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, Nikolaos G. Frangogiannis

***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+/Ly6C^{hi} 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 Vasc Biol*. 2012;32:2598-2608.)**

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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From the Department of Medicine (Cardiology), The Wilf Family Cardiovascular Research Institute, Albert Einstein College of Medicine, Bronx, NY (W.C., A.S., N.L., J.S., D.-W. L., M.D., N.G.F.); Division of Cardiology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China (W.C.,); and Department of Medicine, Baylor College of Medicine, Houston, TX (W.C., A.G., Q.T., N.G.F.).

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Correspondence to Nikolaos G. Frangogiannis, MD, The Wilf Family Cardiovascular Research Institute, Albert Einstein College of Medicine, 1300 Morris Park Avenue Forchheimer G46B, Bronx, NY 10461. E-mail nikolaos.frangogiannis@einstein.yu.edu

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with increased mortality and a high incidence of arrhythmias and heart failure.[6] 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.[7,8]

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.[9–11] 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.[12] 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.[13,14] IRAK-M is primarily expressed in monocytes and macrophages and plays an important role in restraining inflammatory activation induced by infectious pathogens[13,15]; 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 and IRAK-M$^{-/-}$ mice 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.[16] 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 by our laboratory.[17]

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.[8]

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.[8]

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 sythesis 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.[8]

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.[17]

Fibroblast Isolation and Stimulation

Fibroblasts were isolated from normal mouse hearts and stimulated as previously described.[17,18]

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 Mg^{2+}, Ca^{2+} 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 mean±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 Mac2+ 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

