Lipopolysaccharide Induced Activation of Toll Like Receptor 4 in Isolated Rat Heart Suggests a Local Immune Response in Myocardium

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ABSTRACT

Background: Myocardial dysfunction is one of the major complications in patients with sepsis where there is a relationship between the blood level of cytokines and the onset of myocardial depression. In many cases of sepsis, the presence of Lipopolysaccharide (LPS) has been established. LPS Binding Protein (LBP) bound endotoxin is recognized by CD14/toll-like receptor-4 (TLR4) complexes in innate immune cells which stimulates TNF-α release. **Objectives:** To investigate whether isolated rat heart is capable of producing TNF-α locally through TLR4 activation by LPS. **Methods:** Using langendorff method, LPS in 120 mL of the modified Krebs-Henseleit buffer solution (KHBS) at final concentration of 1 μg/mL was perfused at recycling mode. The effect of LPS on cardiac function was evaluated. To assess the TLR4 activity and TNF-α release, western blotting, real time-PCR, and ELISA were used. **Results:** Compared with control, coronary perfusion pressure (CPP) as well as left ventricular developed pressure (LVDP), maximum and minimum rates of the left ventricular developed pressure (dP/dt_{max}; dP/dt_{min}; $p<0.001$) were depressed to a maximum level after 180 minutes recycling with LPS. This myocardial depression was associated with a significant increase in TLR4 expression ($p<0.01$), MyD88 activity ($p<0.01$) and TNF-α ($p<0.05$) concentration in the heart tissue. **Conclusion:** The results of this study show that heart is capable of producing TNF-α through TLR4 and MyD88 activation independent of classic immune system and suggest a local immune response.

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INTRODUCTION

Sepsis induced myocardial dysfunction is one of the major complications in septic patients with mortality rates close to 70% (1). In some studies obvious relationships were reported between the blood level of cytokines and the onset and severity of myocardial failure in septic patients (2). In the recent years, considerable attention on the cellular mechanisms that cause myocardial depression in sepsis has been focused on the role of inflammatory cytokines, especially on the role of TNF-α. However, it is not clear whether the source of these cytokines is exclusively circulatory immune cells or the heart tissue also is involved in their production. In many cases of sepsis, the presence of Lipopolysaccharide (LPS) has been established. The decreased myocardial compliance due to LPS in sepsis seems to depend on the presence of cell wall receptor TLR4 (3) and CD14 (4).

Toll-like receptors (TLRs) are a family of receptors that play a critical role in activation of the innate immune system in response to various molecules produced by microbes (5,6). Innate immune system which is mediated by macrophages and dendritic cells generates the first line of defense principle role in rapid but non-specific response against infection. Thereinafter, a second layer of protection, adaptive immune system is activated to limit infection (5,6). Besides, microbial pathogens (PAMPs) and endogenous signals (DAMPs) which appear after tissue injury such as myocardial infarction also are able to activate the innate immune system through TLRs and lead to detrimental inflammatory response. TLRs activate a number of signaling pathways, among them MyD88 (myeloid differentiation primary response protein) is the critical adaptor protein. The activation of MyD88 promotes the NFκB translocation to the nucleus and induces the gene transcription of proinflammatory cytokines, particularly TNF-α and IL-6 (7). As many as thirteen murine and ten human TLRs have been identified so far. Among them the TLR family members, TLR 2, 3, 4, 5, 6, 7, and 9 are found in cardiomyocytes (8).

Lipopolysaccharide (LPS) is the most important part of the outer membrane of gram-negative bacteria (9). Although, activation of innate immune response by LPS is adaptive at first but its failure to regulate immune responses leads to immunoparalysis and sepsis (10). Immune responses normally protect the host against infection but its dysregulation causes cell and tissue damage and hence multiple organ failure (11). Clinically, the event results in septic shock and depresses myocardial contractility in two distinct phases. In the first phase immediately after receiving LPS, pulmonary hypertensions and systemic hypotension associated with fall in cardiac output are the hall marks. A few hours later, hypotension, tissue hypoperfusion, microvessel injury, and multiple organ injury occur (12). In septic patients reduction in left ventricular ejection fraction goes along with increase in plasma levels of TNF-α (13). During LPS contamination, TLR4 in conjunction with CD14 on circulating cells senses the presence of LPS (10). LPS-binding protein (LBP), which is a serum protein, along with LPS receptors have a prominent role in the activation of TLRs by LPS. The macrophage scavenger receptor, CD14, is the main LPS receptor (12). Of these, association of LPS with CD14 is facilitated by LBP (14). CD14 has a pivotal role in the LPS sensing complex (15) and LPS presentation to TLR4 on macrophages and dendritic cells (14) where TLR4 is predominantly expressed (16). The interaction of LPS with macrophages results in the release of pro-inflammatory molecules and so systemic toxicity. Altogether, LPS is recognized by CD14/TLR4 complex in circulatory immune cells and

delivers a signal through adaptor protein of myeloid differential protein 88 (MyD88) (14) which leads to nuclear translocation of NF-κB and then production of TNF-α and IL-6 (14).

