Endogenous IRAK-M Attenuates Postinfarction Remodeling Through Effects on Macrophages and Fibroblasts

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Objective—Effective postinfarction repair requires timely suppression of innate immune signals to prevent the catastrophic consequences of uncontrolled inflammation on cardiac geometry and function. In macrophages, interleukin-1 receptor-associated kinase (IRAK)-M acts as a functional decoy preventing uncontrolled toll-like receptor/interleukin-1–mediated responses. Our study investigates the role of IRAK-M as a negative regulator of the postinfarction inflammatory response and as a modulator of cardiac remodeling.

Methods and Results—In wild-type mouse infarcts IRAK-M was upregulated in infiltrating macrophages and fibroblasts exhibiting a biphasic response. When compared with wild-type animals, infarcted IRAK-M−/− mice had enhanced adverse remodeling and worse systolic dysfunction; however, acute infarct size was comparable between groups. Adverse remodeling in IRAK-M−/− animals was associated with enhanced myocardial inflammation and protease activation. The protective actions of IRAK-M involved phenotypic modulation of macrophages and fibroblasts. IRAK-M−/− infarcts showed increased infiltration with proinflammatory CD11b+/Ly6Chi monocytes; leukocytes harvested from IRAK-M−null infarcts exhibited accentuated cytokine expression. In vitro, IRAK-M expression was upregulated in cytokine-stimulated murine cardiac fibroblasts and suppressed their matrix-degrading properties without affecting their inflammatory activity.

Conclusion—Endogenous IRAK-M attenuates adverse postinfarction remodeling suppressing leukocyte inflammatory activity, while inhibiting fibroblast-mediated matrix degradation. (Arterioscler Thromb Vascl Biol. 2012;32:00-00.)

Key Words: cardiac remodeling ■ cytokines ■ macrophages ■ metalloproteinases ■ immune system

Tissue necrosis triggers an intense inflammatory reaction that serves to clear the wound from dead cells and matrix debris, while activating reparative pathways. Because of the catastrophic consequences of excessive or prolonged inflammation on tissue architecture and organ function, repair of injured tissues requires timely activation of endogenous inhibitory pathways that restrain and suppress proinflammatory signals. Initially believed to be a passive process because of the dissipation of the injurious stimulus, it is now clear that suppression and resolution of the acute inflammatory response is a biosynthetically active response that requires activation of molecular mediators that inhibit the inflammatory reaction.1,2

Because the mammalian heart has negligible regenerative capacity, repair of the infarcted myocardium is dependent on the activation of an inflammatory reaction that ultimately results in the formation of a collagen-based scar. Perhaps more so than in any other tissue or type of injury, negative regulation of myocardial inflammation in the ischemic heart is of critical significance for the reparative process.3 Why is the myocardium so vulnerable to the consequences of an accentuated reparative inflammatory reaction? First, the selective pressures responsible for the evolution of the response to injury were likely driven by the potentially catastrophic effects of bacterial contamination in insults associated with traumatic breakdown of epithelial barriers. Thus, mammals may have evolved to interpret necrotic host cells as a sign of infection,1 mounting a robust inflammatory response that may be excessive in a context of ischemic injury and in the absence of microbial contamination. Second, in the myocardium, function is dependent on the optimal maintenance of tissue architecture and chamber geometry. Even relatively subtle alterations in myocardial architecture have profound consequences on cardiac geometry and mechanics leading to contractile dysfunction and chamber dilation, a process termed ventricular remodeling.4,5 In the infarcted heart, an excessive or prolonged inflammatory reaction could be catastrophic by enhancing cardiomyocyte hypocontractility and apoptosis, while promoting protease activation and matrix degradation, thus, markedly accentuating remodeling. More extensive cardiac remodeling after myocardial infarction is associated

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with increased mortality and a high incidence of arrhythmias and heart failure. Emerging evidence suggests that defects in the molecular signals implicated in suppression and resolution of the inflammatory response may be involved in the pathogenesis of postinfarction cardiac remodeling.

Toll-like receptor–(TLR) and interleukin (IL)-1–mediated pathways are critically involved in the postinfarction inflammatory response and in the pathogenesis of cardiac remodeling. Considering the broad proinflammatory actions of TLR/IL-1 activation and the potentially catastrophic effects of uncontrolled inflammation, it is not surprising that several distinct pathways have evolved to restrain TLR and IL-1 responses. One such endogenous inhibitory signal, IL-1 receptor-associated kinase (IRAK)-M, is the only IRAK that lacks endogenous kinase activity, acting as a functional decoy that inhibits TLR and IL-1 responses. IRAK-M is primarily expressed in monocytes and macrophages and plays an important role in restraining inflammatory activation induced by infectious pathogens; however, its potential involvement in regulating the inflammatory and reparative process in ischemic injury has not been studied. We hypothesized that IRAK-M expression may be upregulated in the infarcted heart preventing uncontrolled proinflammatory signaling and protecting it from the development of adverse remodeling after myocardial infarction. Our findings provide the first evidence of the role of IRAK-M in cardiac remodeling and suggest that, in addition to its effects on macrophage inflammatory activity, IRAK-M is induced in cytokine-stimulated fibroblasts regulating their matrix-degrading properties.

**Materials and Methods**

A detailed description of the methodology is provided in the online-only Data Supplement.

**Animal Protocols**

C57BL/6, IRAK-M−/− mice, and IL-1RI−/− animals in a C57BL/6 background were purchased from Jackson Laboratories. All protocols were approved by the committee on animal research care at Baylor College of Medicine and at Albert Einstein College of Medicine. A total of 150 C57BL6 mice and 156 IRAK-M−/− mice were used in the study. Mice that were 2 to 3 months old were anesthetized by isoflurane inhalation (isoflurane 2%–3% vol/vol). Myocardial infarction was induced using a closed-chest mouse model of reperfused myocardial infarction, as previously described. After 6 hours to 28 days of reperfusion, the chest was opened and the heart was immediately excised, fixed in zinc-formalin, and embedded in paraffin for histological studies or snap frozen in liquid nitrogen and stored at −80°C for RNA and protein extraction. Sham animals were prepared identically without undergoing coronary occlusion/reperfusion. In additional mice, infarct size was assessed after 24 hours of reperfusion using triphenyltetrazolium chloride/Evans Blue staining, as previously described.

**Echocardiography**

Echocardiographic studies were performed before instrumentation and after 7 days and 28 days of reperfusion (wild type [WT], n=14; IRAK-M−/−, n=18) using a 25-MHz probe (Vevo 770; Visualsonics, Toronto, ON) as previously described.

**Perfusion Fixation and Assessment of Ventricular Volumes**

Systematic morphometric assessment of ventricular dimensions, ventricular volumes, and scar size in perfusion-fixed hearts was performed as previously described.

**Immunohistochemistry and Quantitative Histology**

Leukocytes were identified in formalin-fixed paraffin-embedded sections using immunohistochemistry with the following primary antibodies: monoclonal antineutrophil antibody (Serotec, Raleigh, NC) and rat anti-mouse Mac-2 (Cedarlane Burlington, Canada) for macrophages. The collagen network was identified using picrosirius red staining.

**RNA Extraction and Quantitative Polymerase Chain Reaction Assay**

Isolated total RNA from the hearts and cultured fibroblasts was reverse transcribed to cDNA using the iScript cDNA synthesis kit (Bio-Rad) following the manufacturer’s guidelines. Quantitative polymerase chain reaction was performed using the SYBR green (Bio-Rad) method on the iQ5 Real-Time PCR Detection System (Bio-Rad).

**Zymography**

Matrix metalloproteinase (MMP) activity in the infarcted myocardium was assessed using gelatin zymography as previously described.

**Preparation of Single Cell Suspensions From Infarcted Mouse Hearts and Flow Cytometric Analysis**

Single cell suspensions were obtained from infarcted WT and knockout hearts as previously described.

**Fibroblast Isolation and Stimulation**

Fibroblasts were isolated from normal mouse hearts and stimulated as previously described.

**Isolation of Fibroblasts and Macrophages From Infarcted Hearts**

Macrophages and fibroblasts were isolated from control and infarcted hearts for immunofluorescent staining and for RNA extraction.

**Immunofluorescent Staining of Isolated Cells and Paraffin-Embedded Sections**

Primary cells were seeded in the chambers of Culture Slides (BD Falcon) and allowed to attach 24 hours to 72 hours. After rinsing with PBS, fibroblasts or macrophages were fixed for 10 minutes in 2% solution of paraformaldehyde (Sigma) in PBS and permeabilized using 0.1% Triton-X (Sigma) in PBS. Paraffin sections were deparaffinized, hydrated, and rinsed in distilled water. Antigen retrieval was performed by heating sections in an antigen retrieval solution (Abcam) for 30 minutes at 95°C. The sections were blocked 30 minutes with Dulbecco PBS with Mg2+, Ca2+ containing 10% rabbit serum. Subsequently, slides were double-stained with goat anti-mouse IRAK-M (1:200; Santa Cruz) and rat anti-mouse Mac2 (1:200; Cedarlane Burlington, Canada) or mouse anti-α-smooth muscle actin (1:200; Sigma, St. Louis, MO). The mouse on mouse (M.O.M) kit (Vector Laboratories) was used for (α-smooth muscle actin staining. Alexa 488–conjugated (Molecular Probes) or Alexa 594–conjugated secondary antibody (Molecular Probes) was used. The immunostained sections were digitally imaged using a Zeiss fluorescence microscope.
Statistical Analysis
Data are expressed as means±SEM. Statistical analysis was performed using unpaired, 2-tailed Student t test using Welch correction for unequal variances and 1-way ANOVA with Tukey multiple comparison test. Paired t test was used to compare echocardiographic parameters before myocardial infarction and after 7 to 28 days of reperfusion. Statistical analyses were performed using Prism software. P<0.05 was considered to be significant. Mortality was compared using the log-rank test.

