):271-7, 2012.

19. Sepúlveda M, Gonano LA, Viotti M, Morell M, Blanco P, LópezAlarcón M, Peroba Ramos I, Bastos Carvalho A, Medei E. et al.: Calcium/Calmodulin Protein Kinase II Dependent Ryanodine Receptor Phosphorylation Mediates Cardiac Contractile Dysfunction Associated With Sepsis. *Crit. Care Med.* 45(4):e399-e408, 2016.

20. Mariathasan S, Weiss DS, Newton K, McBride J, O' Rourke K, Roose-Girma M, Lee WP, Weinrauch Y, Monack DM, Dixit VM: Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* 440(7081): 228-32, 2006.

21. Traeger T, Koerner P, Kessler W, Cziupka K, Diedrich S, Busemann A, Heidecke CD, Maier S: Colon ascendens stent peritonitis (CASP) a standardized model for polymicrobial abdominal sepsis. *J. Vis. Exp.*18(46):pii 2299, 2010.

22. Rittirsch D, Huber-Lang MS, Flierl MA, Ward PA: Immunodesign of experimental sepsis by cecal ligation and puncture. *Nat Protoc* 4(1):31-6, 2009.

23. Lara-Tejero M, Sutterwala FS, Ogura Y, Grant EP, Bertin J, Coyle AJ, Flavell RA, Galán JE: Role of the caspase-1 inflammasome in *Salmonella typhimurium* pathogenesis. *J. Exp. Med.* 203(6):1407-12, 2006

24. Opal SM, Fisher CJ Jr, Dhainaut JF, Vincent JL, Brase R, Lowry SF, Sadoff JC, Slotman GJ, Levy H, Balk RA, et al.: Confirmatory interleukin-1 receptor antagonist trial in severe sepsis: a phase III, randomized, double-blind, placebo-controlled, multicenter trial. The Interleukin-1 Receptor Antagonist Sepsis Investigator Group. *Crit. Care Med.* 25(7):1115-24, 1997.

25. Werdan K, Schmidt H, Ebelt H, Zorn-Pauly K, Koidl B, Hoke RS, Heinroth K, Muller-Werdan U: Impaired regulation of cardiac function in sepsis, SIRS, and MODS. *Can J Physiol Pharmacol* 87(4):266-74, 2009.

26. Mogensen TH: Pathogen Recognition and Inflammatory Signaling in Innate Immune Defenses. *Clin. Microbiol. Rev.* 22(2):240-273, 2009.

27. Monnerat G*, Alarcón ML*, Vasconcellos LR, Hochman-Mendez C, Brasil G, Bassani RA, Casis O, Malan D, Travassos LH, Sepúlveda M, Burgos JI. et al.: Macrophage-dependent IL-1β production induces cardiac arrhythmias in diabetic mice. *Nat.Commun.*7:13344, 2016.

28. Levick SP, Goldspink PH: Could interferon-gamma be a therapeutic target for treating heart failure?.*Heart Fail Rev.*19(2):227-36, 2014.

29. Oikonomou E, Tousoulis D, Siasos G, Zaromitidou M, Papavassiliou AG, Stefanadis C:The role of inflammation in heart failure: new therapeutic approaches. *Hellenic J.Cardiol.* 52(1):30-40, 2011.

FIGURE LEGENDS

Figure 1. Impairment of electrical and mechanical cardiac function after CASP.A) Representative ECG traces of C57BL/6 WT mice highlighting QJ interval prolongation in CASP compared to sham mice (left). RR and QJ intervals 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 per cent 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 WT mice. SF (%): shortening fraction. EF (%): ejection fraction; LVEDV: left ventricular end diastolic volume; LVESV: left ventricular end systolic volume. WT sham n = 8 and CASP n = 5. D) IL-1β serum levels. All parameters were analyzed 24 h post-CASP induction. Results were expressed as mean ± SEM. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