As discussed above, to stimulate TLRs by LPS the presence of the intact immune system and blood circulation is critical. However, it is not known whether LPS in the absence of circulating macrophages and serum binding proteins such as LBP may express and stimulate TLRs in the isolated heart. It was shown that administration of LPS to isolated rat hearts stimulates the release of TNF-α (17). Few studies show the presence of extravascular macrophages (cardiomacrophages) in the healthy heart located among myocytes (18,19). Furthermore, TLR4 is expressed on cardiomyocytes as well (8). These observations provide a possible link between TNF-α production and a local immune response in the heart in sepsis induced myocardial depression.

This study is designed to answer whether isolated rat heart, which is detached from the circulatory immune system, is capable of producing TNF-α locally through TLR4 activation by LPS.

MATERIALS AND METHODS

Animals. Healthy male Wistar rats (270 ± 30 g) were used in this study. They were housed in standard polypropylene cages, six per cage, under a 12-hours light/dark at a controlled ambient temperature of 22 ± 2°C with 50 ± 10% relative humidity. The present study was performed in accordance with the Guide for the Care and Use of Laboratory Animals of Tabriz University of Medical Sciences, Tabriz, Iran (National Institutes of Health Publication No. 85-23, revised 1985).

Chemical Reagents. *Escherichia coli* (serotype k235) lipopolysaccharide was purchased from sigma (Germany). Rabbit monoclonal antibody against MyD88 was obtained from Cell Signaling Technology (Danvers, MA). Mouse monoclonal antibodies against GAPDH, peroxidase-conjugated goat anti-rabbit and rabbit anti mouse secondary antibodies were obtained from Abcam (Cambridge, MA). Trizol from Sigma (USA) was used For RNA extraction. For enzyme-linked immunosorbent assay, Rat TNF-α was obtained from Bendermed (Austria). Protease inhibitor cocktail was bought from Roche (Mannheim, Germany). The other reagents were of a commercial analytical grade.

Preparation and Isolated Heart Perfusion. Materials and techniques of preparation, perfusion, and monitoring of physiological parameters have been described previously in detail (20). Briefly, rats were heparinized and anesthetized with kethamin/xylasin. The ribs at the right and left anterior axillary lines were cut to create a clamshell thoracotomy. Harvested heart was transferred immediately to a dish containing ice cold modified Krebs-Henseleit buffer solution (KHBS) and then was cannulated immediately to a langendorff apparatus. The time delay between opening the thorax for harvesting the heart till the heart being completely perfused with buffer by langendorff apparatus was less than 5 minutes to avoid damage or preconditioning (21).

A ML176-V Langendorff Apparatus (AD Instruments; Australia) was used for recycling. The hearts were retrogradely perfused at a constant flow (10 mL/min.g) with a KHBS containing (in mmol/L) NaCl 125, KCl 4.3, KH_2PO_4 1.1, $MgCl_2$. $6H_2O$ 1.3, $CaCl_2$. 2 H_2O 2.4, $NaHCO_3$ 25, and glucose 13.32. The perfusate was gassed with carbogen (5% CO_2/95% O_2). The pH was 7.38-7.56 at 37°C. All hearts during

stabilization period were initially rinsed with 150- 200 mL KHBS in a non-recirculating mode and after that were switched to recirculating mode (total volume of 120 mL). For monitoring coronary perfusion pressure (CPP), the aortic cannula was connected to a pressure transducer (MLT844 physiological pressure, ADInstruments; Australia). To measure left ventricular contractility, a home-made latex balloon attached to a second pressure transducer was inserted into the left ventricular cavity via the mitral valve after removing the atrial appendage. Left ventricular developed pressure (LVDP) was calculated as the difference between peak-systolic and end-diastolic pressure, the maximum and minimum rate of left ventricular pressure (dP/dt_{max}, dp/dt_{min}) as an index of left ventricular contractility and heart rate (HR) were continuously recorded by PowerLab 8/35. At the end of stabilization time, in case with either poor LVDP (was not in range 70-130 mmHg) or prolonged arrhythmia (>3 minutes), the heart was detached and excluded (21).

Experimental Protocols of Isolated Heart Perfusion. After stabilizing, time was set to zero, and hearts were perfused at recycling mode with 120 ml LPS solution at a final concentration of 1 µg/mL. Control hearts recycled by KHBS in the absence of LPS exactly the same as the LPS groups. Hemodynamic variables were monitored during whole period of the experiment.

