Innate immune modulator MyD88 mediates neutrophil recruitment and myocardial injury after ischemia-reperfusion in mice

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MYOCARDIAL ISCHEMIA-REPERFUSION (I/R) leads to significant myocardial inflammation and injury. Evidence from several lines of investigation suggests that inflammation may be an important functional contributor to the pathogenesis of ischemic myocardial injury (11, 15, 48) although some anti-inflammatory interventions have yielded disappointing results (38, 48).

Following myocardial I/R, there is a significant inflammatory response that leads to downstream activation of multiple soluble and cellular factors such as the activation of endothelial cells, release of chemoattractants (e.g., reactive oxygen species, cytokines, and the activated complements) and adhesion molecules, increased vascular permeability, and rapid recruitment of neutrophils into ischemic myocardium. Although many of the downstream events leading to inflammatory injury have been identified (48), the proximal signaling mechanisms that control the critical events during I/R remain incompletely defined.

Innate immune systems such as those mediated via Toll-like receptors (TLRs) represent the first line of defense against microbial infection. There are at least 10 TLRs identified so far, and they recognize and specifically bind to a variety of pathogenic agonists such as lipopolysaccharide (LPS) (via TLR4), lipopeptide (via TLR2), flagellin (via TLR5), cytokine-phosphorothioate-guanine DNA (via TLR9), and dsRNA (via TLR3) by “molecular pattern recognition” (22). In addition to their pivotal role in host immune defense, recent studies have demonstrated that TLRs can recognize endogenous mediators and modulate tissue inflammation and injury in response to noninfectious injury in the lung (20), liver (37), and heart (7, 35, 42, 46).

The heart expresses at least three receptors involved in TLR signaling, CD14, TLR2, and TLR4 (12, 13, 25, 34, 51, 52). These receptors are in part responsible for cardiac dysfunction in some pathological conditions such as endotoxemia (25, 34) and peptidoglycan-associated lipoprotein (51). The role of TLRs in ischemic myocardial injury is, however, incompletely defined, and prior studies seem conflicting. For example, systemic TLR4 activation by LPS reduced subsequent ischemic myocardial infarction (MI) and improved cardiac functions both in vivo and in isolated hearts (3, 5, 26, 29, 31, 43, 47, 49, 50). The activation of TLR4-MyD88 signaling also protects cardiomyocytes against apoptosis and improved cardiomyocyte functions (6, 52). However, in the absence of systemic TLR4 stimulation, mice deficient for TLR4 exhibited reduced myocardial inflammation and infarction in an in vivo model of I/R injury compared with WT mice, suggesting that TLR4 may mediate ischemic injury in the heart (7, 35). Interestingly, TLR2 deficiency led to a reduced myocardial remodeling with improved LV function but had no impact on the infarct size and degree of inflammation in the heart after ischemia (42).

MyD88, originally isolated as one of the 12 myeloid differentiation primary response genes (27), is an adaptor protein that is critical for transducing signals from all 10 TLR family members (19, 22, 30, 33), except TLR3, and interleukin-1 (IL-1) receptor family members, including IL-1R and IL-18R (1, 10). MyD88 has a NH2-terminal death domain (DD) and a...
COOH-terminal Toll/IL-1R-related domain, which interacts with another Toll/IL-1R-related domain found in TLRs. Upon binding to TLRs and IL-1R, MyD88 recruits the downstream kinase IL-1 receptor-associated kinases via their DD-DD interaction. Like TLR4-deficient mice (36), MyD88−/− mice (21, 52) lack the ability to respond to LPS although MyD88-independent pathways (e.g., Toll/IL-1 receptor domain containing adaptor inducing IFN-β production) exist in TLR4 signaling (23).