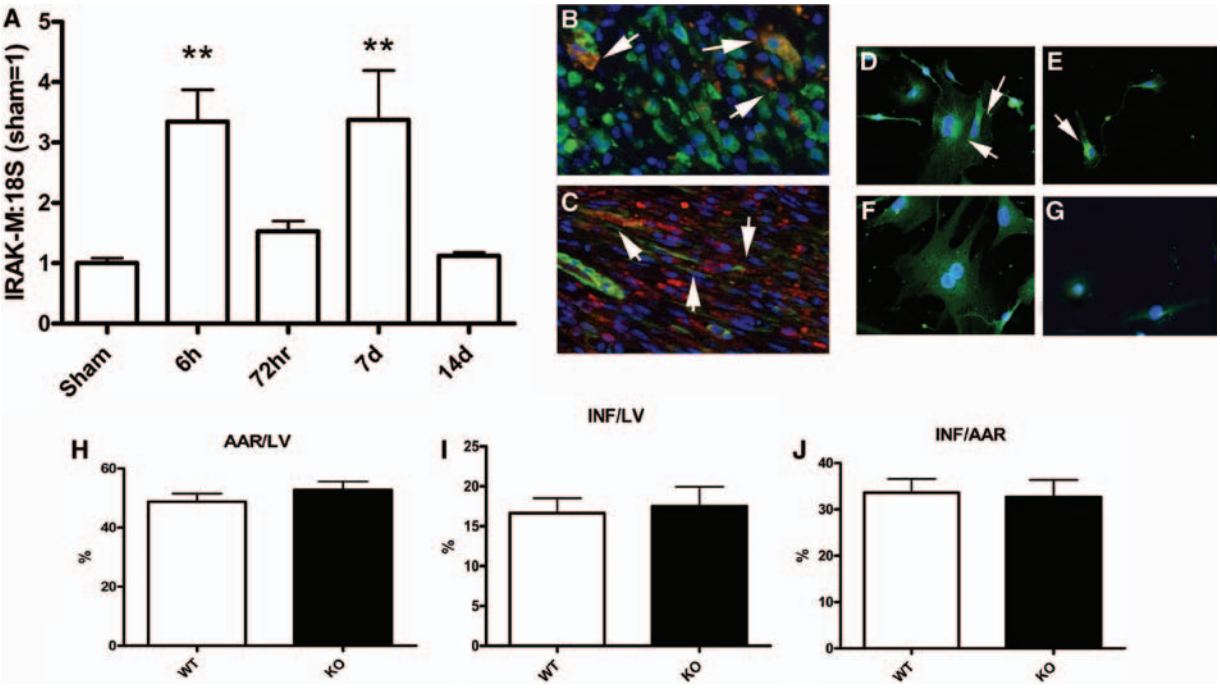

Figure 1. Interleukin-1 receptor-associated kinase (IRAK)-M upregulation in the infarcted mouse heart. **A**, IRAK-M mRNA expression in the infarcted myocardium showed a biphasic response—significant upregulation was noted after 6 hours of reperfusion followed by a second peak after 7 days of reperfusion (**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 Mac2+ 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 reperfusiocn 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 volume; Figure 2A–2G) and morphometrically-derived left ventricular end-diastolic volume 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 (FS), 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 Accentuated 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 upregulation was markedly accentuated in IRAK-M–null infarcts (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 metalloproteinase-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 4G, 4H). A trend toward increased active MMP-9 levels was noted in IRAK-M–null animals (P=0.19). Accentuated 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 Ly6C^{hi} 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 monocytic cells harvested from the infarcted myocardium. After 72 hours of reperfusion, the absolute number of CD45+ leukocytes and of