Western Blot Analysis. Western blot analyses were performed, as previously described by Soraya *et al*. (22) with minor modifications. The tissue sample was homogenized in the ice-cold solution, pH 7.4, containing 50 mM Tris–HCl, 150 mM NaCl, 5 mM Sodium Pyrophosphate (NaPPi), 50 mM NaF, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.1% SDS (w/v), 1% TXT-100 (v/v), and protease inhibitor cocktail. Homogenized sample was centrifuged at 10,000 rpm at 4°C for 10 minutes. The supernatant was aliquoted and stored at -70°C for further analysis. Bradford Protein Assay kit was used to evaluate the protein content of the supernatant. Fifty µg of the homogenate protein was loaded to the SDS-Polyacrylamide gel electrophoresis using Bio-Rad mini protean tetra system (Hercules, CA). The separated proteins were transferred to an Immobilon-P membrane (Millipore, Billerica, MA) and blocked in 5% non-fat milk. The membranes were probed using a range of primary antibodies raised against MyD88 (1:1000 dilution) and GAPDH (1:5000) at 4ºC with gentle shaking, overnight. The membranes then were washed and incubated with peroxidase-conjugated goat anti-rabbit and rabbit anti-mouse secondary antibodies (1:5000 dilutions) at room temperature with gentle shaking. The antibodies were visualized using the BM Chemiluminescence kit (Roche, Mannheim, Germany). Densitometric analysis of the immunoblots was performed using image j software (Wayne Rasband, National Institute of Health, USA). The densitometric values for MyD88 were normalized to GAPDH.

Detection of the TLR-4 mRNA in Cardiac Tissue by Quantitative Real-Time PCR. Myocardium total RNA was extracted using Trizol according to the manufacturer's recommendations. Agarose electrophoresis was used to evaluate the integrity of the extracted RNA. Purity of RNA was determined by optical density measurement (A260/A280 Ratio) with nanodrop instrument (ND 1000, Wilmington, USA). For cDNA synthesis one µg of the extracted RNA from each sample was used. Real time PCR was performed by dNTP mix (Biofluxbiotech), random hexamer primer, Ribonuclease Inhibitor, and M-MuLV Reverse Transcriptase (Cinnagen, Iran). All reactions were performed in a total volume of 25 µl reaction mixtures, triplicate using the iQ5 optical system (Bio-Rad laboratories, Inc, Hercules, and CA). Negative control as well as no template control (NTC) was included in each experiment.

Reaction mixture contained: 1 µl cDNA, 1 µl primer (100nM each primer), 12.5 µl 2X Power SYBR Green PCR Master Mix (Applied Biosystems, Foster city, USA), and 10.5 µl RNase/DNase free water. Target mRNA expression was normalised to GAPDH expression. The cycling conditions were as follow:
For TLR4: 1 cycle at 94°C for 10 min, 40 cycles at 94°C for 15 s, 53°C (annealing temperature) for 30 s and 72°C for 30 s, and 1 cycle at 72°C for 5 s.
For GAPDH: 1 cycle at 94°C for 10 min, 40 cycles at 94°C for 15 s, 60°C (annealing temperature) for 30 s and 72°C for 30 s, and 1 cycle at 72°C for 5 s. The primers were designed for detection, as given below:

For TLR4,
forward: 5′-AAGTTATTGTGGTGGTGTCTAG-3′;
reverse: 5′-GAGGTAGGTGTTTCTGCTAAG-3′.

For GAPDH,
forward: 5′-AAGCTCATTTCCTGGTATGACAACG-3′;
reverse: 5′-TCTTCCTCTTGTGCTCTTGCTGG-3′.

The relative amount of TLR4 mRNA to the reference GAPDH gene was calculated using the $2^{-\Delta\Delta c}{}_{T}$ method (23) and expressed as an n-fold variation of TLR4 levels in the treated samples with respect to control samples (n=6).
Measurements of Cardiac TNF-α Concentration by ELISA. Cardiac concentration of TNF-α was quantified with the use of enzyme-linked immunosorbent assay (ELISA) kit according to the manufacture's instruction. Briefly, cardiac tissue samples were homogenized in lysis buffer. Samples were centrifuged twice at 10,000 rpm for 10 minutes at 4°C. The resulting supernatants were used for assay and concentration of the cytokines was expressed as pg/100 mg cardiac tissue.
Statistical Analysis. Data are presented as mean ± SEM. For hemodynamic analysis, one way ANOVA was used to make comparisons among the groups. If the ANOVA analysis indicated significant differences, LSD *post hoc* test was performed to compare the mean values between the treatment groups and the control group. For the real time PCR, Pair Wise Fixed Reallocation Randomization Test using REST software was used to make comparisons between the groups. In the other cases, two independent samples *t-test* was performed to make comparisons. Differences between groups were considered significant if $p<0.05$.
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RESULTS

Effects of LPS on Hemodynamic Parameters in the Isolated Rat Heart. Perfusion of heart with 120 mL total volume of KHBS enriched by LPS (1µg/mL) in recycling mode was started 15 minutes after stabilizing. As demonstrated in Figure 1 perfusion of isolated hearts with KHBS containing LPS (1µg/mL) gradually elevated CPP which reached a significant level after 120, 150 ($p<0.05$), and 180 minutes ($p<0.001$).