The role of MyD88 in cell death and tissue injury is incompletely understood, and prior results have been conflicting (2, 20). Using MyD88−/− mice, Jiang et al. (20) have recently found that MyD88 mediates an survival signal in lung epithelial cells and plays a critical role in tissue repair as well as inflammation in an acute lung injury model. In cardiomyocytes, we found that although MyD88 deficiency has no impact on cardiomyocyte death in either normal or apoptosis-inducing conditions, it is essential for TLR4-activated survival in mouse cardiomyocytes (52). Moreover, although adenovirus-mediated expression of MyD88 modulates TLR2-induced cytokine production, MyD88 overexpression without upstream TLR2 activation is not sufficient to produce survival benefit (unpublished data) or cytokine production in cardiomyocytes (51). However, other investigators have reported that the overexpression of dominant negative mutant of MyD88 leads to reduced cardiomyocyte apoptosis and tissue injury (16).

Given its critical role in innate immune signaling and the demonstrated roles of TLR4 and TLR2 in ischemic myocardial injury and remodeling, we set out to test the hypothesis that signaling via MyD88 is an important determinant of consequences after ischemic myocardial injury and contributes critically to myocardial inflammatory injury and LV dysfunction after I/R.

METHODS

Animal Models

All animal experiments were performed with the approval of the Animal Care Committee of Massachusetts General Hospital. MyD88−/− mice were provided by Dr. Mason Freeman at Massachusetts General Hospital and backcrossed at least 6 generations into the C57BL/6J strain.

I/R injury model. Male mice (8–12 wk old; 25–30 g) were anesthetized with pentobarbital sodium, intubated, and ventilated in a volume-control mode (inspired O2 fraction equilibrated with pentobarbital sodium, intubated, and ventilated in a C57BL/6J strain. Settts General Hospital and backcrossed at least 6 generations into the

Measurement of AAR and MI

Fourteen hours after I/R, mice were euthanized and the hearts isolated and sectioned from apex to base into four 2-mm sections. Under a fluorescence microscope, the perfused myocardium was identified as the areas filled with red microsphere and the AAR identified as areas devoid of red microsphere. To examine MI, the sections were incubated in 1% (wt/vol) triphenyltetrazolium chloride (TTC) in Tris-HCl (pH 7.4) at 30°C for 10 min followed by fixation with 4% paraformaldehyde. For each section, the AAR and MI areas were measured from enlarged digital microscopics images using Adobe Photoshop. The percentage of MI/AAR was calculated as the infarction area (MI/LV × 100%) divided by the AAR(AAR/LV × 100%).

Echocardiography in Mice

On days 1, 3, and 7 after I/R, mice were lightly anesthetized with ketamine (0.016 mg/g). Ultrasonic transmission gel was applied to the thorax. Thoracic echocardiograms were obtained and interpreted by an echocardiographer (X. Xu) blinded to the experimental groups using a 13.0-MHz linear probe (Vivid 7, GE Medical System, Milwaukee, WI). LV end-diastolic and -systolic dimensions (LVID and LVIS, respectively) were measured on an M-mode obtained from a parasternal short-axis view at mid-papillary level. The fractional shortening (FS) was defined as (LVID – LVIS)/LVID. The ejection fraction (EF) was calculated from the M-mode (LVID3 – LVIS3) (9, 14).

Measurements of Tail-Cuff Blood Pressure in Awake Mice

Systolic blood pressure was measured with a noninvasive tail-cuff machine (XB 1000, Kent Scientific, Torrington, CT) in awake WT and MyD88−/− mice. Mice were first subjected to one or two practice sessions to acclimate to the device. Briefly, the mouse was initially placed in a restrainer for a short period (approximately <1 min to start) and then maintained in the restrainer for longer times to acclimate to the device. The degree of acclimatization was judged by the absence of agitation in the device. After a few days of practice sessions, mice remained comfortably restrained for longer periods in the restraint device (Kent Scientific).