Results
Biphasic Upregulation of IRAK-M in Reperfused Mouse Infarcts
Quantitative polymerase chain reaction analysis demonstrated significant IRAK-M mRNA upregulation in the infarcted myocardium. The time course of IRAK-M induction showed a biphasic response (Figure 1), characterized by marked early upregulation after 6 hours of reperfusion, followed by a second peak after 7 days of reperfusion (Figure 1A).

IRAK-M Is Localized in Infarct Macrophages and Myofibroblasts
Dual immunofluorescence was used to study IRAK-M localization in the infarcted myocardium. IRAK-M immunoreactivity in the infarcted heart was localized in Mac-2+ infarct macrophages and in spindle-shaped, α-smooth muscle actin–positive myofibroblasts (Figure 1B and 1C). Moreover, infarct myofibroblasts and CD11b+ leukocytes isolated from the infarcted heart after 72 hours of reperfusion exhibited IRAK-M expression (Figure 1D–1G). To study cell-type specific changes in the timing of IRAK-M expression, we assessed IRAK-M mRNA levels in cardiac fibroblasts and CD11b+ leukocytes harvested from the infarcted heart. Isolated fibroblasts had a 3-fold increase in IRAK-M mRNA levels after 24 hours to 72 hours of reperfusion in comparison with control cardiac fibroblasts. When compared with control CD11b+ cells harvested from normal hearts, leukocytes isolated after 6 hours of reperfusion showed a trend toward increased IRAK-M mRNA expression (Figure I in the online-only Data Supplement).

IRAK-M Loss Is Associated With Enhanced Adverse Remodeling Despite the Absence of Effects on the Size of the Infarct
IRAK-M–null and WT animals had comparable mortality after myocardial infarction (P=NS). Triphenyltetrazolium chloride/Evans blue staining demonstrated that IRAK-M loss does not affect the size of the infarct after 1 hour of ischemia and 24 hours of reperfusion (Figure 1H–1J). Two independent techniques, echocardiographic imaging (Figure 2A–2G; Table I in the online-only Data Supplement) and quantitative

Figure 1. Interleukin-1 receptor-associated kinase (IRAK)-M upregulation in the infarcted mouse heart. A, IRAK-M mRNA expression in the infarcted myocardium showed a biphasic response—significant upregulation was noted after 6 hours of reperfusion followed by a second peak after 7 days of reperfusion (**P<0.01 vs sham). B and C, Dual immunofluorescence combining IRAK-M staining (red) and Mac-2 immunofluorescence (B; green) to identify macrophages, or α-smooth muscle actin SMA staining to label myofibroblasts and smooth muscle cells (C; green). Dual immunofluorescent staining localized IRAK-M (red) in Mac-2+ macrophages (B; arrows) and spindle-shaped α-smooth muscle actin+ myofibroblasts (C; arrows) infiltrating the infarcted myocardium (1-hour ischemia/7-day reperfusion). Counterstained with 4′,6-diamidino-2-phenylindole (DAPI; blue). D–G, Fibroblasts (D) and macrophages (E) isolated from infarcted hearts 3 days after reperfusion expressed IRAK-M (green; arrows). IRAK-M immunofluorescence in fibroblasts (F) and macrophages (G) isolated from IRAK-M–null infarcts served as negative controls. Cells were counterstained with DAPI (blue). H–J, IRAK-M loss did not affect the size of the infarct after 1-hour ischemia and 24 hours of reperfusion. Triphenyltetrazolium chloride/Evans Blue staining was used to measure the area at risk (AAR) and the infarcted area (INF) in the ischemic and reperfused heart. WT and IRAK-M–null animals had comparable AAR (H), infarcted area (I), and INF:AAR ratio (J). LV indicates left ventricle; KO, knockout; WT, wild type.
morphometry (Figure 2H–2L), demonstrated that IRAK-M loss was associated with enhanced adverse remodeling after myocardial infarction. Systolic and diastolic chamber dimensions measured through echocardiography (left ventricular end-diastolic dimension, left ventricular end-systolic dimension, left ventricular end-systolic volume, and left ventricular end-diastolic pressure; Figure 2A–2G) and morphometrically-derived left ventricular end-diastolic pressure and left ventricular end-diastolic dimension (Figure 2H–2L) were significantly higher in IRAK-M−null mice after 7 and 28 days of reperfusion, indicating increased chamber dilation. Left ventricular mass was also significantly higher in infarcted IRAK-M−null hearts, suggesting accentuated hypertrophic remodeling. Increased adverse remodeling in the absence of IRAK-M was associated with reduced fractional shortening, reflecting worse systolic dysfunction (Figure 2D). Because acute infarct size was comparable between WT and IRAK-M−null mice (Figure 1H–1J), accentuated adverse remodeling in IRAK-M−null hearts was not a result of more extensive cardiomyocyte injury. Moreover, scar size after 7 to 28 days of reperfusion was comparable between IRAK-M−− and WT animals (Figure 2I).

**IRAK-M+/− Mice Have Enhanced Postinfarction Inflammation Exhibiting Increased Myocardial Cytokine mRNA Expression and Accented Macrophage and Neutrophil Infiltration**

Increased chamber dilation in infarcted IRAK-M−null hearts was associated with an accentuated inflammatory response. Immunohistochemical staining demonstrated that IRAK-M−null infarcts had enhanced macrophage and neutrophil infiltration after 3 to 7 days of reperfusion (Figure 3). Moreover, myocardial IL-1β mRNA expression was markedly accentuated in IRAK-M−null hearts (Figure 3I).

**IRAK-M−− Mice Have Increased MMP Expression and Activity in the Infarcted Heart Associated With Reduced Collagen Deposition in the Healing Scar**

Adverse dilative remodeling in IRAK-M−deficient mice was accompanied by enhanced MMP expression and activity. Quantitative polymerase chain reaction analysis demonstrated that myocardial MMP-3, MMP-8, and MMP-9 mRNA levels were significantly higher in IRAK-M−null infarcts when compared with infarcted WT hearts after 24 hours of reperfusion (Figure 4A–4D). MMP-2 mRNA expression levels were also 50% higher in IRAK-M−null infarcts; however, the difference in comparison with WT infarcts did not reach statistical significance (P=0.28). Myocardial tissue inhibitor of matrix metalloprotease-1 and TIMP-2 mRNA expression was also higher in infarcted IRAK-M−null animals (Figure 4E and 4F). Gelatin zymography demonstrated that IRAK-M−null infarcts had significantly higher latent and active MMP-2 levels when compared with WT animals after 72 hours of reperfusion (Figure 4H). A trend toward increased active MMP-9 levels was noted in IRAK-M−null animals (P=0.19). Accented activation of MMPs in IRAK-M−− infarcts resulted in a marked reduction in collagen deposition in the healing scar (Figure 4I and 4J).

**Cytokine-Stimulated IRAK-M Expression in Cardiac Fibroblasts**

Because myofibroblasts are an important source of IRAK-M in the healing infarct (Figure 1), we explored the mechanisms of IRAK-M regulation in mouse cardiac fibroblasts. Stimulation with IL-1β, tumor necrosis factor-α and platelet-derived growth factor-BB, cytokines, and growth factors released in the infarcted myocardium upregulated IRAK-M synthesis in WT mouse cardiac fibroblasts (Figure 5A). Moreover, the TLR agonist lipopolysaccharide induced marked IRAK-M upregulation in cardiac fibroblasts. IRAK-M−null cells were used as a negative control and exhibited negligible IRAK-M mRNA expression.

**IRAK-M Expression Limits the Matrix-Degrading Potential but Not the Proinflammatory Capacity of IL-1β–Stimulated Cardiac Fibroblasts**

To examine whether the effects of IRAK-M loss on cardiac remodeling are attributable to defective regulation of fibroblast inflammatory and matrix-degrading capacity, we compared baseline and IL-1β–stimulated expression of MMPs and inflammatory mediators between IRAK-M−null and WT cardiac fibroblasts (Figure 5). Upon IL-1β stimulation MMP-2 and MMP-8 mRNA expression was significantly higher in IRAK-M−null cardiac fibroblasts, when compared with WT cells (Figure 5B). Moreover, the supernatant collected from IRAK-M−null cells exhibited accentuated baseline MMP-2 activity in comparison with the supernatant obtained from WT cells (Figure II in the online-only Data Supplement). In contrast, IRAK-M loss was not associated with accentuation of fibroblast inflammatory gene synthesis. Baseline and IL-1β–stimulated tumor necrosis factor-α and monocyte chemoattractant protein-1 mRNA expression was comparable between IRAK-M−null and WT fibroblasts, whereas IL-1–induced IL-6 mRNA expression was significantly lower in IRAK-M−null cells (Figure 5C). Because IRAK-M may act by stabilizing mitogen-activated protein kinase (MAPK) phosphatase-1, thus negatively regulating TLR2-induced p38 MAPK phosphorylation,19 we compared the effects of IRAK-M loss on p38 MAPK phosphorylation in stimulated mouse cardiac fibroblasts. IL-1β and lipopolysaccharide–stimulated cardiac fibroblasts exhibited comparable activation of p38 MAPK signaling in response to IL-1β and lipopolysaccharide (Figure III in the online-only Data Supplement).