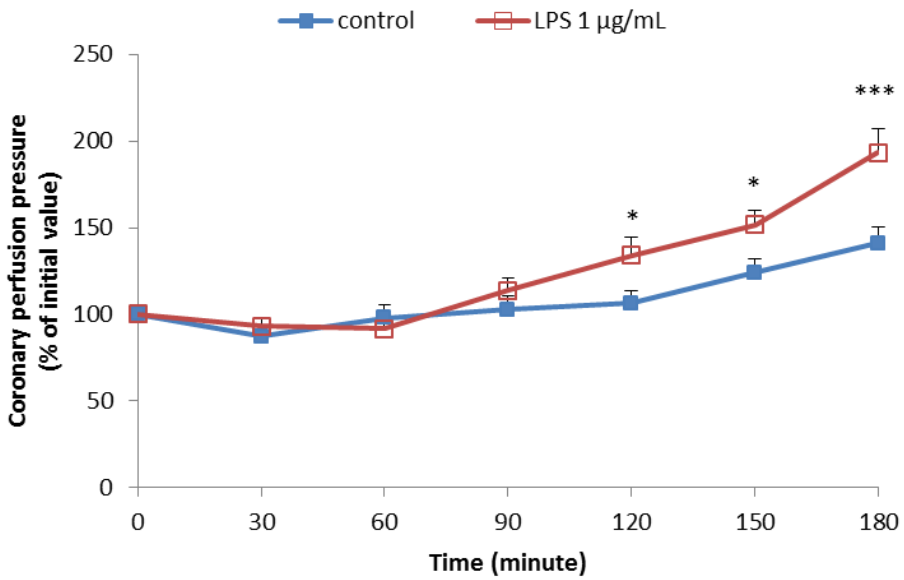

Figure 1. Coronary perfusion pressure (CPP) in the isolated rat hearts perfused with Krebs-Henseleit buffer enriched with lipopolysaccharide (LPS; 1 µg/mL; open square) or without LPS (control; solid squire) in recycling mode. n=14. Values are mean ± SEM. $^{*}p< 0.05$ and $^{***}p<0.001$ vs. control using one way ANOVA with LSD *post hoc* test.

Developed pressure was also significantly decreased by LPS by 26.24% ($p<0.01$) after 150 minutes and 30.66% ($p<0.001$) after 180 minutes (Figure 2).

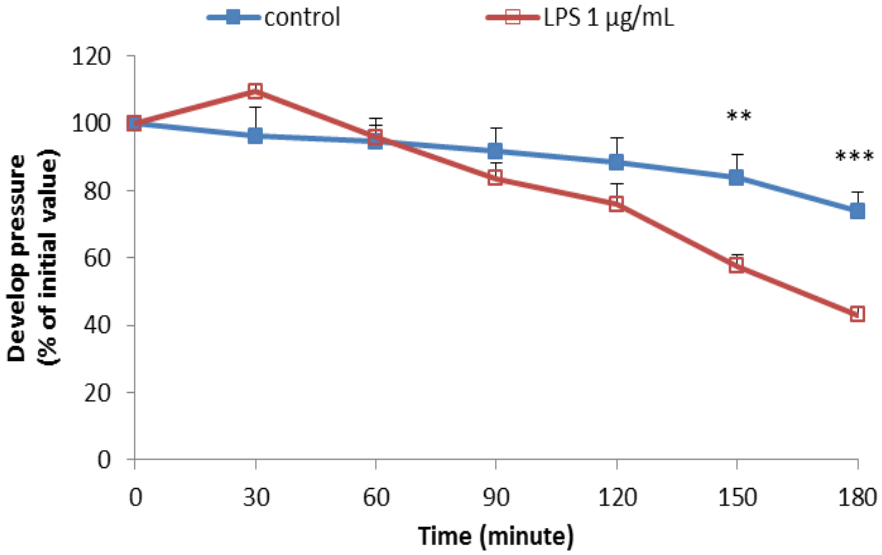

Figure 2. Left ventricular developed pressure in the control group and in the isolated hearts treated with LPS (1 µg/mL) in recycling mode. Control: modified Krebs-Henseleit buffer solution (solid square; n=14); LPS: lipopolysaccharide (open square; n=14). Values are mean ± SEM. $^{**}p< 0.01$ and $^{***}p<0.001$ vs. control using one way ANOVA with LSD *post hoc* test.

Besides, administration of LPS caused a delayed significant depression in the left venricular pressure. When compared with the control group, after 150 minutes LV dP/dt_{min} ($p<0.05$) and both LV dP/dt_{max} and LV dP/dt_{min} after 180 minutes were depressed by LPS perfusion ($p<0.05$ and $p< 0.001$, respectively; Figure 3). LPS had no significant effect on the HR during perfusion period (data are not shown).