Invasive Cardiac Hemodynamic Measurements

Three days after I/R, mice were anesthetized by intraperitoneal injection with ketamine (0.1 mg/g), fentanyl (50 ng/g), and pancuronium (0.002 mg/g); intubated; and mechanically ventilated. An SPR-839 pressure-volume Millar catheter (Millar Instruments, Houston, TX) was inserted into the left carotid artery and advanced into the LV. Heart rate and LVESP and LVEDP were measured, and dP/dtmax were calculated (14, 40).

Immunohistochemistry

Twenty-four hours after I/R or sham operation, hearts were immersed in freeze medium in plastic cubes, frozen in liquid N2, and cut

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from apex to base into 12-μm thin sections. The sections were stained with hematoxylin and eosin to examine myocardial morphology and any evidence of inflammatory cell infiltration. Immunohistochemical staining of neutrophils was performed using immunoperoxidase detecting systems (Vector). Myocardial neutrophils were detected using a biotin-labeled-specific rat anti-mouse Ly-6G/Ly-6C antibody (Gr-1, Clone RB6–8C5, BD Pharmingen, 1:500). Biotin signals were then detected using horseradish peroxidase-coupled streptavidin (ABC reagent, Vector) and AEC peroxidase substrate (32).

Myeloperoxidase Activity

Myeloperoxidase (MPO) activity was measured with a MPO assay kit according to the manufacturer’s recommendations (Cytostore, Calgary, Alberta, Canada).

RNA Extraction and Quantitative RT-PCR Analysis

Total RNA was extracted from mouse myocardial tissues using TRIzol reagent, and cDNA was synthesized by reverse-transcriptase reaction. cDNA gene sequences corresponding to 18S rRNA were amplified by PCR and quantitated using an ABI Prism 7000 (Applied Biosystems) with the forward primer 5’-TATCTGTGTTGAGGAAAGC-3’ and the reverse primer 5’-TTAGCCTTCCGCTGCCTCCAAGACTC-3’. To detect keratinocyte chemotactrant (KC) transcripts (45), forward primer: 5’-CAGCGGCTGATCGGAACCAA-3’, reverse primer: 5’-CTTGGGGACACCCTT TTAGCATCTTT-TGG-3’ were used. For monocyte chemotactrant protein-1 (MCP-1) transcripts, forward primer: 5’-TTAAAAAACTCGGATCGGAACCAA-3’, reverse primer: 5’-GCATTAGCTCTCAAGTTTG-3’ were used. For ICAM-1 transcripts, forward primer: 5’-TGATGCTACAG- GATCCAACATCCA-3’, reverse primer: 5’-ACAAGTCACAAAGCA- CAGCG-3’ were used. For macrophage inflammatory protein-1 (MIP-1) transcripts (45), forward primer: 5’-CACCTCTTCATTGGCTCAACA CATC-3’, reverse primer: 5’-GGCTTCTCTCGCCCTCCAGAGCTC T-3’ were used. For MIP-2 transcripts (45), forward primer: 5’-CCGCTTTGGGACAGTAATCAGCGG-3’, reverse primer: 5’-TTAGCACTTC GCTTCTTGTCATAT-3’ were used. Changes in relative gene expression normalized to 18S rRNA levels were determined using the relative threshold cycle method (52).

Statistical Analysis

Unless stated otherwise, all data are expressed as means ± SE and analyzed with two-way ANOVA for statistic significance. For the serial echocardiographic studies where multiple measurements were performed, a two-way ANOVA for repeated measures with Bonferroini post hoc tests were used to analyze for statistical significance. The null hypothesis was rejected for \( P < 0.05 \).

RESULTS

MyD88−/− Mice Have Smaller MI Sizes Compared with WT Mice after I/R

Figure 1A shows representative photographs of myocardial tissues staining with fluorescent microsphere to delineate AAR and with TTC to show myocardial infarct area in WT and MyD88−/− mice subjected to 30 min of ischemia and 24 h of reperfusion. As indicated in Fig. 1B, the ratios of ischemic area (AAR) to LV are similar between WT and MyD88−/− mice. However, the ratios of MI to AAR are decreased by 58% in MyD88−/− mice compared with that in WT mice (14 ± 2% vs. 33 ± 6%, respectively, \( P < 0.05 \)).