**IRAK-M Absence Is Associated With Increased Leukocyte Inflammatory Activity and Enhanced Recruitment of Proinflammatory Ly6Ch Monocytes**

To examine whether accentuated inflammation in IRAK-M−null hearts is a result of uncontrolled inflammatory activity in macrophages, we used flow cytometry to assess phenotype and cytokine expression in monocyctic cells harvested from the infarcted myocardium. After 72 hours of reperfusion, the absolute number of CD45+ leukocytes and of CD45+/
Figure 2. Echocardiography (A–G) and quantitative morphometry (H–L) demonstrate that interleukin-1 receptor-associated kinase (IRAK)-M absence results in accentuated dilative remodeling and worse systolic dysfunction after reperfused myocardial infarction. A, Representative images of long-axis B mode and short-axis M-mode echocardiography in wild-type (WT) and IRAK-M knockout (KO) mice at baseline and after 7 to 28 days of reperfusion. B–G, IRAK-M−null mice had worse systolic dysfunction and accentuated remodeling of the infarcted heart when compared with WT animals. Quantitative analysis of echocardiographic parameters demonstrated that IRAK-M null hearts had increased chamber dilation (indicated by higher left ventricular end-diastolic dimension [LVEDD; B], left ventricular end-systolic dimension [LVESD; C], left ventricular end-diastolic pressure [LVEDP; E], and left ventricular end-systolic volume [LVESV; F]), worse systolic dysfunction (indicated by lower FS; D) and accentuated hypertrophy (evidenced by higher LV mass; G) after 7 to 28 days of reperfusion (**P<0.01, *P<0.05 vs corresponding WT). H, Quantitative morphometric analysis demonstrates that IRAK-M disruption is associated with increased postinfarction remodeling. Hearts were perfusion-fixed, and systematic morphometric analysis of the geometry of the left ventricle was performed through assessment of sections cut at 250-μm intervals from base to apex. Representative images show cross sections of WT and IRAK-M−null sham and infarcted hearts (after 7 and 28 days of reperfusion). I–L, Quantitative analysis demonstrated that although scar size was comparable between IRAK-M−null and WT animals after 7 to 28 days of reperfusion (B), IRAK-M−/− mice had increased LVEDD and LVEDV and higher LV mass when compared with WT animals (**P<0.01, *P<0.05 vs corresponding WT mice).
CD11b+ monocytes per weight of infarcted myocardium was markedly higher in IRAK-M−null animals when compared with WT mice (Figure 6). Moreover, the number of IL-1β+ cells and the number of CD45+/CD11b+ monocytes expressing IL-1β in the infarcted heart was markedly higher in IRAK-M−null mice when compared with WT animals. IRAK-M loss was associated with selectively increased infiltration of the infarcted myocardium with proinflammatory Ly6C+ monocytes; in contrast, the number of reparative Ly6Clo monocytes was comparable between IRAK-M−/− and WT infarcts (Figure 6; Table II in the online-only Data Supplement).

**Discussion**

Our study demonstrates, for the first time, the critical role of endogenous negative regulation of the innate immune response in preventing uncontrolled inflammation after ischemic injury and in protecting the infarcted myocardium from adverse remodeling. We found that upregulation of the endogenous suppressor of TLR/IL-1 signaling IRAK-M in macrophages and fibroblasts infiltrating the infarcted heart is an important inhibitory mechanism that limits chamber dilation, protecting the heart from uncontrolled inflammation and excessive matrix degradation. Beyond the established role of IRAK-M in suppression of macrophage-derived inflammatory responses, our study reveals novel cellular actions of IRAK-M in the inhibition of the matrix-degrading capacity of cytokine-stimulated fibroblasts.

TLR signaling is critically involved in the response to injury after myocardial infarction.20 Generation of danger-associated molecular patterns,21 such as heat shock proteins22 and fibronectin fragments,23 stimulates TLR signaling in the infarcted heart. TLR-2 activation in infarct leukocytes
Chen et al. IRAK-M in Myocardial Infarction

accentuates inflammatory injury after myocardial infarction and promotes cardiac fibrosis and adverse remodeling, whereas TLR-4 signaling exerts proinflammatory actions stimulating lymphocyte infiltration and enhancing chamber dilation. Activation of cell surface TLRs recruits the adaptor molecule myeloid differentiation factor 88.

Figure 4. Adverse remodeling in infarcted interleukin-1 receptor-associated kinase (IRAK)-M−/− hearts is associated with increased matrix metalloproteinases (MMP) mRNA expression and enhanced protease activity. A–F, Quantitative polymerase chain reaction demonstrated increased expression of MMPs (A, MMP-2; B, MMP-3; C, MMP-8; and D, MMP-9) and tissue inhibitor of matrix metalloproteinases (TIMPs) (E, TIMP-1; F, TIMP-2) in IRAK-M−/− hearts after 24 hours of reperfusion. G, Zymography was used to quantitatively assess MMP activity in the infarcted myocardium after 72 hours of reperfusion. H, Quantitative analysis demonstrated that levels of active MMP-2 were markedly higher in IRAK-M−/− hearts (**P<0.01 vs wild type [WT]). A trend toward increased MMP-9 activity was also noted (P=0.19). I and J, Picrosirius red staining was used to assess collagen deposition in the scar (1-hour ischemia/7-day reperfusion). Increased MMP activity in IRAK-M−/− mice was associated with less collagen deposition. (**P<0.01, *P<0.05 vs corresponding WT). KO indicates knockout.
subsequent binding of members of the IRAK family induces proinflammatory pathways in the infarcted heart. Studies using genetically targeted animals suggested an important role for IRAK-4 in mediating the postinfarction inflammatory response.25 Moreover, rapid activation of IRAK-1 in the ischemic26 and infarcted heart 27 plays an important role in mediating inflammatory and apoptotic responses. Although the innate immune response is important for recognition of ischemic injury and for subsequent recruitment of inflammatory leukocytes in the infarcted myocardium, tight regulation of the TLR cascade is needed to prevent uncontrolled inflammation and excessive matrix degradation.

Both in vivo and in vitro studies support the role of IRAK-M in the negative regulation of TLR-dependent responses. In contrast to IRAK-1 and IRAK-4, IRAK-M lacks kinase activity, but functions as a decoy, inhibiting TLR/IL-1-driven proinflammatory signaling. IRAK-M–null mice exhibit accentuated inflammatory responses after bacterial and viral infections.13,15,28 In vitro, IRAK-M–deficient macrophages display enhanced activation of IL-1/TLR signaling.14,28 Several distinct mechanisms have been suggested to explain the inhibitory effects of IRAK-M on the TLR response. First, IRAK-M is thought to bind myeloid differentiation factor 88/IRAK-4 inhibiting IRAK-4 phosphorylation of IRAK-1. Second, IRAK-M may act by preventing IRAK-1 from dissociating from the myeloid differentiation factor 88 complex, thus inhibiting nuclear factor-κB activation. Third, IRAK-M may exert IRAK-1–independent actions by stabilizing MAPK phosphatase-1, thus negatively regulating TLR2-induced p38 phosphorylation.29

Consistent with a critical role for IRAK-M in regulation of the inflammatory response, IRAK-M loss was associated

