Original Article Gender disparity in the role of TLR2 in post-ischemic myocardial inflammation and injury

Jilin Li^{1,2}, Lihua Ao¹, Yufeng Zhai¹, Joseph C Cleveland Jr¹, David A Fullerton¹, Xianzhong Meng^{1,2}

¹Department of Surgery, University of Colorado Denver, Aurora, Colorado 80045, USA; ²Division of Cardiology, The First Affiliated Hospital, Shantou University Medical College, Shantou 515041, China

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Abstract: It is unclear whether Toll-like receptor (TLR) 2 plays a role in post-ischemic myocardial inflammatory response and cardiac dysfunction in both males and females. Permanent ischemia was induced in male and female C57BL/6J (wild-type, WT) and TLR2 knockout (KO) mice. Infarct size and left ventricular (LV) function were analyzed at day 7. Myocardial levels of monocyte chemoattractant protein-1 (MCP-1) and intercellular adhesion molecule-1 (ICAM-1), as well as neutrophil infiltration, were assessed at day 3, and mononuclear cell accumulation was determined at day 7. Lower MCP-1 and ICAM-1 levels, and reduced leukocyte accumulation correlated with smaller infarct size and improved LV function in male TLR2 KO mice. Female WT mice exhibited attenuated myocardial inflammatory response and injury, and TLR2 KO in females did not provide a protective effect although myocardial TLR2 levels in female WT mice were unaltered, and their cardiac cells responded to bacterial TLR2 agonist properly. TLR2 KO in male mice reduced post-ischemic myocardial inflammatory response, resulting in smaller infarct sizes and improved cardiac function. However, TLR2 KO was not beneficial in female mice. The gender disparity in the role of TLR2 in post-ischemic myocardial inflammatory response and myocardial inflammatory response and myocardial inflammatory response may have therapeutic potentials only in males.

Keywords: Gender disparity, TLR2, TLR4, myocardial inflammatory response, LV remodeling, heart failure

Introduction

Gender difference in cardiovascular diseases is recognized in humans [1]. Women are known to benefit from the cardioprotective actions of female sex hormones that discontinue with the onset of menopause [2, 3]. This gender difference in cardiovascular conditions is also demonstrated in animal studies. Several reports confirmed that female mice have better cardiac function after myocardial infarction (MI) that is associated with attenuated early inflammation, improved repair response and attenuated adverse left ventricular (LV) remodeling [4-6]. Furthermore, female mice exhibit myocardial resistance to tumor necrosis factor receptor (TNFR) 1 signaling during ischemia [7]. In addition, female sex hormones appear to be antiinflammatory and are responsible for cardiovascular protection in females [8, 9].

Toll-like receptors (TLRs) are innate immune receptors that sense the presence of pathogen-associated molecular patterns. Accumulat-

ed evidence demonstrates that TLR2 and TLR4 play important roles in the signaling mechanisms that contribute to post-ischemic myocardial inflammatory response and myocardial injury [10, 11]. Several studies found that TLR2 and TLR4 also respond to endogenous proteins. such as heat shock proteins (HSP) and high mobility group box 1 (HMGB1), released from stressed and/or injured cells [12-16]. TLR2deficient mice are protected against post-ischemic coronary endothelial dysfunction [17]. In addition, reduced myocardial fibrosis in TLR2 knockout (KO) mice after myocardial ischemia indicates TLR2 is involved in maladaptive LV remodeling [18]. Thus, TLR2 appears to play a role in post-ischemic myocardial injury and cardiac dysfunction. However, these previous studies determined the role of TLR2 only in male mice. It is unknown whether TLR2 has a similar role in females.

Sex hormones are important modifiers of the tissue and cellular inflammatory response to injury. Several studies indicate the interaction

of female sex hormones with TLRs. Lipopolysaccharides (LPS)-induced inflammation in murine airway is reduced in female, but is exaggerated in females administered testosterone [19]. In addition, 17beta-estradiol inhibits IL-8 release induced by TLR agonists via ERbeta in cystic fibrosis bronchial epithelial cells [20]. However, removal of endogenous estrogens has been shown to decrease the production of both pro- and anti-inflammatory cytokines, with a concomitant reduction in cell surface expression of TLR4 in macrophages [21]. Similarly, testosterone is found to reduce macrophage expression of TLR4 [22]. Although these studies suggest that sex hormones could alter TLR expression and the TLR-mediated inflammatory responses, it remains unknown whether the TLR2-mediated myocardial inflammatory responses to ischemia are gender-dependent.

Using a permanent ischemia model, the purposes of this study were to determine 1) whether TLR2 knockout (KO) reduces the myocardial inflammatory response in both male and female mice, 2) whether TLR2 KO attenuates myocardial injury and improves cardiac function in the late phase of ischemia, and 3) whether myocardial TLR2 levels are altered in female mice.

Material and methods

Animals

Male and female C57 BL/6J wild-type (WT) and TLR2 KO mice were purchased from Jackson Laboratory (Bar Harbor, MA). Mice were acclimated in a quarantine room for 2 weeks before experiments, and maintained on a standard pellet diet. Their age ranged from 12 to 14 weeks, and body weight was 26.3 ± 1.12 g in males and 22.9 ± 0.81 g in females when used for the experiments. All experiments were approved by the Animal Care and Research Committee of University of Colorado Denver, and this investigation conforms to *The Guide for the Care and Use of Laboratory Animals* (National Research Council, revised 1996).