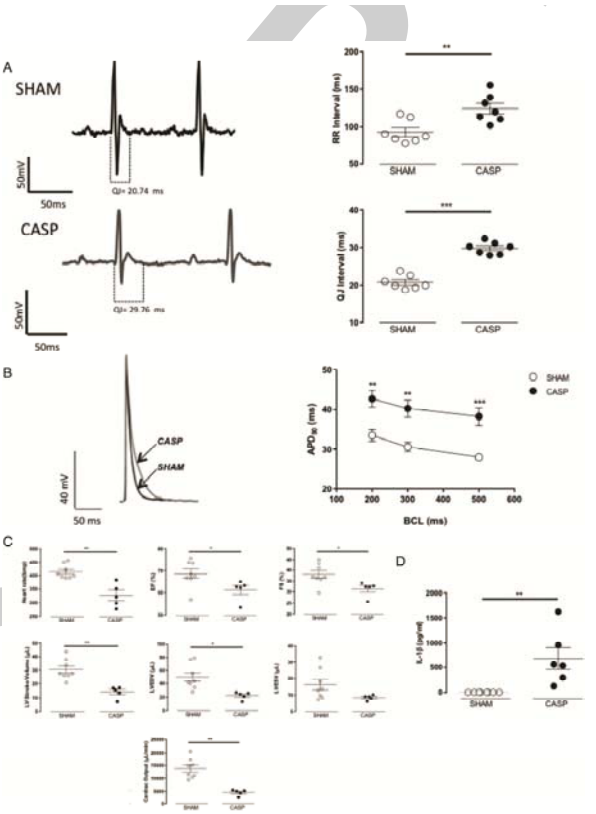

Figure 2. The genetic ablation of TLR4 does not prevent CASP-induced cardiac dysfunction. A) Representative ECG traces of *Tlr4*$^{-/-}$sham and CASP mice showing QJ interval prolongation after CASP (left). RR and QJ intervalS duration of WT and *Tlr4*$^{-/-}$sham and CASP groups (right). WT sham n = 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 **** $P < 0.0001$.

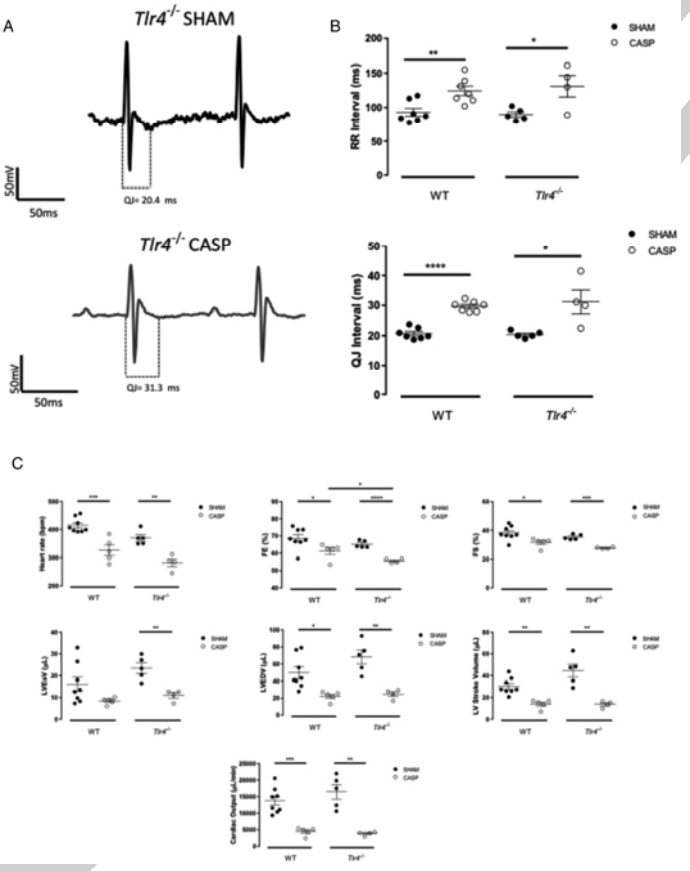

Figure 3. The genetic ablation of *Nlrp3* does not prevent CASP-induced cardiac dysfunction. A) Representative ECG traces of *Nlrp3*$^{-/-}$sham and CASP mice highlighting QJ interval prolongation in *Tlr4*$^{-/-}$after CASP (left). RR and QJ interval duration of WT and *Nlrp3*$^{-/-}$sham and CASP groups (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.