MyD88−/− Mice Have Better Preserved LV Function Compared with WT Mice after I/R

We tested whether or not systemic MyD88 deficiency has an impact on cardiac function after I/R injury in vivo. We first examined the baseline of cardiac function in WT and MyD88−/− mice before I/R. At the cellular level, our previous studies have shown that cardiomyocytes isolated from adult WT and MyD88−/− mice shared similar morphology and sarcomere length and had similar cellular function as measured by calcium transients and sarcomere shortening in response to pacing (52). Consistent with these findings, transthoracic echocardiographic studies indicated that cardiac contractile function was similar between WT and MyD88−/− mice before I/R injury with the EF at 90 ± 1% and 91 ± 1% and the FS at 55 ± 2% and 57 ± 2%, respectively. The LVIS and LVID were similar between the two groups of mice as well (LVIS, 1.2 ± 0.1 vs. 1.1 ± 0.1 mm, and LVID, 2.7 ± 0.1 vs. 2.6 ± 0.1 mm, respectively) (Fig. 2). After 24 h of I/R, the EF and FS in WT mice were decreased by 44% and 62%, respectively (EF, 51 ± 2%, and FS, 22 ± 1%, \( P < 0.001 \) vs. baseline) and remained depressed for up to 7 days after reperfusion (EF, 51 ± 1% and FS, 22 ± 1%, \( P < 0.001 \)). The LVIS and LVID were significantly increased by 133% and 37%, respectively, at 24 h (\( P < 0.001 \)) and by 150% and 40%, respectively, 7 days (\( P < 0.001 \))
MyD88 MEDIATES ISCHEMIC MYOCARDIAL INJURY

Fig. 2. MyD88-deficient (MyD88−/−) mice have better preserved cardiac function after I/R injury (IRI). All mice underwent 30 min of myocardial ischemia and various periods of reperfusion (1, 3, and 7 days). A: representative M-mode echocardiogram of WT and KO mice before and 24 h after I/R injury. B: quantitative data of echocardiographic measurements. Serial echocardiograms were measured before and 1, 3, and 7 days after I/R (n = 12 to 13). *P < 0.01 and †P < 0.001 vs. WT mice. EF, ejection fraction; FS, fraction shortening; LVIS, left ventricular dimensions at end systole; LVID, left ventricular dimensions at end-diastole; AW, anterior wall; PW, posterior wall.

Table 1. Invasive hemodynamic measurements of WT and MyD88−/− mice at baseline and 3 days after I/R

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<th>Baseline</th>
<th>I/R</th>
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<td>WT</td>
<td>MyD88−/−</td>
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<tr>
<td>LV mass/BW, mg/g</td>
<td>3.0±0.1</td>
<td>2.9±0.1</td>
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<tr>
<td>HR, beats/min</td>
<td>625±13</td>
<td>619±20</td>
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<tr>
<td>LVESP, mmHg</td>
<td>118±2</td>
<td>115±1</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>4±1</td>
<td>4±1</td>
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<tr>
<td>LV dP/dtmax, mmHg/s</td>
<td>14,600±1,300</td>
<td>15,000±1,800</td>
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<tr>
<td>LV dP/dtmin, mmHg/s</td>
<td>−15,100±800</td>
<td>−14,700±700</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>MyD88−/−</td>
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<td></td>
<td></td>
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<tr>
<td>LV mass/BW, mg/g</td>
<td>3.5±0.2</td>
<td>3.4±0.1</td>
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<tr>
<td>HR, beats/min</td>
<td>647±12</td>
<td>648±22</td>
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<tr>
<td>LVESP, mmHg</td>
<td>63±2†</td>
<td>68±2</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>4±0.3</td>
<td>4±0.3</td>
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<tr>
<td>LV dP/dtmax, mmHg/s</td>
<td>5,447±514†</td>
<td>7,853±524*</td>
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<tr>
<td>LV dP/dtmin, mmHg/s</td>
<td>−5,370±424†</td>
<td>−7,239±526*</td>
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Values are means ± SE; n = 5 mice in each group. The baseline cardiac functions were measured in 12–16 wk-old mice without left anterior descending coronary artery (LAD) ligation. Separate groups of mice were subjected to LAD ligation for 30 min followed by days of reperfusion (ischemia-reperfusion I/R) as described in METHODS. LV, left ventricular; BW, body weight; HR, heart rate; LVESP, LV end-systolic pressure; LVEDP, LV end-diastolic pressure; +dP/dtmax, the maximum first derivative of developed LV pressure; −dP/dtmin, the minimum first derivative of developed LV pressure. *P < 0.05 vs. wild-type (WT) I/R; †P < 0.01 vs. WT baseline.