Chemicals and reagents

Staphylococcus aureus peptidalglycan (PGN) was purchased from Sigma-Aldrich (St. Louis, MO). Rabbit anti-mouse intercellular adhesion molecule (ICAM)-1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-mouse HSP25 was purchased from Enzo Life Science (Plymouth Meeting, PA). Rabbit anti-mouse TLR2 and rabbit anti-mouse β -actin were purchased from Cell Signaling (Boston, MA). Goat anti-mouse MyD88 and mouse monocyte chemoattractant protein (MCP)-1 Enzyme-linked immunosorbent assay (ELISA) kit were purchased from R&D Systems (Minneapolis, MN). All other chemical and reagents were purchased from Sigma Chemical Co (St Louis, MO).

Induction of myocardial infarction

Myocardial infarction was induced by coronary ligation in male WT, male TLR2 KO, female WT and female TLR2 KO mice. Animals were anesthetized by intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg). When animals became unconscious, they were placed on a heating pad and their body temperature was kept at 37°C. The trachea was intubated through oral route with a cannula sized either 22 G or 20 G according to the body weight of animal. Animals were ventilated with a rodent ventilator at a rate of 130 breaths/min and a tidal volume of 0.012 ml/g body weight, using 100% oxygen.

A left thoracotomy was performed to expose the heart. The major anterior descending branch of the left coronary artery was ligated with a 8-0 silk suture at the position of approximately 2.0 mm distal to the lower edge of the left atrium. Sham operation was performed without ligating the left coronary artery.

Hearts were collected at the end of the experiments. Apex was separated for myocardial for analysis of ICAM-1 and MCP-1 levels, and the middle portion between apex and the suture was used for histology analysis.

Measurement of LV function

LV function was determined using a pressurevolume catheter (Millar Instruments, Houston, TX) as previously described [23]. Mice were anesthetized and anticoagulated with pentobarbital (60 mg/kg, i.p.) and heparin (1,000 U/ kg, i.p.). The right carotid artery was cannulated and a microcatheter (1F) inserted until a ventricular waveform was obtained. The pressure-volume loops were recorded using the MPVS-400 System with the aid of PVAN software. Pressure-volume loops were recorded at steady-state for the following parameters: heart rate (HR), end-diastole volume (EDV), end-systolic volume (ESV), stroke volume (SV), end-diastole pressure (EDP), end-systolic pressure (ESP), ejection fraction (EF) and cardiac output (CO). Six to ten pressure-volume loops during inferior vena cava occlusion were sampled and used to analyze maximum elasticity (Emax), which is the slope of the end-systolic pressure-volume relationship independent of changes in preload and afterload.

Histological analysis of infarction size

Hearts were embedded in freezing medium and frozen in dry ice. Heart specimens were sectioned along the short axis into 6.0-µm thick section and stained for hematoxylin-eosin (H.E.). Photos were taken under a low power microscope. The sizes of infarction were assessed by the NIH ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda, MD) and expressed as infarct% [(infarct area/LV area) ×100%].

Isolation, culture and treatment of mouse coronary vascular endothelial cells

Mouse cardiac microvascular endothelial cells (MCVECs) were isolated using a previously reported method [24] with modification [25]. Briefly, hearts were briefly dipped into 70% ethanol to devitalize epicardial mesothelial cells and endocardial endothelial cells. Ventricular tissue was minced into approximately 1.0 mm³ pieces, and digested at 37°C for 10 min in 2.0 ml of norminally calcium-free Hank's balanced salt solution (HBSS) supplemented with collagenase II (1.0 g/I), glucose (2.0 g/I), taurine (2.5 g/l), bovine serum albumin (BSA, 0.1%), and MgCl₂ (1.4 mM). Then, the tissue pellet was re-suspended in a second digestion solution containing 0.125% trypsin, 0.1 mM EDTA and 2.0 g/l glucose dissolved in HBSS, and incubated at 37°C for 10 min with shearing by pipetting once every 3 min. At the end of this digestion, the supernatant was transferred into a 15 ml falcon tube containing 1.0 ml of FBS, and cells were separated from tissue debris and remaining myocytes by spinning at 500 rpm for 5 min. The supernatant was centrifuged at 1,200 rpm (4°C) for 8 min to collect endothelial cells. The cells were resuspended in 10 ml Dulbecco's modified Eagle's medium (DMEM, Mediatech, Mannassas, VA) supplemented with 20% FBS, penicillin (50,000 U/I) and streptomycin (50 g/I).

Cells were seeded in 500 μ I DMEM in 24-well plates. After growing to confluent, PGN (10 μ g/ml) was added to the cells. After treatment for 24 h, MCAECs were washed three times with cold PBS, and then lysed with lysis buffer (protease inhibitor cocktail and Mammalian Protein Extraction Reagent, Thermo Scientific, Waltham, MA).

