## SUPPLEMENTAL MATERIAL:

## SUPPLEMENTAL METHODS:

### Animals

C57BL/6, IRAK-M -/- mice[1] and IL1RI -/- animals[2] 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[3].

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

previously described [4].

**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[5]. 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[5].

**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 sythesis 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) GCCAGTACCAGTGTCAGTA; MMP-3 (forward) GGAAATCAGTTCTGGGCTATACGA (reverse) TAGAAATGGCAGCATCGATCTTC;

MMP-8 (forward) GATTCAGAAGAAACGTGGACTCAA, (reverse) CATCAAGGCACCAGGATCAGT; MMP-9 (forward) TGTTCCCGTTCATCTTTGAG, (reverse) ATCCTGGTCATAGTTGGCTGT; TIMP-1 (forward) CAGTAAGGCCTGTAGCTGTGC, (reverse) AGGTGGTCTCGTTGATTTCTG; TIMP-2 (forward) GGAATGACATCTATGGCAACC, (reverse) GGCCGTGTAGATAAACTCGAT; MCP-1(forward) GCTGGAGCATCCACGTGT, (reverse) CTGCTGCTGGTGATCCTCTT; TNF-α (forward) GCCAACGGCATGGATCTC, (reverse) GCAGCCTTGTCCCTTGAAGAG; IL-6 (forward) GCTAAGGACCAAGACCATCCAAT, (reverse)GGCATAACGCACTAGGTTTGC and 18S (forward) ACCGCAGCTAGGAATAATGGA, (reverse) GCCTCAGTTCCGAAAACC. 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[5].

**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[6]. Cells harvested from the infarcted heart were counted and reconstituted in staining buffer (BD Biosciences) to a concentration of $1\times10^6$cells/ml. Subsequently cells were incubated with LIVE/DEAD® Fixable Dead Cell Stain single-color dyes (Invitrogen) for 30min 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: Percp-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/permabilization 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$^{low}$CD19$^{neg}$CD45$^{high}$CD11b$^{high}$ cells. Within this population, subsets were identified as either F4/80+ or F4/80-. Further, each F4/80 subgroup was identified as either Ly-6C$^{hi}$ or Ly-6C$^{lo}$. 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[7],[6]. 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 $Ca^{2+}$ and $Mg^{2+}$ (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 $Ca^{2+}$ and $Mg^{2+}$, 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 EDTA, incubated with CD11b+ microbeads (Miltenyi Biotec) 10µl/$10^7$ 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+ 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+ 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 $Mg^{2+}$, $Ca^{2+}$ (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

| | WT (n=14) | | | KO (n=18) | | |
|---|---|---|---|---|---|---|
| | Pre | 7day | 28 day | pre | 7day | 28 day |
| HR(bpm) | 557±66 | 527±64 | 541±70 | 576±73 | 596±61 | 472±43 |
| LVESD(mm) | 3.20±0.08 | 3.61±0.12 | 4.01±0.15 | 3.33±0.08 | 4.14±0.08* | 4.72±0.16* |
| LVEDD(mm) | 4.34±0.07 | 4.33±0.09 | 4.71±0.11 | 4.48±0.07 | 5.00±0.02* | 5.31±0.04* |
| FS(%) | 51.75±1.60 | 34.62±3.68 | 31.62±2.76 | 50.52±1.20 | 25.61±2.30¶ | 24.38±2.06¶ |
| LVESV(μl) | 85.52±3.27 | 85.12±3.97 | 103.65±5.67 | 91.75±3.32 | 119.36±6.36* | 137.66±8.44* |
| LVEDV(μl) | 41.44±2.37 | 55.97±4.04 | 72.30±6.20 | 45.70±2.26 | 88.83±3.46* | 105.58±9.07* |
| LVAWd(mm) | 0.60±0.03 | 0.69±0.02 | 0.63±0.04 | 0.64±0.03 | 0.64±0.05 | 0.68±0.04 |
| LVAWs(mm) | 1.04±0.05 | 0.98±0.06 | 0.89±0.05 | 1.06±0.03 | 0.89±0.08 | 0.90±0.05 |
| LV mass(mg) | 104.45±6.93 | 114.19±6.30 | 117.22±6.12 | 115.38±8.10 | 136.59±5.25* | 152.20±8.42* |
| 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 |