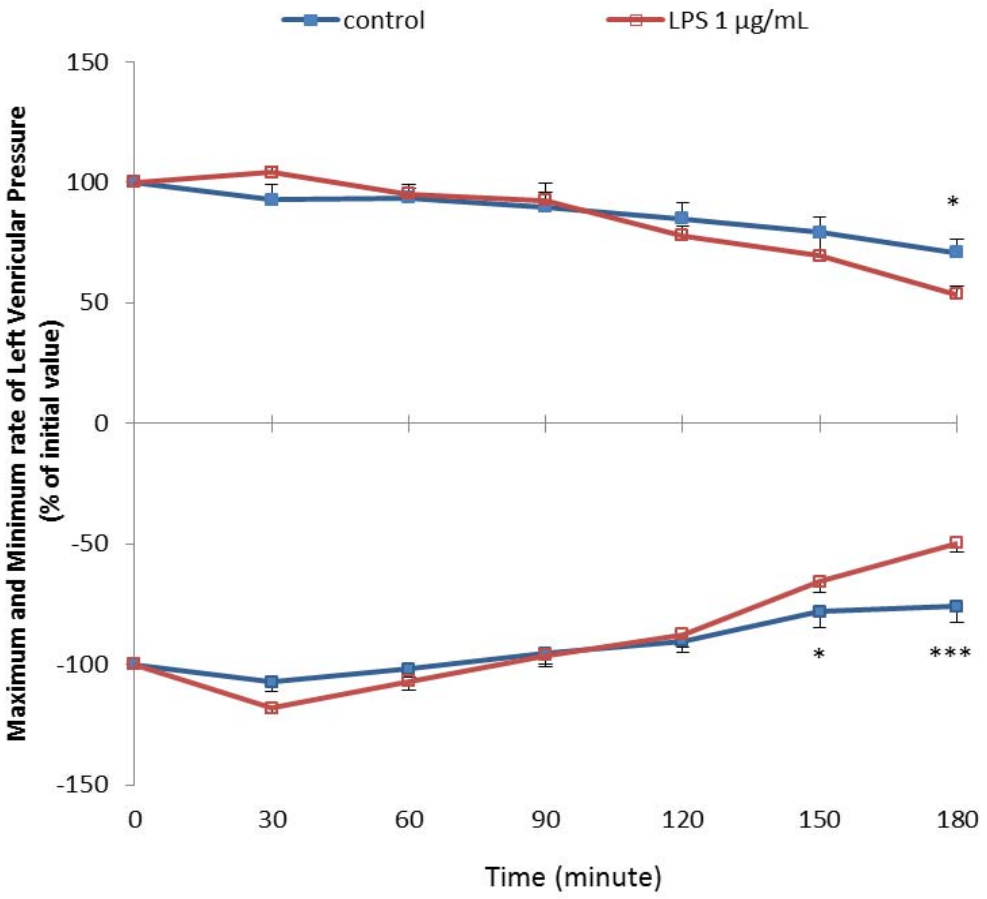

Figure 3. Maximal rates of positive and negative changes in LV pressure (LV dP/dt_{max}; LV dP/dt_{min}) in the control and in the isolated heart treated with LPS (1µg/mL) in recycling mode. Control: modified Krebs-Henseleit buffer solution (solid square; n=14); LPS: lipopolysaccharide (open square; n=14). Values are mean ± SEM. *$p< 0.05$ and ***$p<0.01$ vs. control using one way ANOVA with LSD *post hoc* test.

Effects of LPS on TLR4 Expression in Isolated Rat Heart. To determine the effect of LPS on the TLR4 activity in isolated heart, TLR4 mRNA content in myocardium was assessed at the time that LPS caused significant changes in the hemodynamic parameters. Real-time quantitative PCR showed a remarkable upregulation in the level of TLR4 mRNA in the heart tissue ($p<0.01$) by perfusion of 1µg/mL LPS for 180 minutes (Figure 4).

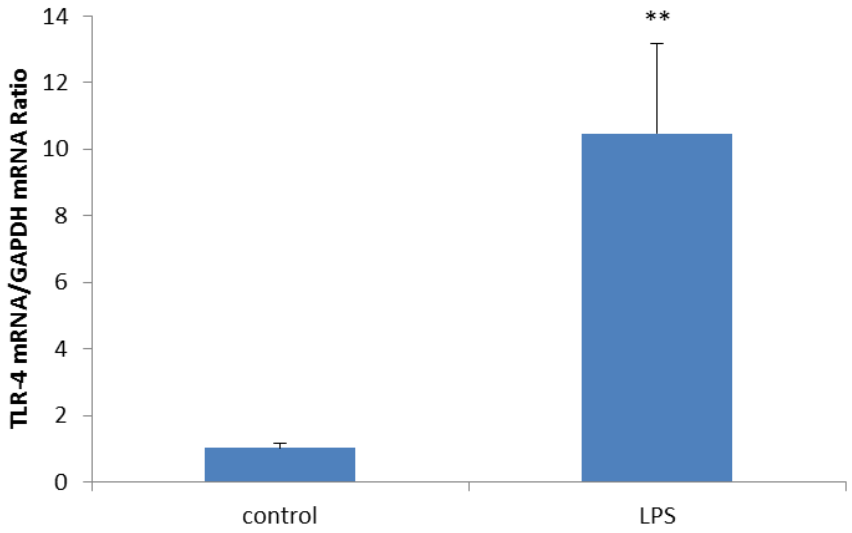

Figure 4. The effect of LPS (1 µg/mL) on TLR4 mRNA expression in isolated rat heart. Values are mean ± SEM (n=6) for the ratio of TLR4 mRNA to GAPDH in the control group (KHBS) and in LPS treated group. The significant of results was tested by a Pair Wise Fixed Reallocation Randomization Test using REST software. $^{**}p< 0.01$ vs. control.