Immunoblotting

TLR2, MyD88, TLR4 and ICAM-1 levels in myocardial homogenate were analyzed by immunoblotting as previously described [26]. Myocardial homogenate was mixed with an equal volume of sample buffer (100 mM Tris-HCL (pH 6.8), 2% SDS, 0.02% bromophenol blue and 10% glycerol). Crude myocardial protein (10 µg) and MCVECs protein were fractioned on 4-20% acrylamide gradient gels (Bio-Rad, Hercules, CA) and transferred onto nitrocellulose membranes. Membranes were blocked for 1 h at room temperature with 5% skim milk in TPBS (PBS containing 0.1% Tween 20), and then incubated with the appropriate primary antibodies diluted with TPBS containing 5% skim milk (TLR2 antibody 1:200, MyD88 antibody 1:500, TLR4 antibody 1:400, β-actin antibody 1:1000, ICAM-1 antibody 1:200 and HSP25 antibody 1:1000) overnight at 4°C. After washing with TPBS, membranes were incubated with horseradish peroxidase (HRP)-linked secondary antibodies (1:5000 dilution with TPBS containing 5% skim milk) at room temperature for 1 h. Bands were developed using enhanced chemiluminescence (ECL, Thermo Scientific, Rockford, IL) and exposed on Fuji X-ray films. Band density was analyzed using the NIH ImageJ software.

MCP-1 assay

Enzyme-linked immunosorbent assay (ELISA) kit was used to analyze MCP-1 in myocardial homogenates. Myocardial homogenate was prepared in 10 volumes of homogenate buffer [0.1 M sodium phosphate (pH 7.5) containing 0.1% Triton ×100, 2 mM EGTA, 1.0 mM benzamidine and 1.0 mM phenylmethylsulfonylfluoride] and centrifuged at 1,000× g for 15 min at



Figure 1. Infarct size at 7 days after coronary ligation is reduced in male TLR2 KO mice, but not in female TLR2 KO mice. Representative histological images and semi-quantitative data show that infarct size was reduced in male TLR2 KO mice compared to male WT mice. Female WT mice had smaller infarct sizes than male WT mice. However, female TLR2 KO mice did not exhibit further reduction of infarct size. Results are expressed as Mean \pm SEM; n=7; **P*<0.05 vs. male WT mice; #*P*<0.05 vs. female TLR2 KO mice.

4°C with an Eppendorf centrifuge (model 5417R, Brinkmann Instruments, Westbury, NY). Supernatant was collected, and protein concentrations were analyzed before analysis of MCP-1. Recombinant murine MCP-1 was used to construct standard curves. Absorbance of standards and samples was determined spectrophotometrically at 450 nm using a microplate reader (Bio-Rad, Hercules, CA). Results were plotted against the linear portion of a standard curve.

Immunofluorescent staining

Immunofluorescent staining was performed as previously described to examine polymorpho-

nuclear leukocytes (PMNs) and mononuclear cells in infracted area [12]. Myocardial sections were fixed in 4% paraformaldehyde, incubated with a rat monoclonal antibody against mouse PMN (clone 7/4, ABD Serotec, Oxford, UK) or a rabbit polyclonal antibody against CD68 (a specific marker for monocytes and macrophages), and then incubated with Cy3-tagged secondary goat anti-rat IgG or goat anti-rabbit IgG (imaged in the red channel). Nuclei were stained with bis-benzimide (DAPI, imaged in the blue channel), and glycoproteins on cell surfaces with Alexa 488-tagged wheat germ agglutinin (imaged in the green channel). Microscopy was performed with a Leica DMRXA digital microscope (Leica Mikroskopie und Systeme GmbH, Wetzlar, Germany). Under ×400 magnifications, the densities of PMNs and macrophages in the infracted zones were determined manually from 5 randomly selected areas.

Statistical analysis

Data are expressed as mean ± standard error

(SEM). Analysis of variance (ANOVA) was performed, and differences were considered significant when P<0.05, as verified by Fisher post-hoc test.

Results

Infarct size is reduced and LV function is improved in male TLR2 KO mice, but not in female TLR2 KO mice

The survival rate is not significantly different between male and female mice (80%, 12 out of 15, in male WT mice; 86% 12 out of 14, in female WT mice; 92%, 12 out of 13, in male TLR2 KO mice and 86%, 12 out of 14, in female TLR2 KO mice). The main causes of mortality

	Male			Female		
Group/Parameter	Sham	WT	TLR2 KO	Sham	WT	TLR2 KO
		Ischemia	Ischemia		Ischemia	Ischemia
HR, beat/min	528 ± 15.3	531 ± 10.3	514 ± 8.15	522 ± 6.25	535 ± 9.54	531 ± 12.8
ESV, μΙ	14.5 ± 2.11	28.1 ± 2.36*	21.7 ± 3.08 ^{*,#}	12.2 ± 3.01	22.2 ± 1.37*,#	23.5 ± 1.36
EDV, µl	27.0 ± 5.60	31.8 ± 2.26	30.2 ± 3.34	23.9 ± 6.12	30.0 ± 2.35*	31.9 ± 1.33
SV, µl	18.9 ± 2.68	8.13 ± 0.86*	15.4 ± 1.03 ^{*,#}	18.8 ± 1.12	13.2 ± 1.63 ^{*,#}	14.7 ± 1.43
ESP, mmHg	98.5 ± 0.85	51.2 ± 4.76*	88.9 ± 11.7#	90.5 ± 1.52	87.9 ± 6.05#	85.3 ± 4.95
EDP, mmHg	6.31 ± 0.12	13.0 ± 2.77*	10.9 ± 9.03#	6.42 ± 0.53	11.8 ± 3.46*	12.1 ± 4.05
EF, %	46.3 ± 3.68	11.7 ± 1.76*	28.3 ± 3.24 ^{*,#}	48.9 ± 5.54	26.0 ± 0.88 ^{*,#}	26.2 ± 0.55
CO, mI/min	10.0 ± 0.69	4.32 ± 0.20*	7.93 ± 0.64 ^{*,#}	9.84 ± 0.46	7.05 ± 0.43 ^{*,#}	7.77 ± 1.88
Emax, mmHg/µl	12.4 ± 0.71	4.20 ± 0.49*	9.39 ± 1.05 ^{*,#}	11.3 ± 0.52	7.84 ± 0.87 ^{*,#}	8.06 ± 0.45