Figure 5. A, Interleukin-1 receptor-associated kinase (IRAK)-M regulation in stimulated cardiac fibroblasts. Interleukin (IL)-1β, the toll-like receptor agonist lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α), and platelet-derived growth factor (PDGF)-BB upregulated IRAK-M mRNA expression in cardiac fibroblasts (**P<0.01, *P<0.05 vs control). mRNA isolated from IRAK-M–null fibroblasts was used as a negative control. B and C, IRAK-M loss enhances cytokine-stimulated matrix metalloproteinases (MMP) expression but does not affect inflammatory cytokine synthesis in isolated cardiac fibroblasts. B, IRAK-M–null fibroblasts exhibited increased MMP-2 and MMP-8 expression on stimulation with IL-1β, when compared with wild-type (WT) fibroblasts. In contrast, MMP-3 and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) expression was comparable between groups. C, IL-1β–mediated upregulation of TNF-α and monocyte chemoattractant protein-1 (MCP-1) was comparable between wild-type (WT) and IRAK-M–null fibroblasts. IRAK-M–null fibroblasts had reduced IL-6 upregulation on IL-1β stimulation. KO indicates knockout.
Figure 6. Interleukin-1 receptor-associated kinase (IRAK)-M loss is associated with the infiltration of the infarcted myocardium with pro-inflammatory monocytes. Flow cytometric analysis of cell suspensions harvested from the infarcted heart after 72 hours of reperfusion demonstrated marked increases in the number of infiltrating mononuclear cells and IL-1β-positive cells. Cell suspensions from infarcted hearts of C57BL/6 and IRAK-M−null mice were stained with LIVE/DEAD Fixable Dead Cell Stain, −CD45, −CD11b, −Ly6C, −F4/80, and −interleukin (IL)-1β mAbs. A and B, Representative dot plots show significantly higher number of live CD45+ hematopoietic cells (A), CD11b+/F4/80− monocytes (gate 1), and CD11b+/F4/80+ macrophages/dendritic cells (gate 2) (B) in IRAK-M−/− infarcts after 72 hours of reperfusion. C, Representative dot plots show the percentage of IL-1β-positive cells in Ly6C− and Ly6C+ subgroups of monocytic cells is shown in representative histograms comparing findings from wild-type (WT; blue) and IRAK-M−null infarcts (red). D, IL-1β expression in Ly6C− and Ly6C+ subgroups of monocytic cells is shown in representative histograms comparing findings from wild-type (WT; blue) and IRAK-M−null infarcts (red). E–L, Quantitative analysis of flow cytometric findings showed that IRAK-M−/− infarcts had markedly higher absolute number of CD45+ hematopoietic cells (E), CD45+/CD11b+ mononuclear cells (F), Ly6C+/CD45+/CD11b+ proinflammatory monocytes (G), and F4/80+ macrophages (H) per mg of weight of the infarcted heart. Monocytes and macrophage subpopulations harvested from IRAK-M−/− infarcts had increased expression of the proinflammatory cytokine IL-1β, reflecting accentuated inflammatory activity. The number of IL-1β+/CD45+ hematopoietic cells (I), IL-1β+/CD45+/CD11b+ mononuclear cells (J), IL-1β+/CD45+/CD11b+ macrophages (K), and IL-1β+/F4/80+ macrophages (L) was markedly higher in IRAK-M−null infarcts (**P<0.01, *P<0.05 vs corresponding WT animals). KO indicates knockout.
with increased cytokine expression in the infarcted heart, leading to enhanced infiltration of the infarcted myocardium with inflammatory leukocytes (Figure 3) and accentuated MMP expression and activity (Figure 4). Defective negative regulation of the inflammatory response did not affect the extent of cardiomyocyte injury after reperfused infarction. However, enhanced inflammation and protease activity in IRAK-M-null infarcts resulted in worse geometric remodeling of the infarcted ventricle and increased systolic dysfunction. Endogenous IRAK-M upregulation functions as a key molecular signal in cardiac repair protecting the infarcted heart from uncontrolled inflammation and excessive matrix degradation.

Although traditionally viewed as a macrophage-specific product, IRAK-M expression is increasingly identified in other cell types. In patients with early-onset persistent asthma, high-level IRAK-M expression has been demonstrated in pulmonary epithelial cells. In mice, the pattern of IRAK-M expression seems broader, as IRAK-M synthesis has been demonstrated in murine neutrophils, National Institutes of Health 3T3 fibroblasts, B cells, dendritic cells, and various types of epithelial cells. However, in vivo biological actions of IRAK-M appear to involve predominantly inhibitory effects on monocytes/macrophages. Our study demonstrates for the first time that effects of IRAK-M in the infarcted heart involve actions in both macrophages and cardiac fibroblasts.

Cardiac fibroblasts expressed significant amounts of IRAK-M both in vivo and in vitro (Figures 1 and 5). A wide range of stimuli, including the TLR agonist lipopolysaccharide, the proinflammatory cytokine IL-1β, and the fibrogenic growth factors transforming growth factor-β and platelet-derived growth factor enhanced IRAK-M expression in cardiac fibroblasts (Figure 5). IRAK-M loss did not affect the inflammatory potential of cardiac fibroblasts (Figure 5C). However, IRAK-M expression played an important role in restraining the matrix-degrading potential of cardiac fibroblasts; in its absence cytokine-induced fibroblast MMP synthesis was markedly accentuated (Figure 5B). Thus, our observations suggest a broader role for IRAK-M in matrix remodeling and tissue repair.

In addition to its effects on cardiac fibroblasts, IRAK-M modulated mononuclear cell phenotype and function. Using flow cytometric analysis of cells harvested from the infarcted heart, we found that IRAK-M deficiency had profound effects on the phenotype and inflammatory activity of monocytes infiltrating the infarcted myocardium. IRAK-M-null infarcts exhibited a markedly higher number of IL-1β-expressing CD45+/CD11b+ leukocytes. Enhanced IL-1β expression by IRAK-M–null infarct monocytes likely reflects their increased capacity to synthesize proinflammatory cytokines upon stimulation with TLR ligands in comparison with WT cells. Moreover, IRAK-M−/− infarcts exhibited alterations in the profile of monocyte subsets recruited in the infarcted myocardium. IRAK-M absence was associated with an abundance of proinflammatory Ly6C+ monocytes in the infarct (Table 2, Figure 6). The increased proportion of inflammatory monocytes in IRAK-M–null infarcts may simply reflect a global accentuation of inflammatory activity leading to increased activation of chemokine-mediated pathways responsible for recruitment of proinflammatory cells.

In addition, because TLR signaling plays an important role in inflammatory monopoiesis, IRAK-M expression may selectively inhibit generation and release of proinflammatory Ly6C+ cells.

Our findings have important therapeutic implications. More than 20 years ago, extensive experimental evidence suggested that inflammatory mediators and infiltrating leukocytes may induce death of surviving cardiomyocytes in the infarcted myocardium, extending ischemic myocardial injury. Unfortunately, the effectiveness of anti-inflammatory strategies in reducing infarct size in large animal models of reperfused infarction did not translate into clinical success; both anti-integrin approaches and complement inhibition failed to reduce acute myocardial injury in patients with acute myocardial infarction. Our experimental findings are consistent with these clinical observations, suggesting limited effects of the inflammatory reaction on cardiomyocyte survival. IRAK-M absence resulted in accentuated inflammation without affecting the size of the infarct (Figure 1). Moreover, marked attenuation of the postinfarction inflammatory response attributable to the disruption of IL-1 signaling, or loss of adhesion molecule-mediated interactions, had no effect on the size of the infarct. If inhibition of inflammation does not protect ischemic cardiomyocytes, is there a future for strategies modulating the inflammatory reaction after myocardial infarction? Our findings underscore the importance of negative regulators of the innate immune response in protection of the infarcted ventricle from adverse remodeling. Enhanced, or prolonged, innate immune signaling in the infarcted myocardium may not acutely increase the extent of ischemic injury but is associated with defective suppression of the inflammatory response and uncontrolled activation of proteases. Excessive protease-mediated matrix degradation induces chamber dilation, promoting systolic dysfunction. In patients surviving an acute myocardial infarction the extent of dilatative remodeling may reflect, at least in part, their ability to control the inflammatory reaction. Genetic defects, or pathological conditions, that disrupt key endogenous regulatory signals (such as IRAK-M) may be responsible for worse remodeling leading to rapid development of heart failure. In this subgroup of patients, anti-inflammatory strategies may attenuate chamber dilation, protecting from progression of heart failure.

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Disclosures

None.

References


Endogenous IRAK-M Attenuates Postinfarction Remodeling Through Effects on Macrophages and Fibroblasts
Wei Chen, Amit Saxena, Na Li, Jinyu Sun, Amit Gupta, Dong-Wook Lee, Qi Tian, Marcin Dobaczewski and Nikolaos G. Frangogiannis

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We are grateful to the reviewers for their kind comments and insightful criticisms that significantly improved the quality of our work. We have extensively revised our manuscript according to their recommendations. New experiments were performed: a) to compare acute infarct size between WT and IRAK-M null mice (Figure 1), b) to examine the effects of IRAK-M loss on cytokine and LPS-simulated activation of p38 MAPK in cardiac fibroblasts (supplemental figure 3), c) to assess IRAK-M expression in the infarcted myocardium at later timepoints (after 14 days of reperfusion) (Figure 1), d) to examine IRAK-M expression in fibroblasts and macrophages harvested from the infarcted heart (supplemental figure 1), e) to assess the effects of IRAK-M loss on the matrix-degrading activity of cardiac fibroblasts (supplemental figure 2). Moreover, extensive work was done to study IRAK-M expression with western blotting (described in detail in the specific responses). The discussion has been revised to address the reviewers’ criticisms.

Specific comments were addressed as follows:

**Reviewer 1:**
1) Is it possible that IRAK-M may play a key role beyond innate immune response (and MMP induction)? Page 2 of the discussion describes several alternate roles for IRAK-M in modulating cell signaling. Perhaps a couple of these could be easily tested (e.g. MAPK).

**Response:** Thank you for this great suggestion. We have performed new in vitro experiments examining the role of IRAK-M in LPS and IL-1β-induced p38 MAPK activation in cardiac fibroblasts. Our findings showed that WT and IRAK-M null cardiac fibroblasts exhibit comparable activation of p38 MAPK upon stimulation with IL-1b and the TLR agonist, LPS. These new experiments are presented in supplemental figure 3.

2) Considering the above, the data in the manuscript, and the fact that IRAK-M is found in cardiac fibroblasts, perhaps the discussion should center more around IRAK-M as a cardiac regulator, and less on innate immunity.