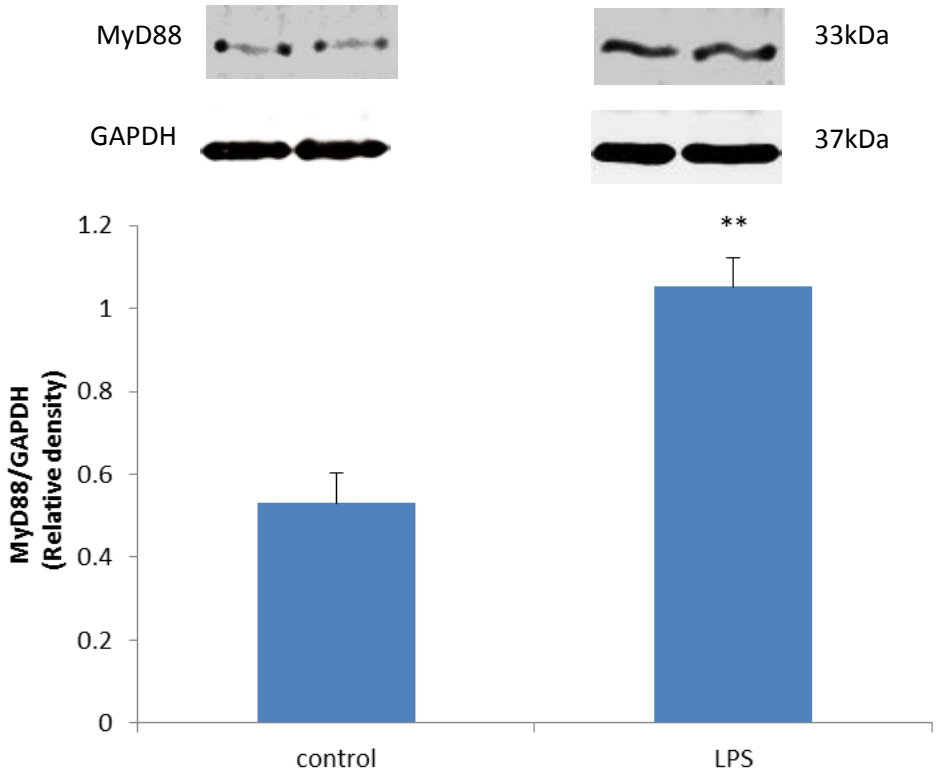

Figure 5. Representative immunoblots of MyD88 protein content following perfusion of the isolated heart in a recycling mode with (LPS) or without LPS (control). Bars represent the ratio of MyD88 to GAPDH. Control: modified Krebs-Henseleit buffer solution (n=6); LPS: lipopolysaccharide (n=6). Values are mean ± SEM. Two independent samples *t-test* was used to make comparisons between the groups. $^{**}p< 0.01$ vs. control.

Effects of LPS on MyD88 Protein Expression in Isolated Rat Heart. MyD88 is a general adaptor protein which is critical for many toll-like receptors activities. To determine whether the elevation of TLR4 mRNA in the isolated rat heart is associated with the increase in the level of MyD88 protein, the content of MyD88 was measured in the heart tissue after LPS perfusion. Perfusion of heart with LPS at 1 µg/mL caused a considerable increase ($p<0.01$) in the level of MyD88 protein (Figure 5).

Effect of LPS on the TNF-α Concentration in Isolated Rat Heart. To find out whether TLR4 activation by LPS and then stimulation of NF-κB transcription factor by MyD88 may lead to increases in the heart tissue concentration of TNF-α, the level of TNF-α in the myocardium was assessed. As demonstrated in Figure 6. perfusion of the isolated rat heart by LPS at 1 µg/mL in recycling mode significantly raised the TNF-α concentration in the heart tissue compared to control group ($p<0.05$).

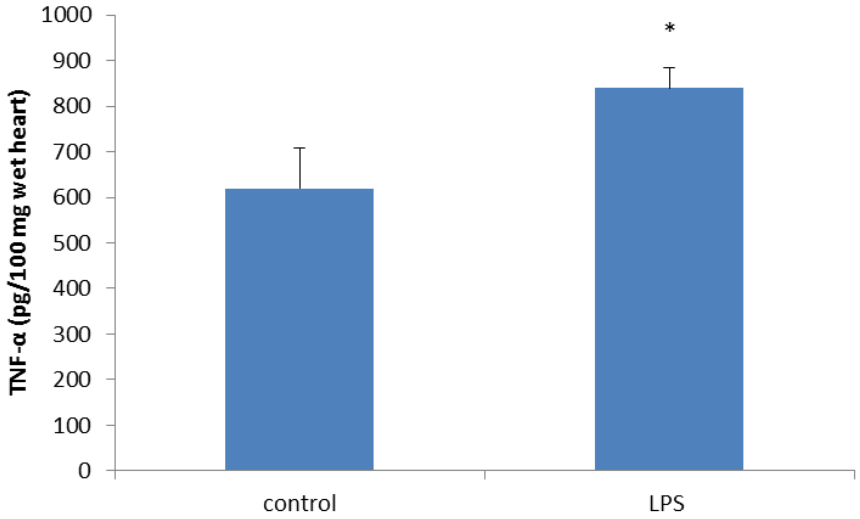

Figure 6. The effect of LPS (1 µg/mL) on the concentration of TNFα in isolated rat heart. Values are mean ± SEM for 6 hearts in each group. Control: modified Krebs-Henseleit buffer Solution; LPS: lipopolysaccharide. Two independent samples *t-test* was used to make comparison between the groups. *p< 0.05 vs. control.