Table 1. LV function at day 7 after coronary ligation

HR, heart rate; ESV, end-systolic volume; EDV, end-diastolic volume; SV, stroke volume; ESP, end-systolic pressure; EDP, enddiastolic pressure; EF, ejection fraction; CO, cardiac output; Emax, end-systolic elastance; Results are expressed as Mean \pm SEM; n = 7; **P*<0.05 vs. correspondent sham (combined WT and TLR2 KO); #*P*<0.05 vs. male WT ischemia. HR.



included blood loss during surgery and severe arrhythmia immediately after coronary ligation. Out of the 12 survivors in each group, 5 animals were sacrificed at day 3 and 7 for analysis.

We measured the infarct size and LV function at 7 days after coronary ligation. In male WT mice, infarct size was $50.01 \pm 1.82\%$ of ischemic myocardium, and it was significantly smaller in male TLR2 KO mice ($33.06 \pm 5.48\%$; P<0.05 vs. male WT, **Figure 1**). Infarct size in female WT mice was $39.12 \pm 2.10\%$ (P<0.05 vs. male WT), and there was no difference in infarct size



Figure 2. Hearts of female mice express functional TLR2. A. Myocardial homogenates from male and female WT mice were analyzed by immunoblotting for TLR2, MyD88 and TLR4 levels. Myocardial levels of TLR2 and MyD88 were comparable between males and females. Similarly, there is no difference in myocardial TLR4 levels between male and female mice. B. Cardiac microvascular endothelial cells from male and female WT mice were stimulated with a TLR2 agonist (PGN, 10 μ g/ml) for 24 h. ICAM-1 levels were analyzed by immunoblotting. A representative immunoblot of two separate experiments shows that cells from female hearts had an intact ICAM-1 response to a bacterial TLR2 agonist. Ctrl = untreated control.

between female WT and female TLR2 KO mice $(41.43 \pm 5.73, Figure 1)$.

Selected LV function parameters were shown in **Table 1**. Since sham-operated WT and TLR2 KO mice had no difference in LV function, sham data were combined. Male TLR2 KO mice exhibited improved LV function and ventricular elasticity in comparison to male WT mice. While female WT mice exhibited improved LV function in comparison to male WT mice, TLR2 KO in females did not further improve LV function. Thus, TLR2 KO reduced infarct size and improved early LV remodeling only in male mice.



Figure 3. TLR2 KO reduces MCP-1 and ICAM-1 expression in ischemic myocardium of male mice. Male and female WT and TLR2 KO mice were subjected to myocardial ischemia for 3 days. MCP-1 levels in ischemic myocardium were assessed by ELISA, and ICAM-1 levels were analyzed by immunoblotting. A. TLR2 KO reduced myocardial MCP-1 levels in male mice. Female WT mice had lower myocardial MCP-1 levels, and TLR2 KO did not affect myocardial MCP-1 levels in female mice. Results are expressed as Mean \pm SEM; n=5; **P*<0.05 vs. male WT mice; #*P*<0.05 vs. female TLR2 KO mice. B. A representative immunoblot of 4 experiments and densitometric data show a reduction in ICAM-1 levels after ischemia in male TLR2 KO mice compared with male WT mice. Female WT mice had lower myocardial ICAM-1 levels. No further reduction was evident in female TLR2 KO mice. Results are expressed as Mean \pm SEM; n=5; **P*<0.05 vs. male WT mice had lower myocardial ICAM-1 levels. No further reduction was evident in female TLR2 KO mice. Results are expressed as Mean \pm SEM; n=5; **P*<0.05 vs. male WT mice had lower myocardial ICAM-1 levels. No further reduction was evident in female TLR2 KO mice. Results are expressed as Mean \pm SEM; n=5; **P*<0.05 vs. male WT mice; #*P*<0.05 vs. female TLR2 KO mice.

There is no gender difference in myocardial TLR2 and MyD88 levels

To determine whether the improved post-ischemic outcomes and the lack of TLR2 KO effect in females are due to altered myocardial TLR2 and/or MyD88 levels, we examined myocardial TLR2 and MyD88 levels in female and male WT mice. In addition, myocardial levels of TLR4 were analyzed in male and female WT mice for contrast. The results in **Figure 2A** show

comparable levels of TLR2, MyD88 and TLR4 in females and males. То determine whether TLR2 is dysfunctional in cardiac cells of female mice, we isolated cardiac microvascular endothelial cells, an important cell type in the inflammatory myocardial response to ischemia, from female and male WT mice and stimulated the cells with a specific TLR2 agonist. A representative immunoblot in Figure 2B shows that cells from female and male mice expressed ICAM-1 in response to TLR2 stimulation and that there was no gender difference in the response to a bacterial TLR2 agonist. Thus, female and male mice have comparable levels of myocardial TLR2 and MyD88, and TLR2 function in cardiac microvascular endothelial cells of female mice is unaltered.

