SUPPLEMENT MATERIAL:

MATERIALS AND METHODS:

1. Murine ischemia/reperfusion protocols.

All animal studies were approved by the animal protocol review committee at Baylor College of Medicine. IP-10 -/- mice in a C57/BL6 background and WT C57/BL/6 controls (both from our own colony) were used for myocardial infarction experiments. IP-10 null mice have been previously characterized \(^1\) and have been used to investigate the role of the chemokine in inflammation and immunity. 8-12 week-old mice (18.0-22.0 g body weight) were anesthetized by an intraperitoneal injection of sodium pentobarbital (60 µg/g). A closed-chest mouse model of reperfused myocardial infarction was utilized in order to avoid the confounding effects of surgical trauma and inflammation, which may influence the baseline levels of chemokines and cytokines \(^2\), \(^3\). The left anterior descending (LAD) coronary artery was occluded for 1h then reperfused for 6h to 28 days. At the end of the experiment, the chest was opened and the heart was immediately excised, fixed in zinc-formalin, and embedded in paraffin for histological studies, or snap frozen and stored at –80°C for RNA isolation. Sham animals (KO, n=5; WT, n=5) were prepared identically without undergoing coronary occlusion/reperfusion. Animals used for histology underwent 24 h, 72 h, 7 and 28 day reperfusion protocols (8 animals per group). Mice used for RNA extraction underwent 6 h, 24 h, and 72 h of reperfusion (8 animals per group). Additional animals were used for perfusion-fixation after 7 days of reperfusion (KO, n=15; WT, n=18) and after 28 days of reperfusion (KO, n=12; WT, n=12) in order to assess remodeling-associated parameters.

2. Infarct size determination.

To assess the size of acute infarcts the Evans blue-triphenyltetrazolium chloride (TTC) staining method was used \(^4\). WT (n=8) and IP-10 null mice (n=7) underwent LAD coronary ischemia and reperfusion protocols and the heart was harvested after 24h of reperfusion. The aorta was canulated, the LAD was reoccluded and 1% Evans Blue was perfused into the aorta.
Subsequently the heart was sectioned from base to apex into 1mm sections. The sections were stained with 1.5% TTC. After TTC staining the viable myocardium is brick red, whereas the infarct appears pale white. All sections were imaged. The area at risk (AAR) and the area of infarction was assessed planimetrically using Image Pro software. The total volume of the AAR and the infarct area were calculated and expressed as a percentage of the total volume of the ventricle. AAR, infarct size and the infarct size/AAR ratio were compared between IP-10 null and WT mice.

3. Immunohistochemistry and quantitative histology

For histopathological analysis murine hearts were fixed in zinc-formalin (Z-fx; Anatech, Battle Creek, MI), and embedded in paraffin. Sections were cut at 5 µm and stained immunohistochemically with the following antibodies: anti-α smooth muscle actin (α-SMA) antibody (Sigma, St. Louis, MO), rat anti-mouse macrophage antibody Mac-2 (Cedarlane), rat anti-neutrophil antibody (Serotec, Raleigh NC), and rat anti-mouse CD31 antibody (Pharmingen, San Diego, CA). Staining was performed using a peroxidase-based technique with the Vectastain ELITE rat, rabbit, or goat kit (Vector Labs, Burlingame, CA) and developed with diaminobenzidine (DAB) + nickel (Vector) ⁵. The Mouse on Mouse (MOM) kit (Vector) was used for α-SMA immunohistochemistry ³. For CD31 staining, sections were pretreated with trypsin and staining was performed using the Tyramide Signal Amplification kit (Perkin Elmer, Boston, MA) ⁵. Quantitative assessment of neutrophil and macrophage density was performed by counting the number of neutrophils and Mac-2-immunoreactive cells respectively in the infarcted area ³. Myofibroblasts were identified as extravascular α-SMA-positive cells and counted in the infarcted and in the non-infarcted remodeling myocardium. Proliferating myofibroblasts were identified using dual immunolabeling for Ki-67, a marker of proliferating cells, and α-SMA. Initially, sections were stained with anti-α-SMA antibody using alkaline phosphatase-based technique and developed with Red substrate kit (Vector Labs).
Subsequently, sections were incubated for 20 min in 95°C non-boiling citrate buffer (Lab Vision) to allow antigen retrieval. To avoid nonspecific binding of the avidin-peroxidase complex in the second step of labeling, sections were pretreated with avidin and biotin blocking kit (Vector Labs). Finally, proliferating nuclei were stained with rat monoclonal TEC-3 anti–mouse Ki67 antibody (Dako) using a peroxidase-based technique and developed with DAB + nickel (Vector Labs).