DISCUSSION

Until recently little attention has been paid to the local immune response in the heart. In the present study we showed for the first time that LPS induced myocardial dysfunction was associated with TLR4 activity and TNF-α production in the isolated rat heart where the circulatory immune system was absent. TLRs participate in the first line of defense against invading pathogens and damage-associated molecular pattern molecules (DAMPs) and play a critical role in the innate immune response and the resulting inflammation outcome in the heart (7,8). Inflammatory responses in the heart have a dual role.

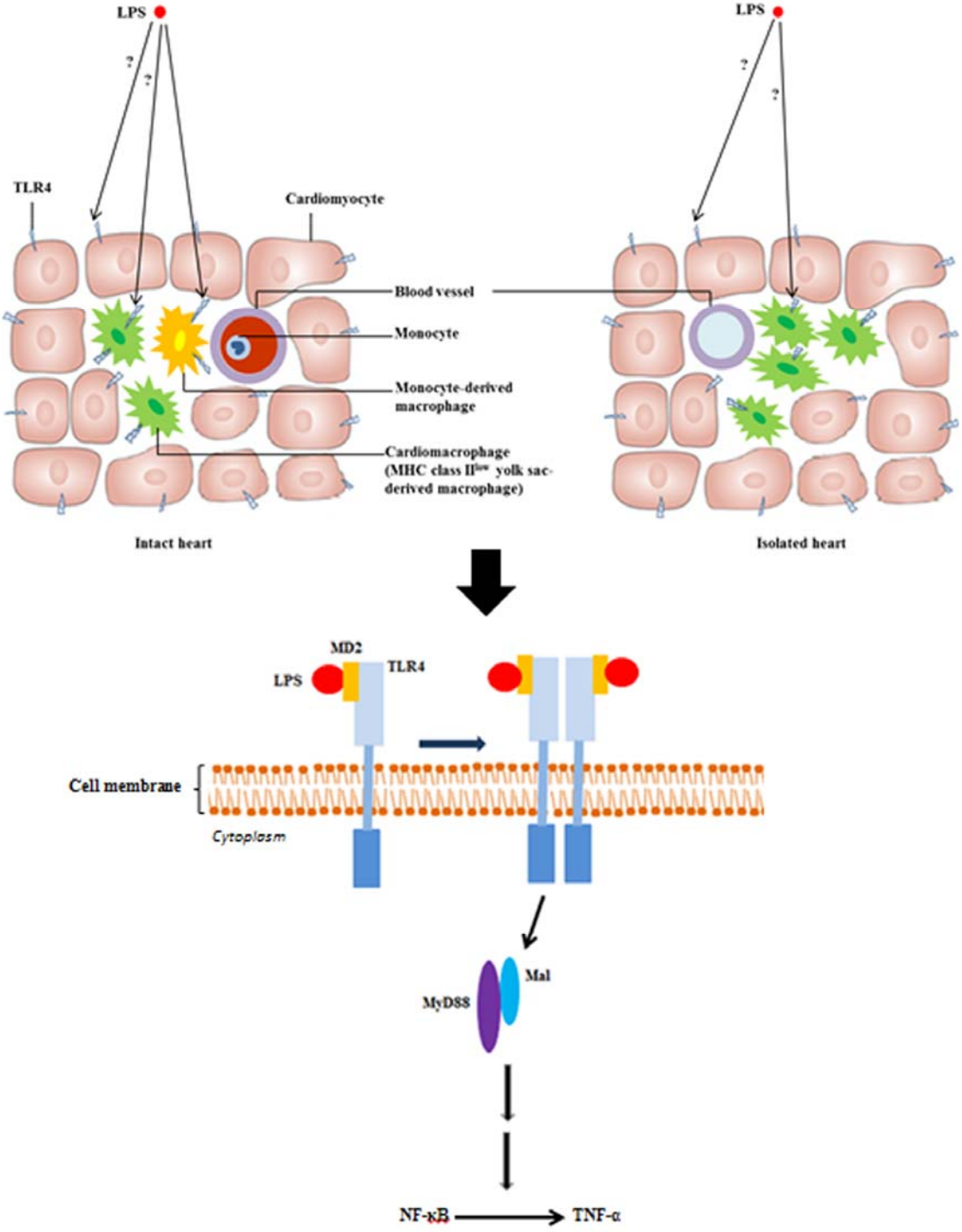

Figure 7. Schematic diagram showing the possible signaling pathways leading to LPS-induced myocardial dysfunction in intact heart tissue in the presence of circulatory immune cells (Left, via presented TLR-4 on the surface of monocyte-derived macrophages, cardiomyocytes or cardiomacrophages) and in isolated heart tissue when the circulatory immune system is absent (Right, via presented TLR-4 on the surface of cardiomyocytes or cardiomacrophages). In this way, TLR-4 activation and therefore MyD88 up-regulation raises the TNF-α concentration.