after reperfusion. In comparison, MyD88−/− mice showed much less ventricular depression after I/R. EF and FS were decreased by 27% and 42%, respectively, at 24 h (EF, 67 ± 3% and FS, 33 ± 2%, P < 0.001 vs. WT) and by 20% and 35% (EF, 73 ± 1%, and FS, 37 ± 1%, P < 0.001 vs. WT), respectively, on day 7 of I/R. LVIS was significantly smaller in the MyD88−/− compared with the WT mice after 7 days of reperfusion (P < 0.01), whereas LVID was similar between the two groups of animals within the same period of time (Fig. 2). These echocardiographic data suggest that cardiac contractile function was better preserved in MyD88−/− mice compared with that in WT mice after I/R. We also examined mouse cardiac function using invasive hemodynamic measurements after I/R. As shown in Table 1, the baseline cardiac functions of WT and MyD88−/− mice were similar. Three days after I/R, LV dP/dtmax and dP/dtmin were remarkably reduced indicating attenuated LV function. However, dP/dtmax and dP/dtmin were significantly higher in mice deficient for MyD88 than that in WT mice, suggesting a significant improvement in both LV systolic and diastolic function in the MyD88−/− mice. The baseline of mean arterial blood pressure was similar between the two mouse strains (Table 2). Six hours after surgery, the mean arterial blood pressure was significantly reduced in both sham-operated and I/R mice and to a similar degree in both WT and knockout mice, suggesting that the reduction in the mean blood pressure was mainly due to the thoracotomy procedure and not I/R injury.

MyD88 Deficiency Has No Impact on MI Sizes in Isolated Mouse Hearts after Global I/R

To avoid the potential systemic contributors associated with extracardiac MyD88 deficiency in the setting of in vivo I/R, we isolated and subjected mouse hearts to I/R in a Langendorff...
Myocardial Neutrophil Recruitment Is Impaired in MyD88-reduced but similar between the two groups of mice (Table 3). of the hearts isolated from WT and MyD88 were significantly 20 min ischemia and 40 min reperfusion, the cardiac function levels of cardiac contractile dysfunction in both groups. After isolated hearts induced 30/H11006 26/H11006 26/H11006 26/H11006 before surgery. Myocardial Neutrophil Recruitment Is Impaired in MyD88−/− Mice Subjected to I/R Thirty minutes of ischemia followed by 24 h of reperfusion induced significant neutrophil infiltration in myocardium in WT mice compared with the sham-operated mice as demonstrated by hematoxylin and eosin staining and immunohistochemical staining with GR-1, a specific antibody for neutrophils. However, in MyD88−/− mice subjected to the same I/R protocol, the level of neutrophil recruitment was much lower compared with that in WT mice (Fig. 3A). Moreover, MPO activity, a marker of neutrophil function, was significantly decreased in the hearts of MyD88−/− mice compared with WT mice after I/R (Fig. 3B). The decrease in the myocardial neutrophil recruitment in MyD88−/− mice after I/R was not due to the altered level of circulating neutrophils since the numbers of blood neutrophils were similar between WT and MyD88−/− mice (data not shown). Together, these data suggest that myocardial neutrophil recruitment was significantly impaired in MyD88−/− mice compared with WT mice subjected to I/R.