**Response:** Thank you for your comment. We have revised our discussion to put more emphasis on the role of IRAK-M in cardiac remodeling. The second part of the discussion has been rewritten.

**Reviewer 2:**
The experimental studies have been performed in a careful manner and do provide some new information regarding myocardial inflammation and remodeling.

**Comments:**
1) The authors suggest that the study is essential for the design of new therapeutic strategies by activating inhibitory pathways and modulate myocardial inflammation and reduce the development of heart failure following infarction. This concept has been promoted for many years with very little clinical translation. As the authors are well aware, myocardial inflammation is required for myocardial healing. The authors should clearly elaborate on how IRAK-M represents a feasible and viable clinical target. What realistic therapeutic approaches could be applied to activate this inhibitory pathway during the evolution of acute MI? How is IRAK-M different from previous targets? How is this approach superior to infarct size reduction and therapies that minimize myocardial infarct size?

**Response:** We thank the reviewer for these insightful comments. As the reviewer points out, 20-25 years ago the concept of post-infarction inflammatory cardiomyocyte injury was first proposed. Extensive experimental evidence (primarily derived from neutralization studies in large animal models) suggested that inhibition of key pro-inflammatory signals markedly reduces the size of the infarct saving a large number of cardiomyocytes from death. It was suggested that early inflammation
following infarction accentuates cardiomyocyte death, in part through cytotoxic effects of leukocytes and their products. Unfortunately, these impressive experimental findings did not translate into therapeutic advances. Clinical trials designed to save cardiac muscle by inhibiting early post-infarction inflammation through blockade of the complement cascade (APEX-AMI) or via inhibition of integrin signaling (HALT-MI) did not improve outcome and did not reduce the size of the infarct.

In recent years, our own work and studies from other labs in genetically targeted mice suggested that inflammatory signals triggered following infarction may not accentuate cardiomyocyte death. We showed no significant differences in infarct size upon disruption of key pro-inflammatory signals (such as IL-1 and MCP-1) (Dewald et al Circ Res 2005; Bujak et al Am J Pathol 2008) despite impressive reduction in inflammation. However, we also found that overactive inflammatory cascades may have late effects, contributing to the development of dilative remodeling following infarction, in part by increasing protease activity in the infarcted heart. As the reviewer points out the inflammatory cascade is closely intertwined with the reparative response. We propose that, although the acute inflammatory reaction does not increase the area of infarction, **timely suppression of the post-infarction inflammatory cascade is essential to protect from adverse remodeling.** Adverse remodeling in patients with myocardial infarction may reflect, at least in part, defective suppression and resolution of inflammation. Our current work identifies for the first time IRAK-M as a key endogenous STOP signal that protects from adverse remodeling by limiting the innate immune response. This is the first demonstration of the consequences of unrestrained innate immune response in the injured myocardium. Regarding the therapeutic implications of the study, we do not propose a therapeutic strategy that specifically targets IRAK-M. In the clinical context, patients suffering an acute infarction have very different remodeling responses despite similar amounts of ischemic injury: some patients with large infarcts show rapid dilation (indicative of high protease activity and enhanced inflammation), while others exhibit hypertrophy/fibrosis and diastolic dysfunction in the absence of dilative remodeling (this may suggest overactive matrix-preserving/profibrotic signals). **In patients exhibiting marked chamber dilation and evidence of uncontrolled inflammation following infarction, defective negative regulation of the innate immune response may be the underlying mechanism responsible for the adverse remodeling response.** These patients may be good candidates for anti-inflammatory interventions targeting TLR/IL-1 signaling (such as administration of IL-1Ra/anakinra). We have recently discussed these concepts in invited reviews (Frangogiannis Circ Res 2012). We agree with the reviewer that more specific statements on the therapeutic implications of the findings are needed in the current manuscript. For this reason, we have rewritten the last paragraph to discuss the therapeutic possibilities (Pages 19-20).

2) It is important to determine if IRAK-M is involved only in cardiac remodeling and not in the progression of myocardial infarct size development. Experimental studies should be performed with myocardial ischemia and reperfusion and myocardial infarct size should be measured.

**Response:** We thank the reviewer for this very important point. We have performed new experiments examining the effects of IRAK-M loss on the size of the acute infarct using TTC/Evans Blue staining. We found that WT and IRAK-M null mice had comparable infarct size after 1h of ischemia and 24h of reperfusion. Thus, the effects of IRAK-M loss on cardiac remodeling are not due to an effect on the size of the infarct. The new findings are reported in figure 1 of the revised manuscript.

**Reviewer 3:**

General: The present manuscript reports a number of novel and interesting new findings with respect to IRAK-M functions in the heart and the data shown are of high quality. However, an important control is missing that is critical to support the author’s conclusion that IRAK-M does not affect infarct size, but mediates subsequent remodeling. To this end, it is necessary to show that the infarct size to area at risk at 24 hr following I/R is comparable between these groups (as assessed by standard Evans blue/TTC staining).
**Response:** Thank you very much for this important comment. We have performed new experiments comparing infarct size between WT and IRAK-M null animals after 1h ischemia and 24h of reperfusion. IRAK-M loss did not affect the size of the acute infarct. These new findings are shown in figure 1 of the revised manuscript.

Comments on Experimental Design, Data, and Interpretation:
1) Figure 1A: Expression of IRAK-M at later time points following I/R should be evaluated (i.e. D10, 14) as such data are required to conclude that induction of this gene is biphasic. Is there a cell-type specific difference in the timing of expression (i.e. cardiac fibroblasts followed by infiltrating monocytes/macrophages)? Fig 1B: Statistical differences between the levels of IRAK-M in sham and injured hearts should be shown. Importantly, why is there such a difference between the levels and timing of IRAK-M expression in the wt hearts shown in this panel and those shown in panel A? What does the elevated peak in IRAK-M expression mean in the ILR1- KO hearts? Is it elevated in fibroblasts but reduced in monocytes/macrophages? How does the phenotype of these mice relate to the phenotypes observed in the IRAK k/o hearts? This should be further discussed. At this juncture, the data presented in Fig1B add little new information to the current manuscript. The findings in panels E-H should be substantiated by Western blotting as the IRAK-M antibody appears to exhibit significant background staining in IRAK-M null cells.

**Response:** We thank the reviewer for these excellent comments.

i) As suggested by the reviewer, we have performed new experiments to assess IRAK-M expression levels after 14 days of reperfusion. At this timepoint, myocardial IRAK-M mRNA expression is comparable with sham hearts, thus supporting the biphasic induction of IRAK-M in the infarcted myocardium. The combined findings are shown in figure 1A.

ii) We agree with the reviewer that the IL-1R1 null experiments add little new information to the current manuscript. The accentuated early IRAK-M response in IL-1R1 null infarcts cannot be explained and may be due to increased TLR-induced IRAK-M expression in these animals. We have previously demonstrated that IL-1R1 null animals have marked attenuation of post-infarction inflammation (Bujak et al Am J Pathol 2008); however, this is unlikely to be related to an accentuated IRAK-M response and rather reflects loss of pro-inflammatory IL-1R1 signaling culminating in NF-kappaB activation. As suggested by the reviewer we decided to omit figure 1B.

iii) In order to address the question regarding cell type-specific differences in the timing of IRAK-M expression, we invested a lot of time and effort in identifying an antibody suitable for IRAK-M western blotting in mouse tissues. Unfortunately, our experiments documented that commercially available anti-mouse IRAK-M antibodies appear to bind non-specifically to other proteins. An overview of our work to address this issue follows:

First, we used both monoclonal and polyclonal anti-IRAK-M antibodies from Chemicon; these antibodies have been used in western blotting experiments for mouse IRAK-M detection. We tested the antibodies in samples from the mouse spleen, an organ that contains a large number of IRAK-M+ cells. To our surprise, although a prominent 68-kd band was present, consistent with the size of mouse IRAK-M, this band was seen in both WT and IRAK-M null spleens (Figure 1)

![Figure 1: Western blotting using a monoclonal anti-IRAK-M antibody (Chemicon) shows a band consistent with the expected size of IRAK-M in both WT and IRAK-M KO spleen.](image)
Similar findings were noted in WT and KO infarcts (Figure 2):

**Figure 2:** Western blotting for mouse IRAK-M using a polyclonal antibody from Chemicon shows bands with size consistent with mouse IRAK-M (68kd) in both WT and IRAK-M KO infarcts. (Symbols: C: control heart, 24: 1h ischemia/24h reperfusion)

In order to confirm the absence of IRAK-M in IRAK-M KO mice (as previously reported in Kobayashi et al Cell 2002), we repeated genotyping of the mice used for these experiments (Figure 3). In addition, we documented the complete absence of IRAK-M mRNA in IRAK-M KO spleens using qPCR (Figure 3)

These findings suggested that the 68kD band noted in KO mice may reflect cross-reactivity of the antibody with other proteins. In order to explore this further we immunoprecipitated the 68kd bands from WT and KO spleens and we used MALDI-TOF mass spectrometry to determine the identity of the protein (Figure 4). The extracted 68-kd protein bands were identified as Lymphocyte Cytosolic Protein-1 (LCP1), a 68-kd cytosolic protein found in lymphocytes) and albumin in both WT and KO mice. IRAK-M was not found. These findings suggested that the Chemicon antibody is not specific for IRAK-M.