TLR2 KO reduces MCP-1 and ICAM-1 levels in ischemic myocardium of male mice

To determine whether smaller infarct sizes and improved LV function in male TLR2 KO mice and female WT mice are associated with a reduced inflammatory response to ischemia,

we analyzed myocardial levels of MCP-1 and ICAM-1, critical mediators for leukocyte infiltration, at day 3. MCP-1 and ICAM-1 levels increased in ischemic myocardium at 3 days after coronary ligation (**Figure 3A** and **3B**). In comparison to male WT mice, myocardial MCP-1 levels decreased by 61.67% (*P*<0.05), and ICAM-1 levels decreased by 54.39% (*P*<0.05) in male TLR2 KO mice (**Figure 3A** and **3B**). Myocardial MCP-1 and ICAM-1 levels were also lower in female WT mice compared to male



0 male WT male TLR2KO female WT female TLR2KO

Figure 4. TLR2 KO reduces neutrophil infiltration in ischemic myocardium of male mice at day 3. Male and female WT and TLR2 KO mice were subjected to myocardial ischemia. Ischemic myocardium was harvested at day 3 for immunofluorescent staining of neutrophils using a specific antibody against mouse PMN (red), the nuclei were counterstained with *bis*-benzimide (blue) and cellular glycoproteins were stained green with wheat germ agglutinin. Images show reduced neutrophil infiltration in injured myocardium in male TLR2 KO mice compared to male WT mice. While female WT mice had fewer neutrophils in injured myocardium, TLR2 KO in females did not further reduce neutrophil infiltration. Neutrophil counts are expressed as Mean \pm SEM; n=5; **P*<0.05 vs. male WT mice.

WT mice. However, TLR2 KO had no effect on myocardial MCP-1 and ICAM-1 levels in females (Figure 3A and 3B).

TLR2 KO reduces neutrophil and mononuclear cell accumulation in ischemic myocardium of male mice

To determine whether the attenuated myocardial inflammatory response to ischemia in male TLR2 KO mice and female WT mice correlated with reduced leukocyte infiltration, we examined neutrophils in ischemic myocardium at 3 days and mononuclear cells in ischemic myocardium at 7 days after coronary ligation. **Figure 4** shows that neutrophil infiltration was reduced in male TLR2 KO mice and female WT mice. In consistent with the MCP-1 and ICAM-1 results, neutrophil infiltration was also not different between female TLR2 KO mice and female WT mice. As shown in **Figure 5**, mononuclear cell accumulation was reduced in male TLR2 KO mice and female WT mice at 7 days after coronary ligation. TLR2 KO had no effect on mononuclear cell accumulation in female mice.

Discussion

The results show that TLR2 KO reduces infarct size and improves LV function only in male mice. These beneficial effects of TLR2 KO are associated with reduced MCP-1 and ICAM-1 levels. and attenuated neutrophil and mononuclear cell accumulation in the ischemic myocardium. Female WT mice exhibit reduced myocardial inflammatory response and myocardial injury following ischemia. However, TLR2 KO has no beneficial effect in female mice although myocardial TLR2 and MyD88 levels in female WT mice are comparable to those of male WT mice, and coronary vascular endothe-

lial cells from female WT mice respond to bacterial TLR2 agonist properly.

It has been reported that infarct size following myocardial ischemia/reperfusion (I/R) is smaller in male TLR2 deficient mice [17]. The results of the present study confirmed that infarct size is reduced in male TLR2 KO mice at 7 days of permanent ischemia. In addition, we observed that male TLR2 KO mice have significantly improved LV function at 7 days of ischemia. The improvement in LV function could be due to both infarct size reduction and adaptive remodeling. Surprisingly, female TLR2 KO mice did not exhibit either infarct size reduction or LV functional improvement in comparison to female WT mice. Previous studies found that female mice have smaller infarct size after myocardial I/R [27]. Our results show that female WT mice are partly protected against



Figure 5. TLR2 KO reduces mononuclear cell accumulation in ischemic myocardium of male mice at day 7. Male and female WT and TLR2 KO mice were subjected to myocardial ischemia. Ischemic myocardium was harvested at day 7 for immunofluorescent staining of mononuclear cells using a specific antibody against mouse CD68 (red), the nuclei were counterstained with *bis*-benzimide (blue), and cellular glycoproteins were stained green with wheat germ agglutinin. Images show reduced mononuclear cell accumulation in injured myocardium in male TLR2 KO mice compared to male WT mice. Female WT mice had attenuated mononuclear cell accumulation in injured myocardium. However, TLR2 KO in females had no effect on mononuclear cell accumulation. Mononuclear cell counts are expressed as Mean ± SEM; n=5; **P*<0.05 vs. male WT mice; #*P*<0.05 vs. female TLR2 KO mice.

myocardial injury and LV dysfunction at 7 days after permanent coronary ligation. It remains unclear why TLR2 KO has no additional benefit in female mice. Apparently, myocardial TLR2 and MvD88 levels are unaltered in female WT mice. Further, cardiac microvascular endothelial cells from female WT mice respond to bacterial TLR2 agonist PGN properly for ICAM-1 expression. The observation that cardiac microvascular endothelial cells, an important cell type in the early myocardial inflammatory response to ischemia, has no gender difference in their response to a TLR2 agonist suggests that the gender disparity in the role of TLR2 in the myocardial inflammatory response and myocardial injury is likely due to an influence on TLR2 activation or signaling in female hearts by myocardial and/or systemic factors associated with myocardial ischemia. Female

hearts may not release a significant amount of endogenous TLR2 activators, such as HSP70 and HMGB1, during ischemia. Alternatively, female sex hormones may modulate TLR2 signaling during ischemia. It is also possible that male sex hormones are required for TLR2 function during ischemia. Nevertheless, females are protected and TLR2 KO would not provide further protection. In this regard, estrogen has been found to be anti-inflammatory in animal models of renal, cerebral and myocardial I/R injury [28-30]. Further investigations are needed to determine the underlying mechanism(s).