Table 2. Mean arterial BP of WT and MyD88−/− mice before and after surgery

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<tr>
<th></th>
<th>Sham, mmHg</th>
<th>I/R, mmHg</th>
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<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>WT</td>
<td>106±8</td>
<td>59±5*</td>
</tr>
<tr>
<td>MyD88−/−</td>
<td>101±12</td>
<td>65±2*</td>
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Values are means ± SE; n = 5–7 mice. Blood pressure (BP) was measured in awake WT and MyD88−/− mice before and 6 h after either sham operation or I/R injury using tail-cuff method (see METHODS) *P < 0.01 vs. baseline BPs before surgery.

MyD88 Mediates the Myocardial Production of Proinflammatory Mediators after I/R

We examined the myocardial levels of ICAM-1, MCP-1, KC, and MIP-1/2, key proinflammatory mediators critical for neutrophil recruitment, in WT and MyD88−/− mice. The baseline levels of ICAM-1, MCP-1, KC, and MIP-1/2 were similar in sham-operated WT and MyD88−/− mice (P > 0.05). Six hours after I/R, there was a significant increase in the levels of MCP-1 and KC transcripts in WT mice compared with those in sham-operated control WT mice (432 ± 103% and 508 ± 124%, respectively, P < 0.05, n = 6). However, MyD88−/− mice had a significantly lower level of KC (174 ± 37%, P < 0.05, n = 6). Twenty-four hours after I/R, there was a significant increase in the level of ICAM-1, MCP-1, and KC transcripts in WT mice compared with the level in sham-operated control WT mice (258 ± 17%, 381 ± 92%, and 2,859 ± 753%, respectively) (Fig. 4). In contrast, MyD88−/− mice had significantly lower levels of ICAM-1, MCP-1, and KC expression after I/R (120 ± 17%, 180 ± 16%, and 267 ± 190%, respectively) compared with those of WT mice subjected to I/R (Fig. 4). There was no significant increase in the level of myocardial MIP-1/2 transcripts after 24 h of I/R or any difference between the two groups of animals (data not shown).

DISCUSSION

It is well documented that inflammation is an important functional contributor to the pathogenesis of ischemic myocardial injury (11, 15, 48) and that the innate immune response to I/R is, by far, the most common cause of myocardial inflammation. Given its critical role in the signal transduction of many innate immune receptors (22), we hypothesized that MyD88 signaling is an important determinant in ischemic myocardial injury. Using a mouse model of I/R injury, we demonstrate that MyD88 is critical for myocardial neutrophil recruitment and proinflammatory mediator production after I/R and significantly contributes to ischemic MI and LV dysfunction.

Emerging evidence from various animal models of tissue injury suggest that TLR-MyD88 signaling plays a critical role in modulating tissue inflammation and injury in the absence of microbial infection. For example, in an acute lung injury model, hyaluronan, produced in response to lung injury, induces a proinflammatory and an antiapoptotic effect in lung epithelial cells via both TLR2- and TLR4-MyD88-dependent