At the beginning, the inflammatory reactions clear the dead cells and matrix debris, while the exaggeration, prolongation, or expansion of the inflammatory responses results in worse remodeling and dysfunction. As a part of the innate immune system, macrophages provide a first line of defense during infection and injury. TLRs are membrane bound receptors mainly expressed in sentinel cells such as macrophages and dendritic cells that recognize specific microbial components derived from pathogens including bacteria, fungi, protozoa and viruses. Cardiac effects of LPS in septic patients have an utmost importance and many of the cardio-depressive effects of LPS in sepsis are induced by TLR4 mediated production of cytokines especially TNF-α (24). Of course, during inflammation most macrophages are derived from inflammatory monocytes that are infiltrated into the site of inflammation from the blood pool (Figure 7). These monocytes are the progeny of haematopoietic progenitors which are residing in the bone marrow. Haematopoietic stem cells (HSCs) express receptors such as TLRs to directly sense circulating danger signals (25,26). However, it is currently known that non-bone marrow-derived cells also take part in inflammatory cascade through TLR signaling.

Several types of parenchymal cells, including cardiomyocytes express TLRs (31). TLR 1, 2, 3, 4, 5, and 6 are expressed in the endothelial and smooth muscle cells and TLR 2-6 are found in cardiomyocytes (4). Besides, the myocardium hosts a significant number of extravascular macrophages (18,19). It is not known whether these cardiomacrophages are renewed independently of circulated monocytes. We believe that cardiac resident macrophages, considered as a local immune system, deserve attention because they contribute to cardiac inflammatory responses and play a potential role in regulating cardiac regeneration and remodeling. Cardiac tissue macrophages (cTMs) display typical macrophage characteristics and express a wide array of pattern recognition receptors such as CD14, CD64 and TLRs that enable rapid responses to a range of DAMPs (27).

In an *in vivo* study we have demonstrated that TLR4 expression and activity in rat hearts and subsequent increase in tissue and serum level of inflammatory cytokines are strongly associated with post myocardial infarction (MI) failures (22,28). Meanwhile we showed that activation of AMPK, a key sensor of cellular AMP:ATP ratio, by metformin prevents cardiac inflammatory responses after myocardial infarction by suppression of TLR4/MyD88 activity (22,28). It has been reported that TLR4 deficient mice have reduced myocardial injury after ischemia-reperfusion (29) and had reduced hypertrophy in pressure overload and MI models (30). In a study on cardiomyocytes, Singh *et al.* (20,12) suggested that TLR/MyD88 pathway is important for adverse responses to MI and contributes to clinically important pro-inflammatory and pro-oxidant responses after MI (30). Recently, we have reported that monocytic expressions of human TLR4 (hTLR4) as well as the serum levels of pro-inflammatory cytokines are positively correlated with the degree of coronary luminal stenosis in patients with stable angina (31). TLRs are the first line of immune system at investigation of pathogenous micro-organisms. Also they play an important role in many pathophysiological processes in human disease that do not involve pathogens (8,32).

The present study showed that LPS is capable of stimulation and activation of TLRs in isolated rat hearts which are detached from the circulatory immune system. To explore the role of TLRs in the heart tissue, in the absence of any blood components, the present study determined the responses of isolated rat hearts to Krebs solution enriched with LPS. In the isolated rat hearts, LPS led to a significant myocardial depression following

120 minutes exposure to 1 μg/mL of the polysaccharide. The negative inotropic effect was accompanied with rise in coronary perfusion pressure. LPS incited a marked release of the vasoconstrictor thromboxane into the coronary bed (24) which may cause heart stiffness and therefore cardiac depressant effects. Further, the myocardial depression under LPS perfusion was associated with a significant increase in TLR4 expression and MyD88 activity as well as TNF-α release from the heart tissue. The finding of TNF-α secretion from the myocardium in response to TLR4 activation by LPS, indicates that this cytokine, at least in part directly originates from the heart tissue in sepsis and suggests a local immune response in the heart tissue. We speculate that cardiomyocytes or cardiomacrophages may be involved in the myocardial TLR4 activation and TNF-α production and therefore in the genesis of LPS induced myocardial dysfunction (Figure 7). Taking all together, the results of the present study demonstrated that LPS induces myocardial dysfunction in isolated rat hearts in the absence of circulatory immune cells and this cardiodepression is associated with the TLR4 and MyD88 up regulation and TNF-α release. TLR4 expression and the production of TNF-α in isolated rat hearts suggests that a local innate immune response may contribute to cytokine mediated myocardial dysfunction in sepsis.

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