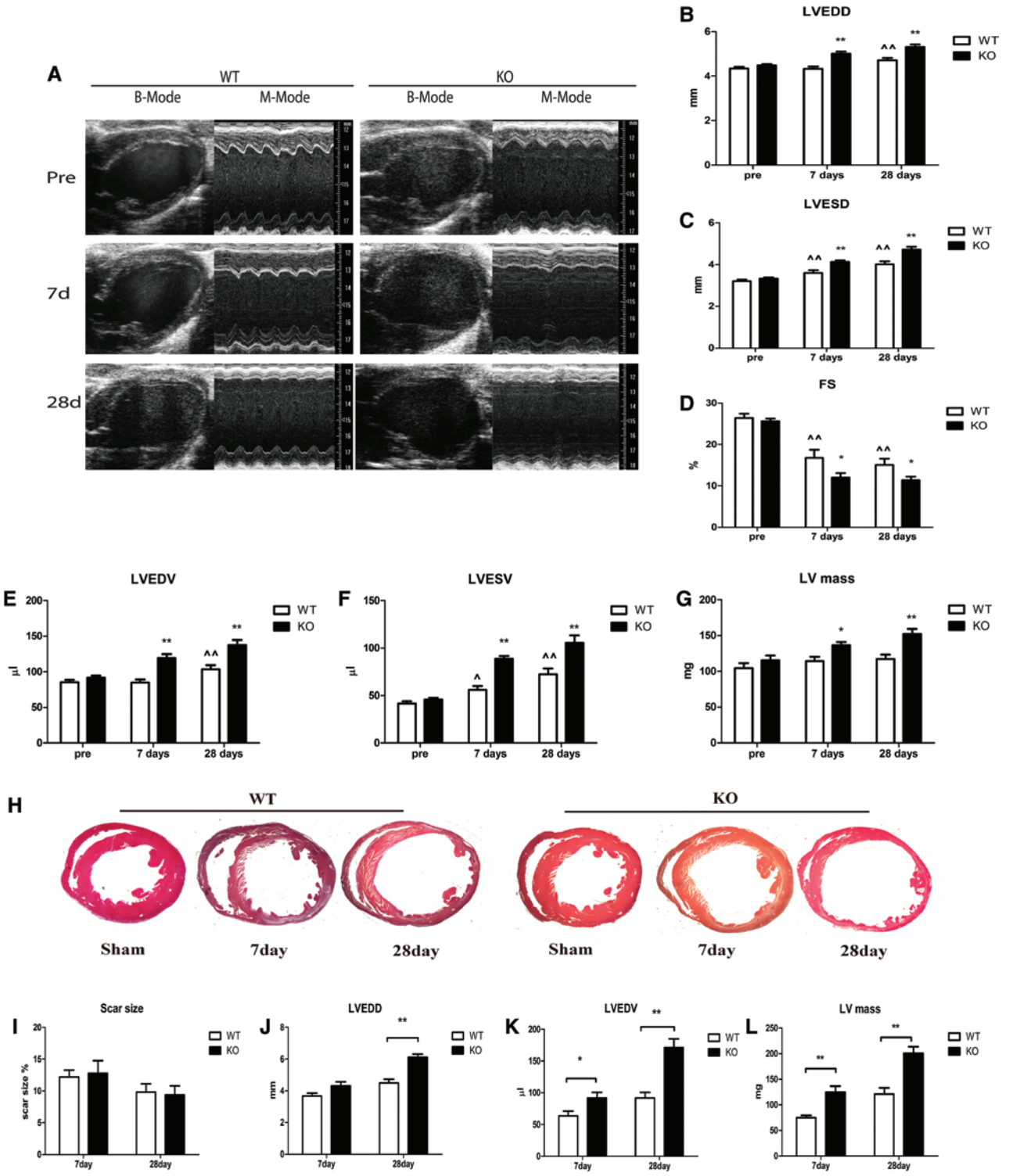

Figure 2. Echocardiography (**A**–**G**) and quantitative morphometry (**H**–**L**) demonstrate that interleukin-1 receptor-associated kinase (IRAK)-M absence results in accentuated dilative remodeling and worse systolic dysfunction after reperfused myocardial infarction. **A**, Representative images 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 volume [LVEDV; **E**], and left ventricular end-systolic volume [LVESV; **F**]), worse systolic dysfunction (indicated by lower fractional shortening [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 (**I**), 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).