Although the myocardial inflammatory response is required for injury healing and scar formation after myocardial infarction [31, 32], excessive myocardial inflammatory response could enlarge infarct size [33]. TLRs are important in regulating the myocardial

inflammatory response to injury [10, 34, 35], and the TLR2-MyD88 pathway contributes to the myocardial inflammatory response and myocardial injury [36-38]. It is known that stressed and injured cells release intracellular proteins, and extracellular HSP70 and HMGB1 activate TLR2 and TLR4 to promote the inflammatory response [12, 39, 40]. Evidence suggests that the TLR2-mediated pathway plays a significant role in triggering the post-infarction inflammatory response. We observed that male TLR2 KO mice have markedly lower levels of MCP-1 and ICAM-1 in ischemic myocardium at 3 days of ischemia. Both MCP-1 and ICAM-1 are important for leukocyte infiltration to ischemic tissue [41-43]. Indeed, reduced myocardial levels of MCP-1 and ICAM-1 in male TLR2 KO mice result in attenuated neutrophil infiltration and mononuclear cell accumulation in ischemic

myocardium at 3 days and 7 days, respectively.

An exaggerated myocardial inflammatory response is implicated in the mechanisms of adverse LV remodeling [44, 45]. We found that the smaller infarct sizes and improved LV function at 7 days of permanent ischemia in female mice were associated with attenuated myocardial inflammatory response at 3 days. Our results support the notion that excessive myocardial inflammatory response to ischemia exaggerates post-ischemic myocardial injury and promotes maladaptive LV remodeling. We found that myocardial function in male TLR2 KO mice was improved at 7 days after ischemia in comparison to WT males. In females, however, no functional improvement was associated with TLR2 KO. Similarly, the myocardial inflammatory response was not further reduced in female TLR2 KO mice. Thus, female mice are not benefited from TLR2 KO in either the myocardial inflammatory response or myocardial injury following ischemia. These results indicate that TLR2 plays an important role in the myocardial inflammatory response and LV dysfunction after MI only in males.

In summary, the results of the present study show: 1) TLR2 KO suppresses the myocardial inflammatory response, reduces infarct size and improves early LV remodeling in male mice after permanent myocardial ischemia, 2) female mice have attenuated myocardial inflammatory response and reduced myocardial injury after ischemia, and are not benefited from TLR2 KO and 3) the lack of TLR2 KO effect in females is not due to myocardial TLR2 deficiency. These findings indicate that there is a gender disparity in the role of TLR2 in post-ischemic myocardial inflammatory response and myocardial injury, and suggest that interception with TLR2 signaling may have therapeutic potentials only in males.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xianzhong Meng, Department of Surgery, Box C-320, University of Colorado Denver, 12700 E 19th Avenue, Aurora, CO 80045, USA. Tel: 303-724-6303; Fax: 303-724-6330; E-mail: xianzhong.meng@ucdenver.edu

References

- Ostadal B, Netuka I, Maly J, Besik J, Ostadalova I. Gender differences in cardiac ischemic injury and protection–experimental aspects. Exp Biol Med (Maywood) 2009; 234: 1011-1019.
- [2] Kam KW, Qi JS, Chen M, Wong TM. Estrogen reduces cardiac injury and expression of beta1-adrenoceptor upon ischemic insult in the rat heart. J Pharmacol Exp Ther 2004; 309: 8-15.
- [3] Litwin SE, Katz SE, Litwin CM, Morgan JP, Douglas PS. Gender differences in postinfarction left ventricular remodeling. Cardiology 1999; 91: 173-183.
- [4] Cavasin MA, Tao ZY, Yu AL, Yang XP. Testosterone enhances early cardiac remodeling after myocardial infarction, causing rupture and degrading cardiac function. Am J Physiol Heart Circ Physiol 2006; 290: H2043-2050.
- [5] Fang L, Gao XM, Moore XL, Kiriazis H, Su Y, Ming Z, Lim YL, Dart AM, Du XJ. Differences in inflammation, MMP activation and collagen damage account for gender difference in murine cardiac rupture following myocardial infarction. J Mol Cell Cardiol 2007; 43: 535-544.
- [6] Wang F, Keimig T, He Q, Ding J, Zhang Z, Pourabdollah-Nejad S, Yang XP. Augmented healing process in female mice with acute myocardial infarction. Gend Med 2007; 4: 230-247.
- [7] Wang M, Tsai BM, Crisostomo PR, Meldrum DR. Tumor necrosis factor receptor 1 signaling resistance in the female myocardium during ischemia. Circulation 2006; 114: I282-289.
- [8] Wang M, Crisostomo P, Wairiuko GM, Meldrum DR. Estrogen receptor-alpha mediates acute myocardial protection in females. Am J Physiol Heart Circ Physiol 2006; 290: H2204-2209.
- [9] Bell JR, Bernasochi GB, Varma U, Raaijmakers AJ, Delbridge LM. Sex and sex hormones in cardiac stress--mechanistic insights. J Steroid Biochem Mol Biol 2014; 137: 124-135.
- [10] Chao W. Toll-like receptor signaling: a critical modulator of cell survival and ischemic injury in the heart. Am J Physiol Heart Circ Physiol 2009; 296: H1-12.
- [11] Sakata Y, Dong JW, Vallejo JG, Huang CH, Baker JS, Tracey K, Takeuchi O, Akira S, Mann DL. Toll-like receptor 2 modulates left ventricular function following ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol 2007; 292: H503-H509.