Table 3. Cardiac function of ex vivo perfused hearts from WT and MyD88−/− mice

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<tr>
<th></th>
<th>Pre-I/R</th>
<th>I/R</th>
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<tr>
<td></td>
<td>WT</td>
<td>MyD88−/−</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>390±11</td>
<td>380±13</td>
</tr>
<tr>
<td>LVESP, mmHg</td>
<td>68±6</td>
<td>72±7</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>2±2</td>
<td>3±1</td>
</tr>
<tr>
<td>LVDP, mmHg</td>
<td>66±6</td>
<td>70±7</td>
</tr>
<tr>
<td>LVRPP, mmHg/min</td>
<td>25,740±2,080</td>
<td>26,600±2,350</td>
</tr>
<tr>
<td>LV dP/dtmax, mmHg/s</td>
<td>2,470±265</td>
<td>2,430±290</td>
</tr>
<tr>
<td>LV dP/dtmin, mmHg/s</td>
<td>2,046±207</td>
<td>2,102±240</td>
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Values are means ± SE; n = 8 mice in each group. After 10 min of control perfusion in a Langendorff perfusion system, the baseline cardiac functions were recorded (Pre-I/R). The isolated hearts were then exposed to 20 min of global ischemia followed by 40 min of reperfusion. LVDP, LV developing pressure (LVESP − LVEDP); LVRPP, LV rate-pressure product (HR × LVDP).
mechanisms (20), suggesting a role for TLR-MyD88 signaling in tissue repair of acute lung injury. However, the role of TLR signaling in myocardial I/R injury is complex and incompletely defined. Systemic administration of LPS, which signals through TLR4 and MyD88, reduces subsequent ischemic MI and improves cardiac function in both in vivo and ex vivo models of I/R injury (3, 5, 26, 29, 31, 43, 47, 49, 50). TLR4 signaling also protects cardiomyocytes from apoptosis via MyD88- and inducible nitric oxide synthase-dependent mechanisms in vitro (6, 52). However, given the multiple systemic reactions in response to in vivo administration of LPS, it is unclear whether the observed cardiac benefits are direct results of TLR4 signaling in the heart or due to other events secondary to systemic activation of the innate immune system. In contrast, in the absence of systemic TLR4 stimulation, mice deficient for TLR4 (7, 35) exhibited reduced myocardial inflammation and infarction compared with WT, suggesting that TLR4 may mediate ischemic injury in the heart. Consistent with these findings, the present study demonstrates that systemic MyD88 deficiency leads to a significant reduction in MI size and impairment in myocardial neutrophil recruitment and proinflammatory mediator production after I/R. Using serial transthoracic echocardiographic and invasive hemodynamic measurements, the present studies also demonstrate that the LV contractile function is better preserved in MyD88−/− mice compared with WT mice as early as 24 h and up to 7 days after I/R. Moreover, in an effort to determine the potential impact of extracardiac MyD88 deficiency on cardiac injury associated with the in vivo model of I/R, we tested whether or not MyD88 deficiency has any effect on myocardial injury in isolated mouse hearts. We found that in isolated hearts subjected to global I/R, MyD88 deficiency has no direct impact on MI and cardiac function following ex vivo I/R. This finding suggests that in the in vivo condition, MyD88 may play a pivotal role in

Fig. 3. MyD88−/− mice have a marked reduction in neutrophil recruitment after I/R. Mice were subjected to 30 min ischemia and 24 h of reperfusion. A: representative photomicrographs (×400) of myocardial sections labeled with hematoxylin and eosin (H&E) stain and anti-Gr-1 mAb (immunoperoxidase method). B: myeloperoxidase (MPO) activity (units × 10⁻²/mg; n = 8). MPO values for sham-operated hearts were 0.46 ± 0.03 for WT and 0.33 ± 0.07 units × 10⁻²/mg for MyD88−/− mice. P = 0.14 (not significant).

Fig. 4. MyD88 deficiency attenuates expression of proinflammatory mediators in the hearts after I/R injury. Both WT and MyD88−/− mice were subjected to either sham operation (light gray bars) or I/R (30 min of ischemia and 24 h of reperfusion) (dark gray bars). At the end of reperfusion, the animals were euthanized and the cardiac tissues processed for RNA extraction as described in METHODS. ICAM-1 (A), monocyte chemoattractant protein-1 (MCP-1; B), and keratinocyte chemoattractant (KC; C) transcripts were analyzed with quantitative RT-PCR (n = 3–5). *P < 0.05; †P < 0.01.
mediating myocardial inflammation that is critical for I/R injury and is consistent with the hypothesis that the cardiac benefits observed in MyD88−/− mice in vivo may require circulating blood components during I/R.