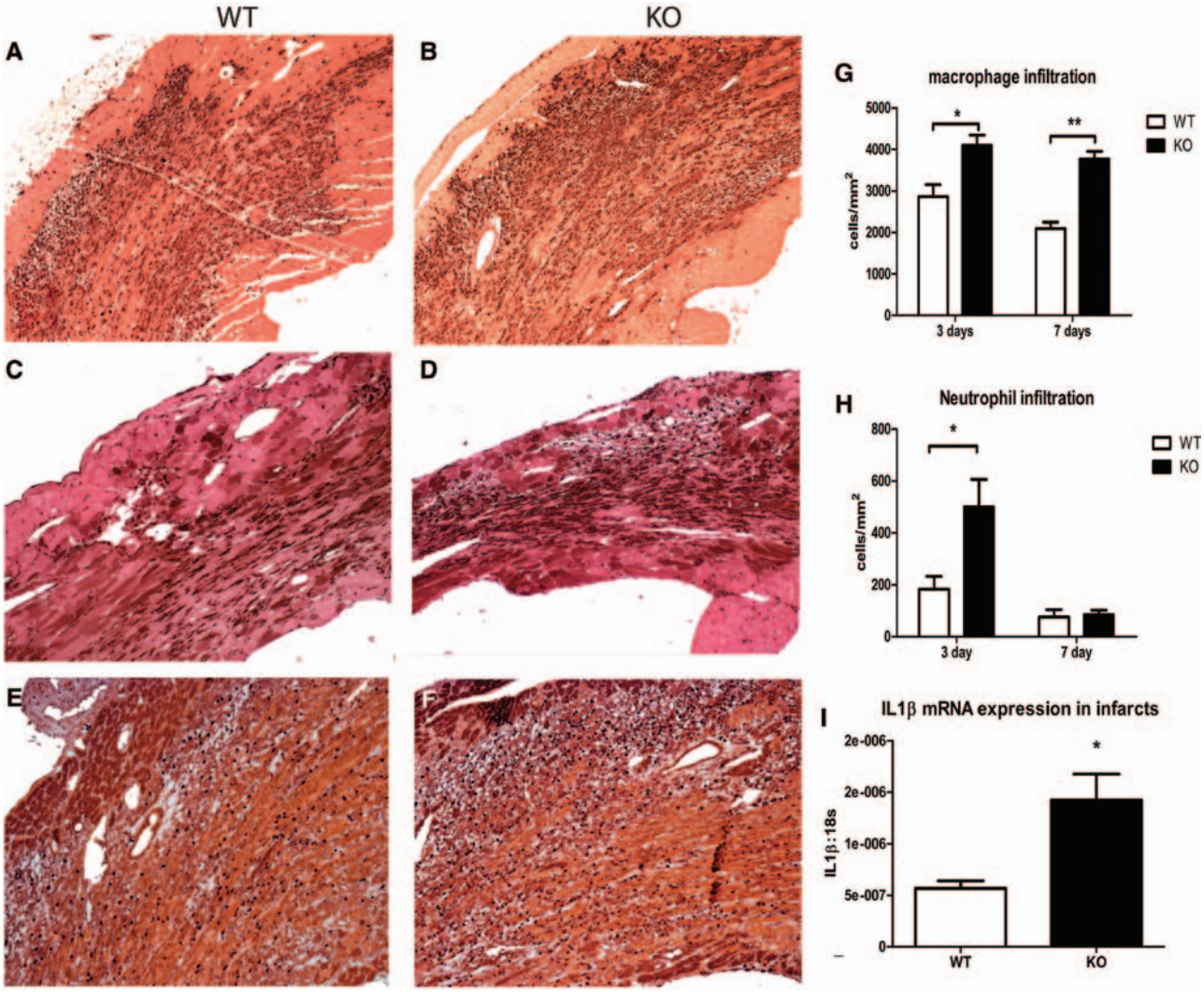

Figure 3. Interleukin-1 receptor-associated kinase (IRAK)-M–null mice exhibit an accentuated inflammatory response after myocardial infarction. **A**–**D**, Representative images show identification of macrophages in the infarcted myocardium using Mac-2 immunohistochemistry after 72 hours (**A** and **B**) and 7 days of reperfusion (**C** and **D**). **E** and **F**, Representative images show immunohistochemical staining with an antineutrophil antibody after 72 hours of reperfusion. **G**, Quantitative analysis showed that macrophage density was significantly higher in IRAK-M–null infarcts (***P*<0.01, **P*<0.05 vs corresponding wild type [WT]). **H**, Neutrophil density was markedly higher in IRAK-M–null infarcts after 72 hours of reperfusion; however, resolution of the neutrophil infiltrate occurred in a timely manner in both groups. **I**, IL-1β mRNA levels were markedly higher in IRAK-M–null infarcts, suggesting accentuation of the inflammatory response. All sections were counterstained with eosin. KO indicates knockout; IL, interleukin.

CD45+/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 Ly6C^{lo} 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 proteins[22] and fibronectin fragments,[23] stimulates TLR signaling in the infarcted heart. TLR-2 activation in infarct leukocytes