- [12] Ao L, Zou N, Cleveland JCJ, Fullerton DA, Meng X. Myocardial TLR4 is a determinant of neutrophil infiltration after global myocardial ischemia: Mediating KC and MCP-1 expression induced by extracellular HSC70. Am J Physiol Heart Circ Physiol 2009; 297: H21-H28.
- [13] Kaczorowski DJ, Nakao A, Vallabhaneni R, Mollen KP, Sugimoto R, Kohmoto J, Zuckerbraun BS, McCurry KR, Billiar TR. Mechanisms of Toll-like receptor 4 (TLR4)-mediated inflammation after cold ischemia/reperfusion in the heart. Transplantation 2009; 87: 1455-1463.
- [14] Zou N, Ao L, Cleveland JC Jr, Yang X, Su X, Cai GY, Banerjee A, Fullerton DA, Meng X. Critical role of extracellular heat shock cognate protein 70 in the myocardial inflammatory response and cardiac dysfunction after global ischemia-reperfusion. Am J Physiol Heart Circ Physiol 2008; 294: H2805-H2813.
- [15] Herzog C, Lorenz A, Gillmann HJ, Chowdhury A, Larmann J, Harendza T, Echtermeyer F, Muller M, Schmitz M, Stypmann J, Seidler DG, Damm M, Stehr SN, Koch T, Wollert KC, Conway EM, Theilmeier G. Thrombomodulin's lectin-like domain reduces myocardial damage by interfering with HMGB1-mediated TLR2 signalling. Cardiovasc Res 2014; 101: 400-410.
- [16] Tian J, Guo X, Liu XM, Liu L, Weng QF, Dong SJ, Knowlton AA, Yuan WJ, Lin L. Extracellular HSP60 induces inflammation through activating and up-regulating TLRs in cardiomyocytes. Cardiovasc Res 2013; 98: 391-401.
- [17] Favre J, Musette P, Douin-Echinard V, Laude K, Henry JP, Arnal JF, Thuillez C, Richard V. Toll-like receptors 2-deficient mice are protected against postischemic coronary endothelial dysfunction. Arterioscler Thromb Vasc Biol 2007; 27: 1064-1071.
- [18] Wang L, Li YL, Zhang CC, Cui W, Wang X, Xia Y, Du J, Li HH. Inhibition of Toll-like receptor 2 reduces cardiac fibrosis by attenuating macrophage-mediated inflammation. Cardiovasc Res 2014; 101: 383-392.
- [19] Card JW, Carey MA, Bradbury JA, DeGraff LM, Morgan DL, Moorman MP, Flake GP, Zeldin DC. Gender differences in murine airway responsiveness and lipopolysaccharide-induced inflammation. J Immunol 2006; 177: 621-630.
- [20] Chotirmall SH, Greene CM, Oglesby IK, Thomas W, O'Neill SJ, Harvey BJ, McElvaney NG. 17Beta-estradiol inhibits IL-8 in cystic fibrosis by up-regulating secretory leucoprotease inhibitor. Am J Respir Crit Care Med 2010; 182: 62-72.
- [21] Rettew JA, Huet YM, Marriott I. Estrogens augment cell surface TLR4 expression on murine macrophages and regulate sepsis susceptibility in vivo. Endocrinology 2009; 150: 3877-3884.