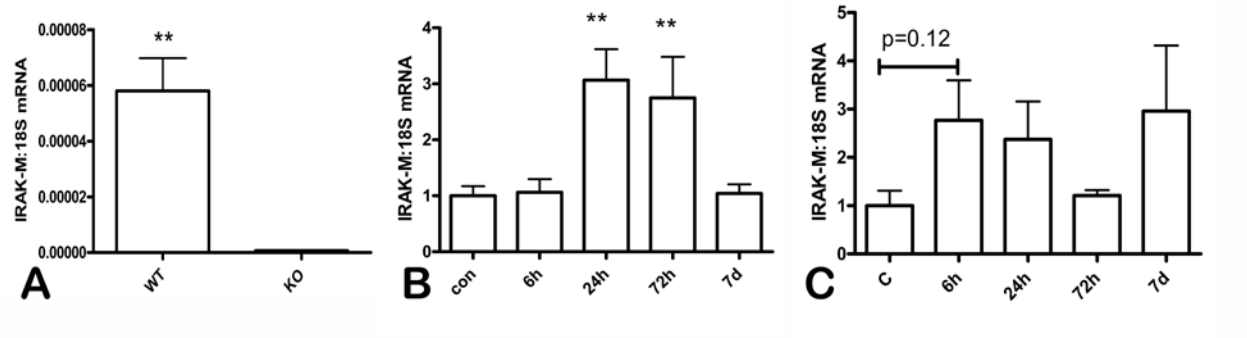
*P <0.01 versus corresponding WT.
¶p<0.05 versus corresponding WT.

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

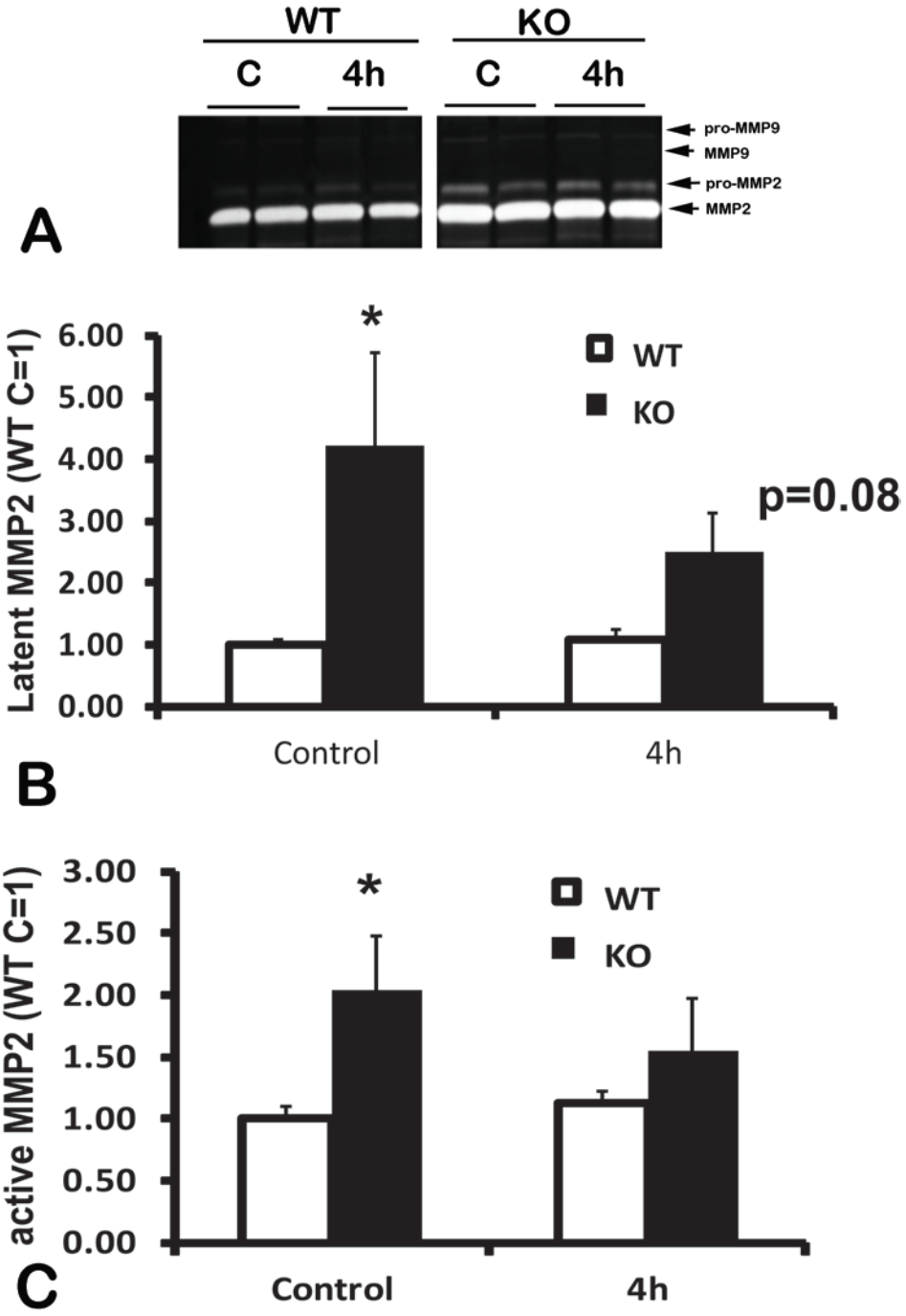
| Absolute cell number (cells/mg) | WT | KO |
|---|---|---|
| CD45+ | 1502±414 | 5269±1408* |
| CD45+/CD11b+ | 1192±430 | 4651±1383* |
| CD45+/CD11b+/Ly6C$^{hi}$ | 780±316 | 4009±1114* |
| CD11b+/Ly6C$^{lo}$ | 174±81 | 319+159 (pNS) |
| CD45+/F4/80+ | 1095±372 | 3484±978* |
| IL-1β+ | 1658±258 | 5430±933* |
| IL-1β+/CD45+ | 1291±362 | 4900±1261* |
| IL-1β+/CD45+/CD11b+ | 1012±371 | 4503±1343* |
| IL-1β+/Ly6C$^{hi}$ | 705±312 | 3856±1071* |