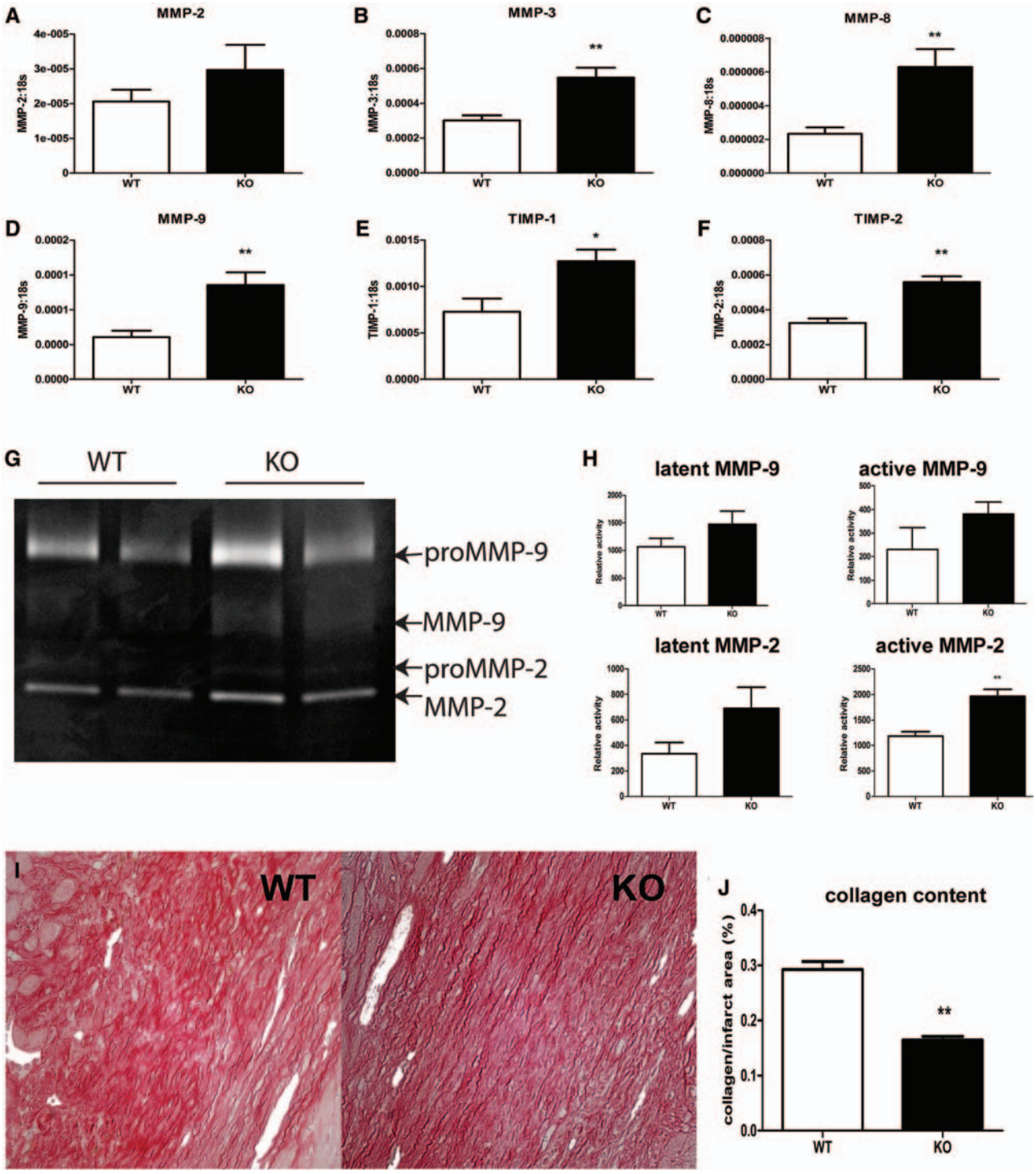

Figure 4. Adverse remodeling in infarcted interleukin-1 receptor-associated kinase (IRAK)-M$^{-/-}$ hearts is associated with increased matrix metalloproteinases (MMP) mRNA expression and enhanced protease activity. **A**–**F**, Quantitative polymerase chain reaction demonstrated increased expression 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–null infarcts 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–null infarcts (**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.

accentuates inflammatory injury after myocardial infarction[9] and promotes cardiac fibrosis and adverse remodeling,[24] whereas TLR-4 signaling exerts proinflammatory actions stimulating lymphocyte infiltration and enhancing chamber dilation.[10] Activation of cell surface TLRs recruits the adaptor molecule myeloid differentiation factor 88[10];