- [22] Rettew JA, Huet-Hudson YM, Marriott I. Testosterone reduces macrophage expression in the mouse of toll-like receptor 4, a trigger for inflammation and innate immunity. Biol Reprod 2008; 78: 432-437.
- [23] Su X, Sykes JB, Ao L, Raeburn CD, Fullerton DA, Meng X. Extracellular heat shock cognate protein 70 induces cardiac functional tolerance to endotoxin: differential effect on TNF-alpha and ICAM-1 levels in heart tissue. Cytokine 2010; 51: 60-66.
- [24] Li JM, Mullen AM, Shah AM. Phenotypic properties and characteristics of superoxide production by mouse coronary microvascular endothelial cells. J Mol Cell Cardiol 2001; 33: 1119-1131.
- [25] Li J, Jin C, Cleveland JC Jr, Ao L, Xu D, Fullerton DA, Meng X. Enhanced inflammatory responses to toll-like receptor 2/4 stimulation in type 1 diabetic coronary artery endothelial cells: the effect of insulin. Cardiovasc Diabetol 2010; 9: 90.
- [26] Ao L, Song Y, Fullerton DA, Dinarello CA, Meng X. The interaction between myocardial depressant factors in endotoxemic cardiac dysfunction: Role of TNF-alpha in TLR4-mediated ICAM-1 expression. Cytokine 2007; 38: 124-129.
- [27] Cavasin MA, Tao Z, Menon S, Yang XP. Gender differences in cardiac function during early remodeling after acute myocardial infarction in mice. Life Sci 2004; 75: 2181-2192.
- [28] Booth EA, Marchesi M, Knittel AK, Kilbourne EJ, Lucchesi BR. The pathway-selective estrogen receptor ligand WAY-169916 reduces infarct size after myocardial ischemia and reperfusion by an estrogen receptor dependent mechanism. J Cardiovasc Pharmacol 2007; 49: 401-407.
- [29] Choi JS, Kim SJ, Shin JA, Lee KE, Park EM. Effects of estrogen on temporal expressions of IL-1beta and IL-1ra in rat organotypic hippocampal slices exposed to oxygen-glucose deprivation. Neurosci Lett 2008; 438: 233-237.
- [30] Shen KH, Lin CH, Chang HK, Chen WC, Chen SH. Premarin can act via estrogen receptors to rescue mice from heatstroke-induced lethality. Shock 2008; 30: 668-674.
- [31] Chen W, Frangogiannis NG. The role of inflammatory and fibrogenic pathways in heart failure associated with aging. Heart Fail Rev 2010; 15: 415-422.
- [32] Frangogiannis NG. The immune system and cardiac repair. Pharmacol Res 2008; 58: 88-111.
- [33] Frenkel D, Pachori AS, Zhang L, Dembinsky-Vaknin A, Farfara D, Petrovic-Stojkovic S, Dzau VJ, Weiner HL. Nasal vaccination with troponin reduces troponin specific T-cell responses and improves heart function in myocardial isch-

emia-reperfusion injury. Int Immunol 2009; 21: 817-829.

- [34] Jenke A, Wilk S, Poller W, Eriksson U, Valaperti A, Rauch BH, Stroux A, Liu P, Schultheiss HP, Scheibenbogen C, Skurk C. Adiponectin protects against Toll-like receptor 4-mediated cardiac inflammation and injury. Cardiovasc Res 2013; 99: 422-431.
- [35] Ha T, Liu L, Kelley J, Kao R, Williams D, Li C. Toll-like receptors: new players in myocardial ischemia/reperfusion injury. Antioxid Redox Signal 2011; 15: 1875-1893.
- [36] Shishido T, Nozaki N, Yamaguchi S, Shibata Y, Nitobe J, Miyamoto T, Takahashi H, Arimoto T, Maeda K, Yamakawa M, Takeuchi O, Akira S, Takeishi Y, Kubota I. Toll-like receptor-2 modulates ventricular remodeling after myocardial infarction. Circulation 2003; 108: 2905-2910.
- [37] Iwata A, Morgan-Stevenson V, Schwartz B, Liu L, Tupper J, Zhu X, Harlan J, Winn R. Extracellular BCL2 proteins are danger-associated molecular patterns that reduce tissue damage in murine models of ischemia-reperfusion injury. PLoS One 2010; 5: e9103.
- [38] Mersmann J, Iskandar F, Latsch K, Habeck K, Sprunck V, Zimmermann R, Schumann RR, Zacharowski K, Koch A. Attenuation of myocardial injury by HMGB1 blockade during ischemia/reperfusion is toll-like receptor 2-dependent. Mediators Inflamm 2013; 2013: 174168.
- [39] Qiu J, Xu J, Zheng Y, Wei Y, Zhu X, Lo EH, Moskowitz MA, Sims JR. High-mobility group box 1 promotes metalloproteinase-9 upregulation through Toll-like receptor 4 after cerebral ischemia. Stroke 2010; 41: 2077-2082.

- [40] Higashikuni Y, Tanaka K, Kato M, Nureki O, Hirata Y, Nagai R, Komuro I, Sata M. Toll-like receptor-2 mediates adaptive cardiac hypertrophy in response to pressure overload through interleukin-1beta upregulation via nuclear factor kappaB activation. J Am Heart Assoc 2013; 2: e000267.
- [41] Birdsall HH, Green DM, Trial J, Youker KA, Burns AR, MacKay CR, LaRosa GJ, Hawkins HK, Smith CW, Michael LH, Entman ML, Rossen RD. Complement C5a, TGF-beta 1, and MCP-1, in sequence, induce migration of monocytes into ischemic canine myocardium within the first one to five hours after reperfusion. Circulation 1997; 95: 684-692.
- [42] Kukielka GL, Hawkins HK, Michael L, Manning AM, Youker K, Lane C, Entman ML, Smith CW, Anderson DC. Regulation of intercellular adhesion molecule-1 (ICAM-1) in ischemic and reperfused canine myocardium. J Clin Invest 1993; 92: 1504-1516.
- [43] Ghigo A, Franco I, Morello F, Hirsch E. Myocyte signalling in leucocyte recruitment to the heart. Cardiovasc Res 2014; 102: 270-280.
- [44] Frangogiannis NG. Targeting the inflammatory response in healing myocardial infarcts. Curr Med Chem 2006; 13: 1877-1893.
- [45] Satoh M, Minami Y, Takahashi Y, Nakamura M. Immune modulation: role of the inflammatory cytokine cascade in the failing human heart. Curr Heart Fail Rep 2008; 5: 69-74.