**Figure 4:** Mass spectrometry findings in 68kD protein bands immunoprecipitated from KO and WT spleens. Analysis identified the bands as Lymphocyte Cytosolic Protein-1 and albumin, not IRAK-M.

In our efforts to identify an anti-IRAK-M antibody suitable for western blotting, we contacted Dr Richard Flavell (Yale University), a renowned expert in the field of innate immunity who developed the IRAK-M null mice and discovered the negative regulatory properties of the protein. Dr Flavell referred us to Dr Koichi Kobayashi (Harvard Medical School) who, as a post-doc in his lab, was the first author in their seminal IRAK-M manuscript (Kobayashi et al Cell 2002). Dr Kobayashi confirmed that he also
had problems with the use of anti-IRAK-M antibodies for western blotting and, on the basis of his experience, could not recommend any anti-IRAK-M antibodies for western blotting. In addition, we purchased a number of commercially available anti-IRAK-M antibodies (Table 1), but had negative results. In western blotting all six IRAK-M Antibodies show 68KD bands in both WT and KO infarcts, probably reflecting the influx of lymphocytes expressing LCP-1.

Table 1: Anti-IRAK-M antibodies tested in western blotting experiments:

<table>
<thead>
<tr>
<th>Company</th>
<th>poly/monoclonal</th>
<th>position</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Chemicon</td>
<td>Poly</td>
<td>C-terminal</td>
<td>Rabbit</td>
</tr>
<tr>
<td>2 Chemicon</td>
<td>Mono</td>
<td>C-terminal</td>
<td>Rabbit</td>
</tr>
<tr>
<td>3 Sigma</td>
<td>Poly</td>
<td>near C-terminal</td>
<td>Goat</td>
</tr>
<tr>
<td>4 Cell Signaling</td>
<td>Poly</td>
<td>near C-terminal</td>
<td>Rabbit</td>
</tr>
<tr>
<td>5 Santa Cruz</td>
<td>Poly</td>
<td>N-terminal</td>
<td>Goat</td>
</tr>
<tr>
<td>6 Invitrogen</td>
<td>Poly</td>
<td>N-terminal</td>
<td>Rabbit</td>
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iv) Because of the lack of suitable antibodies for western blotting, qPCR seems to be the most reliable method for quantitative assessment of IRAK-M expression in the mouse. In order to examine cell-type specific differences in timing of the IRAK-M response we isolated fibroblasts and CD11b+ macrophages from control and infarcted hearts after 6h-7days of reperfusion and extracted RNA from the cells. Fibroblasts isolated from infarcted hearts after 24h-72h of reperfusion exhibited a 3-fold increase in IRAK-M mRNA levels. CD11b+ macrophages also had increased IRAK-M mRNA expression after 6h and 7 days; however, the difference in comparison to control macrophages was not significantly different. Thus, no cell-type specific patterns explaining the biphasic IRAK-M response ion infarcted hearts were apparent. These findings are reported in Supplemental figure 1.

2) Figure 2. As mentioned above, infarct size should be evaluated at 24 hr post-I/R. As well, it would be nice to show cross-sections from un-injured hearts in panel H.

Response: We thank the reviewer for this suggestion. We have revised figure 2H to include cross-sections from sham hearts.

3) Figure 4: Details should be provided as to the time points evaluated for MMP expression. As noted above, the relevance of these findings to changes in scar size should be further discussed. mRNA levels for collagen should be shown to substantiate that the decreased content is likely due to elevated degradation.

Response: In the revised version we have indicated the timepoints evaluated for MMP mRNA expression (24h R) and activity (72h R). Moreover, we assessed collagen type I and type III mRNA expression in Wt and IRAK-M null infarcts after 24h of reperfusion. The findings suggest differential effects of IRAK-M loss on collagen I and III transcription. Collagen I mRNA levels were lower in IRAK-M null infarcts, whereas there was a trend for higher type III collagen mRNA expression (Table 2). The basis for these findings is unclear. Systematic analysis of collagen levels at several different timepoints would be needed, but was not possible within the time constrains of a revision.
Table 2: Collagen mRNA levels in WT and IRAK-M null infarcts (1h ischemia/24h reperfusion).

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>IRAK-M KO</th>
</tr>
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<tbody>
<tr>
<td>Collagen I:GAPDH</td>
<td>0.000160+0.00004*</td>
<td>0.000026+0.00002</td>
</tr>
<tr>
<td>Collagen III:GAPDH</td>
<td>0.0000014+0.00000016</td>
<td>0.0000043+0.0000014 p=0.07</td>
</tr>
</tbody>
</table>

4) Fig 5: The authors conclude in the Abstract that IRAK-M expression was up-regulated in cytokine-stimulated murine cardiac fibroblasts and suppressed their matrix-degrading properties…” This conclusion is overstated. The authors should show that the enhanced MMP message actually leads to a change in matrix degrading activity. Do isolated IRAK-M null fibroblasts exhibit defects in FITC-collagen or gelatin-degradation in comparison to similarly treated wt cells?

**Response:** We agree with the reviewer that increased MMP mRNA expression does not necessarily imply accentuated activity. In order to examine the effects of IRAK-M loss on fibroblast matrix-degrading capacity we compared gelatinase activity in the supernatant of stimulated WT and IRAK-M null cardiac fibroblasts. We found that, when compared to supernatants harvested from WT cells, supernatants from IRAK-M null fibroblasts exhibited increased baseline MMP-2 activity. A trend towards increased MMP-2 activity was present in supernatants obtained from IRAK-M -/- cells stimulated with IL-1β. These findings are reported in Supplemental figure 2.

5) More details should be provided for the cardiac function analysis performed by echocardiography. The referenced manuscript does not contain details for this method. It appears by the images shown that these were performed on anesthetized mice. Heart rates should be included as significant differences in levels of cardiac depression can lead to alterations in performance (unrelated to the genotype).

**Response:** Thank you very much for your comment. Details on the echocardiographic imaging analysis are provided in the online supplement. In the detailed description of the methodology we have indicated that imaging was performed in anesthetized animals. Moreover, supplemental table 1 provides full echocardiographic data (including heart rate data), as suggested by the reviewer. No significant differences in heart rate were observed between groups.
SUPPLEMENTAL MATERIAL:

SUPPLEMENTAL METHODS:

Animals

C57BL/6, IRAK-M -/- mice and IL1RI -/- animals in a C57BL/6 background were purchased from Jackson Laboratories. All protocols were approved by the committee on animal research care at Baylor College of Medicine and at Albert Einstein College of Medicine.

Murine Model of Reperfused Myocardial Infarction

A total of 130 C57BL6 mice, 146 IRAK-M -/- mice and 27 IL1RI -/- were used in the study. Two to three month-old mice were anesthetized by isoflurane inhalation (isoflurane 2-3% vol/vol). Myocardial infarction was induced using a closed-chest mouse model of reperfused myocardial infarction, as previously described.

After 6h-28d of reperfusion, the chest was opened and the heart was immediately excised, fixed in zinc-formalin and embedded in paraffin for histological studies, or snap frozen in liquid nitrogen and stored at -80°C for RNA and protein extraction. Sham animals were prepared identically without undergoing coronary occlusion/reperfusion. Animals used for histology underwent 24hr, 3-day and 7-day reperfusion protocols. Mice used for RNA extraction underwent 6 hours (WT, n = 8; KO, n =8), 24 hours, 72 hours and 7 days of reperfusion (WT, n = 9; KO, n =10 per group). Mice used for assessment of MMP activity were killed after 72 hours of reperfusion (WT, n = 8; KO, n = 8). IRAK-M KO and wild-type mice used for flow cytometric analysis underwent 24hr and 72hr of reperfusion (n =7 per group). Additional animals were used for perfusion-fixation and systematic morphometric analysis after 7 days and 28 days of reperfusion (WT, n=15; KO, n=17 per group) to assess remodeling-associated parameters as
Echocardiography

Echocardiographic studies were performed in anesthetized mice before instrumentation and after 7 days and 28 days of reperfusion (WT, n=14; IRAK-M-/-, n = 18) using a 25-MHz probe (Vevo 770; Visualsonics. Toronto ON) as previously described. Long-axis B-mode was used to assess the geometric characteristics of the left ventricle after myocardial infarction. Short-axis M-mode was used for measurement of systolic and diastolic ventricular and wall diameters. The left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), left ventricular end-systolic volume (LVESV), and left ventricular end-diastolic volume (LVEDV) were measured as indicators of dilative remodeling. Left ventricular mass (LV mass) was measured as an indicator of hypertrophic remodeling. Fractional shortening (FS = [LVEDD - LVESD] ×100/LVEDD) was calculated for assessment of systolic ventricular function. The percent change in these parameters after infarction was quantitatively assessed using the following formulas: ΔLVEDD=(LVEDD 7 days or 28 days-LVEDD pre)×100/LVEDD pre, ΔLVESD=(LVESD 7 days or 28 days-LVESD pre)×100/LVESD pre, ΔFS=(FS pre-FS 7 days or 28 days)×100/FS pre, ΔLVESV=(LVESV 7 days or 28 days-LVESV pre)×100/LVESV pre, ΔLVEDV=(LVEDV 7 days or 28 days-LVEDV pre)×100/LVEDV pre.