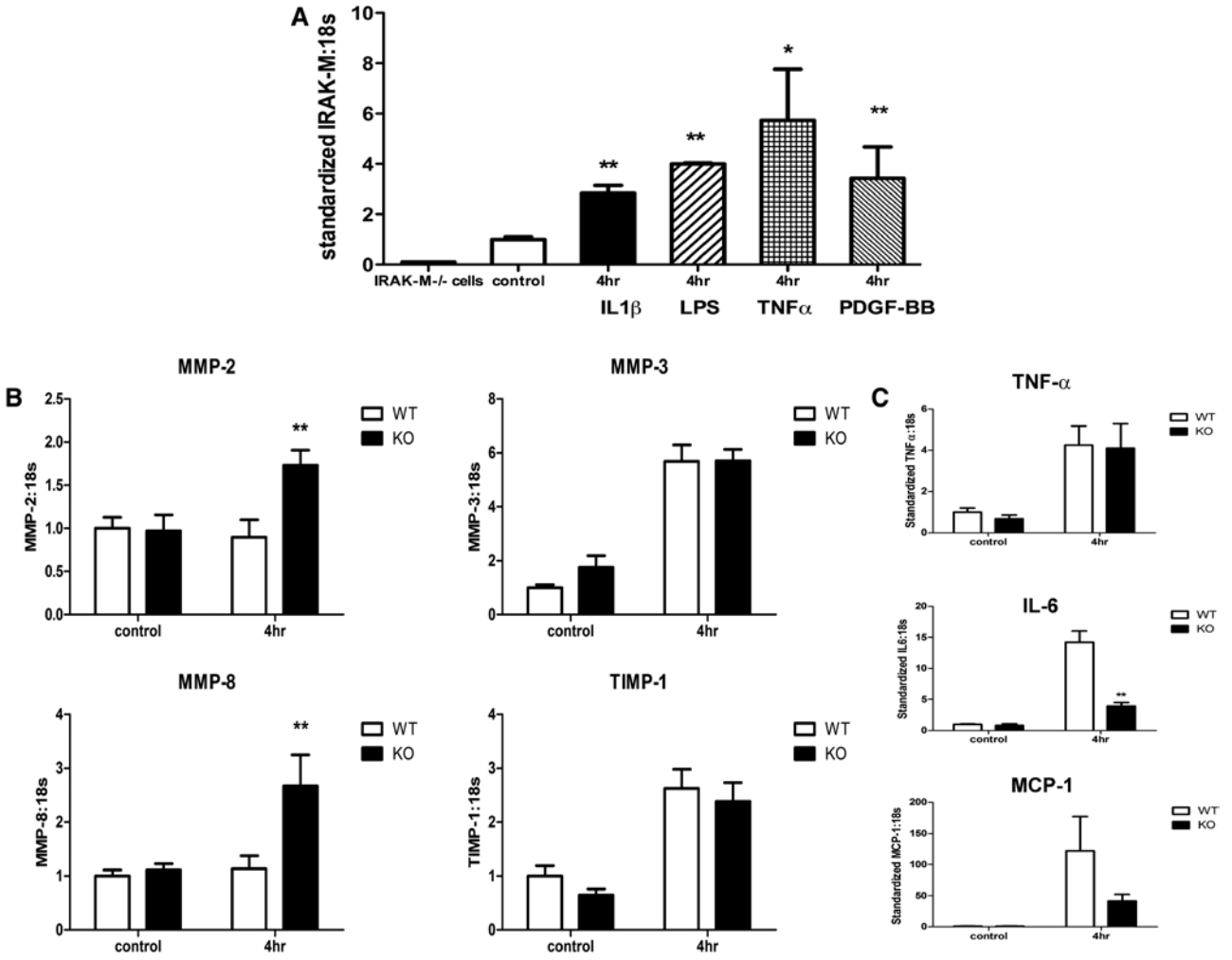

Figure 5. **A**, Interleukin-1 receptor-associated kinase (IRAK)-M regulation in stimulated cardiac fibroblasts. Interleukin (IL)-1β, the toll-like receptor agonist lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α), and platelet-derived growth factor (PDGF)-BB upregulated IRAK-M mRNA expression in cardiac fibroblasts (**P<0.01, *P<0.05 vs control). mRNA isolated from IRAK-M$^{-/-}$ 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$^{-/-}$ 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 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.

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 ischemic[26] 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.[29] These interactions prevent IRAK-1–mediated activation of inhibitor of kappa B (IκB) kinase and MAPK inhibiting nuclear factor-κB and activator protein-1 signaling.[13] 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.[19]