*P <0.05 versus corresponding WT.

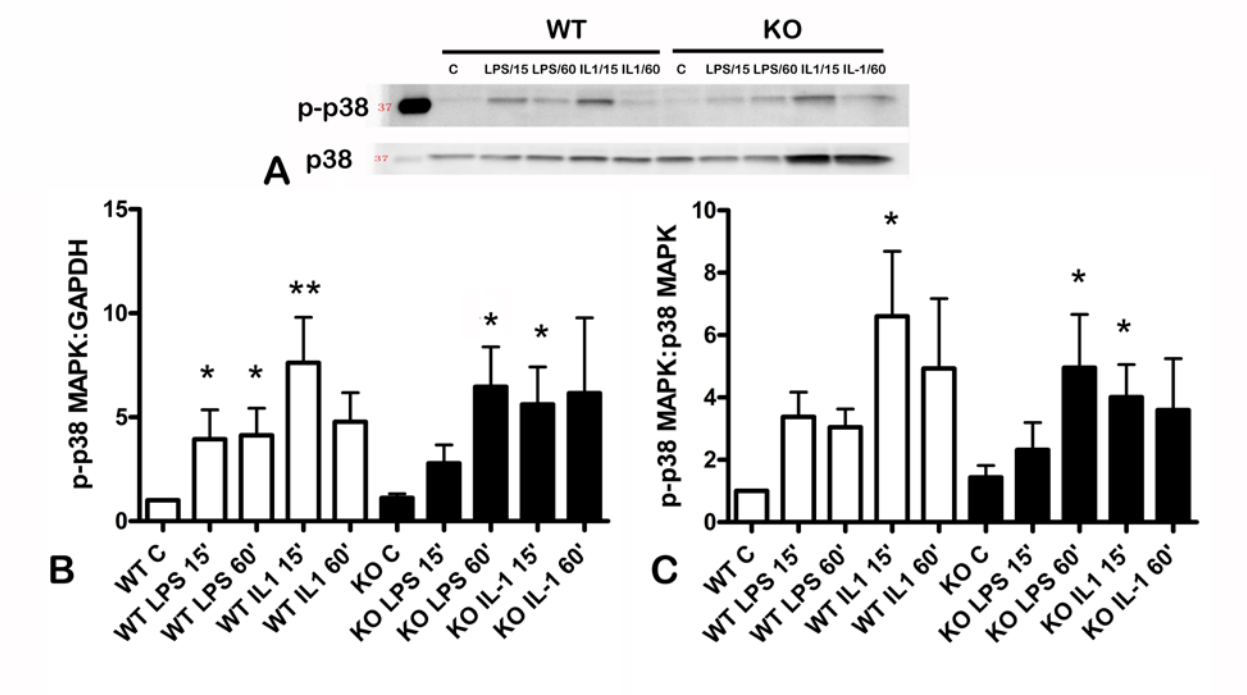
**SUPPLEMENTAL FIGURES:**




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).

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