Perfusion Fixation and Assessment of Ventricular Volumes

Murine hearts were perfused with cardioplegic solution through the jugular vein to promote relaxation. After excision and rinsing in cold cardioplegic solution, a PE-50 catheter was pushed into the left ventricle via aorta. Hearts were fixed for 10 minutes with 10% zinc buffered formalin (Z-fix; Anatech, Battle Creek, MI) by aortic perfusion. After paraffin embedding, the entire heart was cross-sectioned from base to apex at 250 µl intervals. Ten serial
5µl sections were obtained at each interval and the first section from each interval was stained for hematoxylin-eosin. The LVEDD, LVEDV, LV mass and scar size were assessed with ImagePro software (Media Cybernetics, Bethesda, MD) using methods developed in our laboratory.

**Immunohistochemistry and Quantitative Histology**

Leukocytes were identified in formalin-fixed paraffin-embedded sections using immunohistochemistry with the following primary antibodies: monoclonal anti-neutrophil antibody (Serotec, Raleigh NC) and rat anti-mouse Mac-2 (Cedarlane Burlington, Canada) for macrophages. Stained sections were scanned using a Zeiss Axioskop microscope equipped with a Zeiss digital camera. Three to six sections from each heart and five fields from each section were used for quantitative analysis using ImagePro. The collagen network was identified using picrosirius red staining. Ten distinct fields from two different sections were used for quantitative analysis of collagen content in the infarcted heart.

**RNA Extraction and qPCR assay**

Isolated total RNA from the hearts and cultured fibroblasts was reverse transcribed to cDNA using the iScript™ cDNA synthesis kit (Bio-Rad) following the manufacturer’s guidelines. Quantitative PCR was performed using the SYBR green (Bio-Rad) method on the iQ™5 Real-Time PCR Detection System (Bio-Rad). Primers were synthesized at the Baylor College of Medicine Child Health Research Center core facility. The following sets of primers were used in the study: IRAK-M (forward) GCCAGAAGAATACATCAGACAGGG, (reverse) TGTTTCGGGTCATCCAGCAC; MMP-2 (forward) AACTTCCGATTATCCCATGAT, (reverse) GCCAGTACCAGTGCAGTA; MMP-3 (forward) GGAAATCAGTCTGGGCTATACGA (reverse) TAGAAATGGGCAGCATCGATCTTC;
MMP-8 (forward) GATTCAGAAGAAACGTGGACTCAA, (reverse) CATCAAGGCACCAGGATCAGT; MMP-9 (forward) TGTTCGCCGTTCATCTTTTGAG, (reverse) ATCTGGTCATAGTTGGCTGT; TIMP-1 (forward) CAGTAAGGCCCTGTATGCTGTG, (reverse) AGGTGGTGCTCGTTGATTTTG; TIMP-2 (forward) GGAATGACATCTATGGCAACC, (reverse) GGCCGTGTGATAAACTCGAT; MCP-1 (forward) GCTGGAGCATCCACGTGT, (reverse) CTGCTGCTGGGTGATCTCTT; TNF-α (forward) GCCAACGGCATGGATCTC, (reverse) GCAGCCTGTCCCTTGAAAGAG; IL-6 (forward) GCTAAGGACCAAGACCATCCAAT, (reverse) GGCATAACGCACACTAGATTGC and 18S (forward) ACCGCAGCTAGGAATAATGGA, (reverse) GCCTCAGTCGAAAC. Each sample was run in triplicate.

**Zymography**

MMP activity in the infarcted myocardium and in the supernatant collected from control and IL-1β-stimulated fibroblasts was assessed using gelatin zymography as previously described.

**Preparation of single cell suspensions from infarcted mouse hearts and flow cytometric analysis**

Single cell suspensions were obtained from infarcted WT and KO hearts as previously described. Cells harvested from the infarcted heart were counted and reconstituted in staining buffer (BD Biosciences) to a concentration of 1×10⁶ cells/ml. Subsequently cells were incubated with LIVE/DEAD® Fixable Dead Cell Stain single-color dyes (Invitrogen) for 30 min at room temperature to evaluate the viability. After one rinse with washing buffer, cells were incubated with anti-FcγIII/II (clone 2.4G2) antibody (BD Pharmingen) for 15 minutes and labeled at 4°C for 30 minutes simultaneously with following antibodies purchased from BD Pharmingen: Perp-labeled anti-CD45, FITC-labeled anti-CD11b, PE-Cy7-labeled anti-F4/80, APC-Cy7-
labeled anti-Ly6C and Pacific blue-labeled anti-CD19. For intracellular staining, cells were fixed and permeabilized for 20 minutes at 4°C with fixation/permeabilization kit (eBioscience). Subsequently, cells were incubated with PE-labeled anti-IL1β (BD). Finally, cells were washed twice, resuspended in staining buffer, and immediately analyzed with a Becton Dickinson LSRII flow cytometer (BD biosciences). Monocytes/macrophages were defined as Live/dead<sub>low</sub>CD19<sup>neg</sup>CD45<sup>high</sup>CD11b<sup>high</sup> cells. Within this population, subsets were identified as either F4/80<sup>+</sup> or F4/80<sup>-</sup>. Further, each F4/80 subgroup was identified as either Ly-6C<sup>hi</sup> or Ly-6C<sup>lo</sup>. The absolute number of cells in each subset was calculated by multiplying cell number by percent of cells in the subset, which was calibrated by heart weight and expressed as cells/mg. Data analysis was performed using FlowJo (Tree Star, Inc.).

**Fibroblast isolation and stimulation.**

Fibroblasts were isolated from normal mouse hearts as previously described<sup>7,6</sup>. Cells were serum-starved for 24h and subsequently stimulated with 10 ng/ml of LPS (Sigma Aldrich), 10 ng/ml TNFα (R&D Systems), 10 ng/ml PDGF-BB (R&D Systems) and 10 ng/ml IL1β (R&D Systems) for 4 to 24h. At the end of stimulation total RNA was extracted using TRIzol (Invitrogen).

**Isolation of fibroblasts and macrophages from infarcted hearts.**

Macrophages and fibroblasts were isolated from control and infarcted hearts for RNA extraction, or for immunofluorescent staining. Briefly, infarct tissue (1h ischemia followed by 6h, 24h, 72h, or 7 days of reperfusion), or healthy hearts were minced, and placed into a cocktail of 0.25 mg/ml Liberase Blendzyme 3 (Roche Applied Science), 20 U/ml DNase I (Sigma Aldrich), 10 mmol/L HEPES (Invitrogen), 0.1% Sodium Azide in HBSS with Ca<sup>2+</sup> and Mg<sup>2+</sup> (Invitrogen) and shaken at 37°C for 40 minutes. Cells were then triturated through 40μm nylon
mesh and centrifuged (10 min, 200 g, 4°C). Finally, cells were reconstituted with staining buffer (dPBS without Ca\textsuperscript{2+} and Mg\textsuperscript{2+}, 2% FBS, 0.1% sodium azide) and total cell numbers were determined with trypan blue (Mediatech, Inc.). The resulting single-cell suspensions were washed with HBSS supplemented with 0.2% (wt/vol) BSA and 1% (wt/vol) FCS and centrifuged.

Single cells were resuspended in a buffer containing phosphate buffered saline (PBS), pH 7.2, 0.5% BSA and 2 mM EDT\textsubscript{A}, incubated with CD11b\textsuperscript{+} microbeads (Miltenyi Biotec) 10\textmu{l}/10\textsuperscript{7} cells at 4°C for 15min, then washed once and centrifuged. Resuspended cells went through a MACS Column (Miltenyi Biotec) set in a MACS Seperator (Miltenyi Biotec). Unlabeled cells which passed through were collected and washed once with PBS. Adherent CD11b- cells (mostly fibroblasts) were harvested for further experiments. The magnetically labeled CD11b\textsuperscript{+} cells were retained on the column. 5ml of buffer was applied onto the column. Cells were flushed out by firmly pushing the plunger and collected into a tube. Isolated CD11b\textsuperscript{+} cells were incubated overnight (mostly monocytes/macrophages) and collected for immunofluorescent staining.

**Immunofluorescent staining of isolated cells and paraffin-embedded sections.**

Primary cells were seeded in chambers of Culture Slides (BD Falcon) and allowed to attach 24hr to 72hr. After rinsing with PBS, fibroblasts or macrophages were fixed for 10 min in 2% solution of paraformaldehyde (Sigma) in PBS and permeabilized using 0.1% Triton-X (Sigma) in PBS. Paraffin sections were deparaffinized, hydrated and rinsed in distilled water. Antigen retrieval was performed by heating sections in an antigen retrieval solution (Abcam) for 30min at 95°C. The sections were blocked 30 minutes with Dulbecco’s phosphate-buffered saline with Mg\textsuperscript{2+}, Ca\textsuperscript{2+} (DPBS) containing 10% rabbit serum. Subsequently, slides were double-stained with goat anti-mouse IRAK-M (Santa Cruz, 1:200) and rat anti-mouse Mac2 (Cedarlane
Burlington, Canada, 1:200) or mouse anti-α-SMA (Sigma, St. Louis, MO, 1:200). The mouse on mouse (M.O.M) kit (Vector Laboratories) was used for α-SMA staining. Alexa 488-conjugated (Molecular Probes) or Alexa 594-conjugated secondary antibody (Molecular Probes) was used. The immunostained sections were digitally imaged using a Zeiss fluorescence microscope.