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

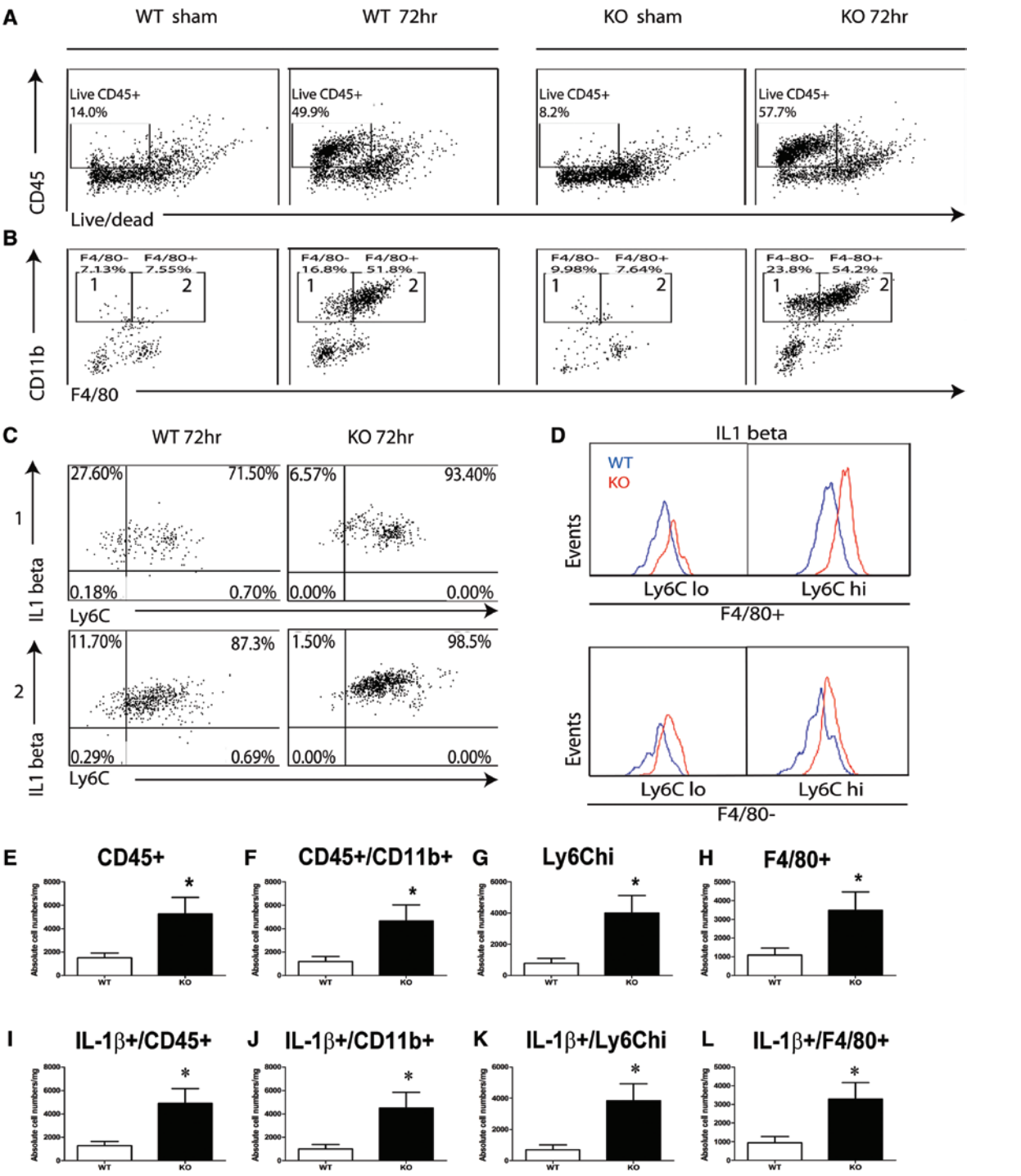

Figure 6. Interleukin-1 receptor-associated kinase (IRAK)-M loss is associated with the infiltration of the infarcted myocardium with pro-inflammatory monocytes. Flow cytometric analysis of cell suspensions harvested from the infarcted heart after 72 hours of reperfusion demonstrated marked increases 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^{hi} and Ly6C^{lo} monocytes (gate 1)/macrophages (gate 2). **D**, IL-1β expression in Ly6C^{lo} and Ly6C^{hi} 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+ monocytic cells (**F**), Ly6C^{hi}/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+ monocytic cells (**J**), IL-1β+/CD11b+/Ly6C^{hi} proinflammatory monocytes (**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.[30] In mice, the pattern of IRAK-M expression seems broader, as IRAK-M synthesis has been demonstrated in murine neutrophils,[31] National Institutes of Health 3T3 fibroblasts,[32] B cells,[33] dendritic cells,[34] and various types of epithelial cells.[35] 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.[13] Moreover, IRAK-M$^{-/-}$ infarcts exhibited alterations in the profile of monocyte subsets recruited in the infarcted myocardium.[36] IRAK-M absence was associated with an abundance of proinflammatory Ly6C^{hi} monocytes in the infarct (Table II in the online-only Data Supplement, 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.[37] In addition, because TLR signaling plays an important role in inflammatory monopoiesis,[38] IRAK-M expression may selectively inhibit generation and release of proinflammatory Ly6C^{hi} 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.[39] 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.[40] 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,[11] or loss of adhesion molecule–mediated interactions,[41] 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 dilative 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 subpopulation of patients, anti-inflammatory strategies may attenuate chamber dilation, protecting from progression of heart failure.

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Disclosures

None.

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