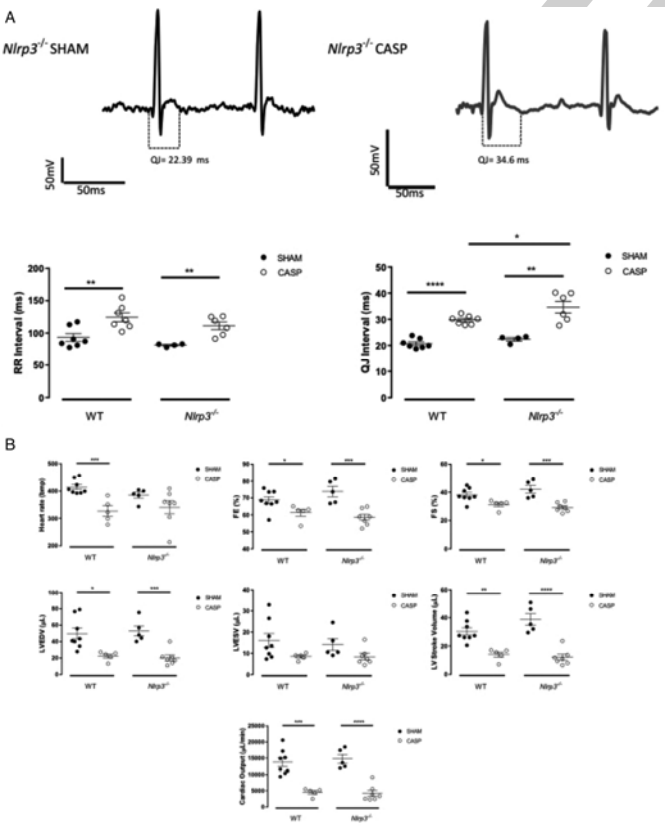

Figure 4. The genetic ablation of *Caspase-1* does not prevent CASP-induced cardiac dysfunction. A) Representative ECG traces of *caspase-1*$^{-/-}$sham and CASP mice highlighting QJ interval prolongation in *caspase-1*$^{-/-}$ after CASP (left). RR and QJ interval duration of WT and *caspase1*$^{-/-}$ 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 **** $P < 0.0001$.

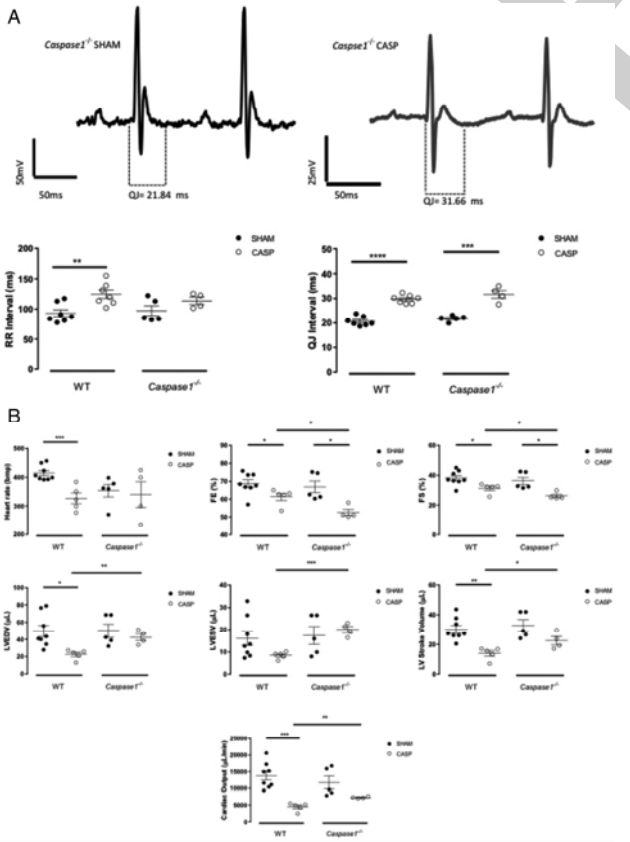

Figure 5. Circulating levels of pro-inflammatory cytokines after CASP depend on *Nlrp3*. Serum levels of cytokines for WT sham, WT CASP, *Nlrp3*$^{-/-}$sham and *Nlrp3*$^{-/-}$ CASP: A) TNF-α, B) IL-6 and C) IL-1β. Results were expressed as mean ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$.

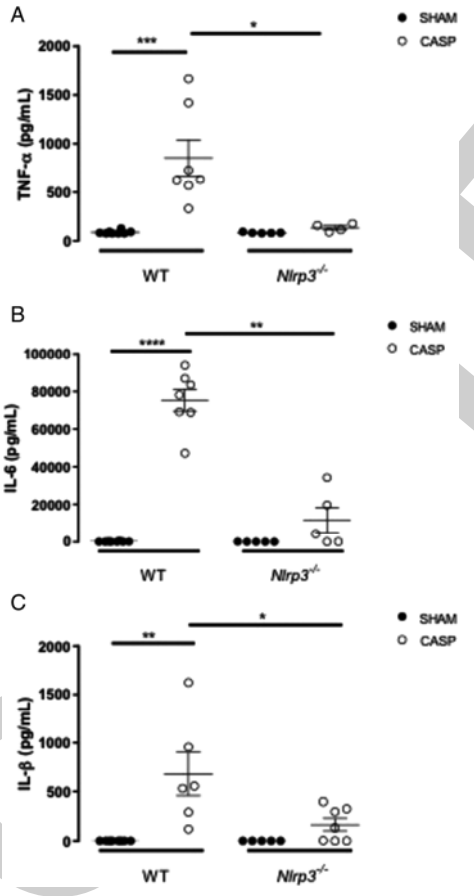

Figure 6. Cardiac function of *Nlrp3*$^{-/-}$ mice respond similarly to high-grade models of sepsis CLP and CASP. A) Left ventricle ejection fraction (EF) and fractional shortening (FS); B) RR and QJ interval duration and C) IL-1β, TNF and IL-6 plasma levels of WT and *Nlrp3*$^{-/-}$ 18 h after sham, CASP, or CLP. * P < 0.05, ** P < 0.01, *** P < 0.001,

P = 0.06.