**Protein extraction and Western blotting**

In order to assess activation of p38 MAPK, fibroblasts harvested from WT and IRAK-M KO hearts underwent stimulation with IL-1β (10 ng/ml) or LPS (100 ng/ml) for 15-60 min. At the end of the experiment cell lysates were used for protein extraction. Western blotting was performed as previously described \(^8\) using antibodies to phospho-p38 MAPK, p38 MAPK (Cell Signaling) and GAPDH. The ratio of p-p38 MAPK expression to GAPDH and to p38 MAPK was assessed.

**Statistical analysis.**

Data are expressed as mean ± SEM. Statistical analysis was performed using unpaired, 2-tailed Student’s t test using Welch’s correction for unequal variances and 1-way ANOVA with Tukey’s multiple comparison test. Paired t test was used to compare echocardiographic parameters before myocardial infarction and after 7 to 28 days of reperfusion. Statistical analyses were performed using GraphPad Prism software. P<0.05 was considered to be significant. Mortality was compared using the log rank test.
SUPPLEMENTAL RESULTS:

1. IRAK-M mRNA expression in fibroblasts and macrophages harvested from control and infarcted hearts.

   mRNA from fibroblasts and CD11b+ macrophages harvested from control and infarcted hearts was used to study cell type-specific changes in temporal regulation of IRAK-M. Fibroblasts isolated from WT hearts, expressed IRAK-M mRNA; in contrast IRAK-M KO hearts showed no IRAK-M mRNA expression (Supplemental Figure IA). When compared with control cardiac fibroblasts, infarct fibroblasts had a 3-fold increase in IRAK-M mRNA expression after 24h-72h of reperfusion (IB). CD11b+ macrophages showed a trend towards increased IRAK-M mRNA expression after 6h of reperfusion (IC). Thus, IRAK-M mRNA upregulation in the infarcted myocardium may reflect both cell-type specific upregulation and an increase in macrophage and fibroblast numbers (n=4 per group).

2. IRAK-M null cardiac fibroblasts exhibit increased matrix-degrading capacity

   WT and IRAK-M null cardiac fibroblasts were stimulated with IL-1β (10 ng.ml) for 4h (n=6). The supernatant was collected and the extracted protein was used for zymography to assess MMP activity (Supplemental Figure II). Supernatants from IRAK-M KO fibroblasts had increased baseline levels of latent and active MMP-2. After stimulation with IL-1β a trend towards increased MMP-2 activity was noted in IRAK-M null cells. MMP-9 activity was negligible.

3. IRAK-M loss does not affect IL-1 or LPS-mediated activation of p38 MAPK

   In order to examine whether the effects of IRAK-M loss on fibroblast MMP expression
are due to alterations in p38 MAPK signaling we compared LPS- and IL-1β-induced p38 MAPK activation between WT and IRAK-M KO fibroblasts (Supplemental Figure III). Protein extracted from cell lysates was assessed after 15-60 min of stimulation with IL-1β or LPS. Both LPS and IL-1β increased p38 MAPK activation in both WT and IRAK-M null cells. IRAK-M KO and WT cells had comparable expression of p-p38 MAPK.
### SUPPLEMENTAL TABLES:

#### Table I. Assessment of Echocardiographic Parameters in the Infarcted Heart

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>KO</th>
<th></th>
<th>WT</th>
<th>KO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=14)</td>
<td>(n=18)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>557±76</td>
<td>527±64</td>
<td>541±70</td>
<td>576±73</td>
<td>596±61</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>3.20±0.08</td>
<td>3.61±0.12</td>
<td>4.01±0.15</td>
<td>3.33±0.08</td>
<td>4.14±0.08*</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>4.34±0.07</td>
<td>4.33±0.09</td>
<td>4.71±0.11</td>
<td>4.48±0.07</td>
<td>5.00±0.02*</td>
</tr>
<tr>
<td>FS (%)</td>
<td>51.75±1.60</td>
<td>34.62±3.68</td>
<td>31.62±2.76</td>
<td>50.52±1.20</td>
<td>25.61±2.30#</td>
</tr>
<tr>
<td>LVESV (μl)</td>
<td>85.52±3.27</td>
<td>85.12±3.97</td>
<td>103.65±5.67</td>
<td>91.75±3.32</td>
<td>119.36±6.36</td>
</tr>
<tr>
<td>LVEDV (μl)</td>
<td>41.44±2.37</td>
<td>55.97±4.04</td>
<td>72.30±6.20</td>
<td>45.70±2.26</td>
<td>88.83±3.46*</td>
</tr>
<tr>
<td>LV mass (mg)</td>
<td>0.60±0.38</td>
<td>1.04±0.48</td>
<td>104.45±6.93</td>
<td>114.19±6.30</td>
<td>117.22±6.12</td>
</tr>
</tbody>
</table>

ΔLVEDD (%) | -0.14±2.0 | 8.63±2.96 | 11.89±2.28* | 18.91±2.62# |
ΔLVESD (%) | 13.41±4.17 | 25.82±4.74 | 32.74±3.99* | 42.23±4.53# |
ΔFS (%)    | -36.30±7.41 | -43.05±4.94 | -52.19±4.97 | -54.99±3.63 |
ΔLVESV (%) | 39.49±11.92 | 78.70±15.57 | 101.58±14.89* | 137.37±18.63# |
ΔLVEDV (%) | 0.48±4.61 | 23.25±7.91 | 31.40±6.27* | 51.52±7.95# |
ΔLV mass   | 15.62±9.34 | 20.14±11.96 | 27.61±10.49 | 40.32±10.26 |

*P <0.01 versus corresponding WT.
#p<0.05 versus corresponding WT.

#### Table II: Flow cytometric analysis of single cell suspensions isolated from infarcted hearts

<table>
<thead>
<tr>
<th>Absolute cell number (cells/mg)</th>
<th>WT</th>
<th>KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45+</td>
<td>1502±444</td>
<td>5269±1408*</td>
</tr>
<tr>
<td>CD45+/CD11b+</td>
<td>1192±430</td>
<td>4651±1383*</td>
</tr>
<tr>
<td>CD45+/CD11b+/Ly6C&lt;hi&gt;</td>
<td>780±316</td>
<td>4009±1114*</td>
</tr>
<tr>
<td>CD11b+/Ly6C&lt;hi&gt;</td>
<td>174±81</td>
<td>319±159 (pNS)</td>
</tr>
<tr>
<td>CD45+/F4/80+</td>
<td>1095±372</td>
<td>3484±978*</td>
</tr>
<tr>
<td>IL-1β+</td>
<td>1658±258</td>
<td>5430±933*</td>
</tr>
<tr>
<td>IL-1β+/CD45+</td>
<td>1291±362</td>
<td>4900±1261*</td>
</tr>
<tr>
<td>IL-1β+/CD45+/CD11b+</td>
<td>1012±371</td>
<td>4503±1343*</td>
</tr>
<tr>
<td>IL-1β+/Ly6C&lt;hi&gt;</td>
<td>705±312</td>
<td>3856±1071*</td>
</tr>
</tbody>
</table>

*P <0.05 versus corresponding WT.
SUPPLEMENTAL FIGURES:

Supplemental Figure I: IRAK-M mRNA expression in fibroblasts and macrophages harvested from WT infarcts. A: Control WT cardiac fibroblasts expressed IRAK-M mRNA; no IRAK-M mRNA expression was observed in fibroblasts isolated from IRAK-M KO animals. B: Fibroblasts harvested from the infarcted WT heart showed a 3-fold increase in IRAK-M mRNA expression after 24-72h of reperfusion. C: CD11b+ macrophages harvested from the infarcted heart showed a trend towards increased IRAK-M expression after 6h of reperfusion (**p<0.01 vs. control cells).
Supplemental Figure II: Cardiac fibroblasts isolated from WT and IRAK-M null animals were stimulated with IL-1β (10 ng/ml) for 4h. The supernatant was collected and zymography was performed to assess MMP activity. A: Representative images show latent and active MMP-2 bands in the supernatant. MMP-9 activity was negligible. B. Quantitative analysis shows that IRAK-M KO cells had a 4-fold higher latent MMP-2 expression. C. Active MMP-2 was also significantly increased in the supernatant from IRAK-M KO fibroblasts (*p<0.05 vs. control, n=6).
Supplemental Figure III: IRAK-M loss does not affect IL-1- and LPS-induced p38 MAPK activation. Cardiac fibroblasts from WT and IRAK-M null (KO) animals were stimulated with IL-1β (10 ng/ml), or LPS (100 ng/ml) for 15-60 min. Cell lysates were used for protein extraction; western blotting was performed with antibodies to p-p38 MAPK, p38 MAPK and GAPDH (A). IL-1β and LPS augmented p-p38 MAPK expression (B) and increased the p-p38MAPK:MAPK (C) ratio in both WT and IRAK-M KO cells. IRAK-M null and WT fibroblasts had comparable responses (*p<0.05, **p<0.01 vs. corresponding controls).
REFERENCES