Evidence from several lines of investigation suggests that inflammation is an important functional contributor to the pathogenesis of ischemic MI (11, 15). In animal models, neutrophil depletion with antibodies (39) or physical filtering (8, 18) as well as inhibition of neutrophil adhesion with anti-CD18 mAb (44), all substantially reduce injury after reperfusion. In addition, interventions targeted at a variety of specific inflammatory mediators such as complement depletion (28) or lipoxigenase inhibitors (41) or antibodies to the proinflammatory cytokine, IL-1 (17), have demonstrated benefits in I/R injury. However, so far, clinical studies investigating the efficacy of anti-inflammatory therapies noted above have failed to show any meaningful cardioprotective effect (48). Moreover, some anti-inflammatory interventions, such as corticosteroids, have yielded disappointing results (38). Failure to translate experimentally effective cardioprotective interventions into clinical therapies is multifactorial. One of these factors could be the use of animal models that do not adequately approximate the clinical setting (4). Interestingly, other investigators have suggested that inflammation may actually play a beneficial role in the healing process after infarction (24). To define the role of MyD88 signaling in controlling the inflammatory processes in the ischemic myocardium, we tested the impact of MyD88 deficiency on myocardial neutrophil recruitment as well as the expression of the NF-κb-dependent chemokine, KC, MCP-1, and MIPs, and the adhesion molecule, ICAM-1, after I/R. We found that both neutrophil recruitment and the expression of KC, MCP-1, and ICAM-1 were attenuated in MyD88−/− mice compared with WT mice after I/R injury. It is unclear, however, whether the decreased myocardial inflammation contributes to, or is the result of, smaller myocardial injury in the MyD88−/− mice. Our finding in isolated perfused heart, a system devoid of circulatory blood cells, that MyD88 deficiency has no impact on myocardial infarction and cardiac function following I/R is consistent with the hypothesis that MyD88 signaling mediates ischemic myocardial injury via mechanisms that are dependent on circulatory blood components such as neutrophils. We speculate that the lack of MyD88 in extracardiac tissues and cells, such as inflammatory cells, could have contributed to the reduction of both myocardial inflammatory injury and LV dysfunction after I/R.

The role of MyD88 in cell survival or death is incompletely understood, and prior results have been conflicting (2, 20). The expression of high levels of exogenous MyD88 in transfected 293 cell line mediates TLR2-induced cell death (2). The overexpression of dominant negative MyD88 led to reduced cardiomyocyte death and injury (16). However, in most cells, the activation of TLR/IL-1R family members does not result in cell death but rather triggers the expression of cell survival and inflammatory genes. For example, MyD88 mediates a cell survival signal in lung epithelial cells and plays a critical role in pulmonary tissue repair (20). Consistent with this observation, we previously reported that although MyD88 deficiency is not sufficient to alter the level of cardiomyocyte apoptosis in vitro, MyD88 is essential for TLR4-activated antiapoptotic signaling in mouse cardiomyocytes (52).

In summary, the present study demonstrates an important role of MyD88 signaling in the pathogenesis of myocardial infarction, infarct, and cardiac dysfunction after I/R. Systemic MyD88 deficiency leads to attenuated neutrophil recruitment and diminished proinflammatory mediator production, reduced MI, and dramatically improved LV contractile function after I/R injury. The observation that isolated MyD88-deficient hearts are not protected from I/R injury suggests that MyD88 may contributes to ischemic myocardial injury via mechanisms involving systemic components. These studies identify MyD88 as a potential therapeutic target in the management of ischemic myocardial injury.

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MyD88 MEDIATES ISCHEMIC MYOCARDIAL INJURY