Macrophage, neutrophil and myofibroblast density was expressed as cells/mm$^2$. The collagen network was identified using picrosirius red staining. Picrosirius red-stained slides from each infarcted heart were scanned using a digital camera. The percentage of the collagen-stained area was assessed in the infarcted myocardium, peri-infarct area and remote remodeling myocardium using ImagePro software. Ten distinct fields from two different sections were used for quantitative analysis of collagen content in each area. Microvascular density was assessed by counting vascular CD31-positive profiles in the infarct, peri-infarct area and remote remodeling myocardium.

4. TUNEL staining and immunofluorescence

Identification of apoptotic cells in myocardial infarction was performed using fluorescent In situ Cell Death Detection Kit (Roche). Briefly, slides were deparaffinized, rehydrated in graded alcohols and permeabilized using 0.1% solution of Triton X in 0.1% sodium citrate. Consequently, slides were incubated with TUNEL reaction mixture for 1h at 37°C. To allow detection of apoptotic myofibroblasts, sections were stained with mouse anti-α-SMA antibody followed by biotinylated anti-mouse IgG secondary antibody (Vector Labs) and steptavidin-Alexa Fluor 594 complex (Invitrogen). Nuclei were stained with DAPI (Invitrogen). The density of TUNEL+ and TUNEL+/α-SMA+ cells was quantitatively assessed in the infarct and in the remote remodeling myocardium and was expressed as cells/mm$^2$. 
5. Preparation of single cell suspensions from myocardial infarction

Single cell suspensions were obtained from infarcted WT (n=6) and KO (n=6) hearts after 72 hours of reperfusion. Briefly, hearts were rapidly excised and placed in ice cold Krebs-Henseleit (KH) buffer containing (in g/L) 2 glucose, 0.141 MgSO₄, 0.16 NaH₂PO₄, 0.35 KCl, 6.9 NaCl, 2.1 NaHCO₃, 0.373 CaCl₂, 1 NaN₃ at pH 7.4. Subsequently, the aorta was cannulated with a 22-gauge tubing adaptor and flushed with ice cold KH buffer to remove residual cells in the coronary vasculature. Two hearts per experiment were minced with fine scissors and placed into a cocktail of 0.25 mg/ml Liberase Blendzyme 3 (Roche Applied Science), 20 U/ml DNase I (Sigma Aldrich), 10 mM HEPES (Invitrogen) , 0.1% Sodium Azide in HBSS with Ca²⁺ and Mg²⁺ (Invitrogen) and shaken at 37°C in 5 min intervals five times. Cells were then passed through a 40 μm nylon mesh (BD Falcon), centrifuged (10 min, 500 g, 4°C), resuspended in red cell lysis buffer (R&D Systems) and incubated for 10 min. Consecutively, cell suspension were diluted with wash buffer (R&D Systems) and centrifuged (10 min, 500 g, 4°C) and reconstituted with staining buffer (dPBS without Ca²⁺ and Mg²⁺, 2% FBS, 0.1% sodium azide) and counted using Trypan blue (Sigma Aldrich). The mean and SEM for live cell recoveries were 20732±1123 cells/mg of tissue vs. 23757±1000/mg of tissue in WT and KO infarcts respectively (p=0.07).

6. Flow cytometry

5×10⁵-10⁶ cells were incubated with either anti-FcγIII/II (clone 2.4G2) antibody (BD Pharmingen) for 5 min and labeled at 4°C for 30 min simultaneously with two of the following antibodies: FITC labeled anti-CD3 (clone BD Biosciences 145-2C11); PE-labeled anti-CXCR3 (R&D Systems); PE-Cy5 labeled anti-CD45 (BD Biosciences, clone 30-F11). For intracellular staining, cells were fixed and permeabilized for 20 min at 4°C with fixation/permabilization kit (BD Biosciences). Subsequently, cells prelabeled with surface markers were incubated with anti-αSMA (Sigma, Clone 1A4). Finally cells were washed twice, resuspended in staining
buffer and immediately analyzed with a Cell Lab Quanta SC flow cytometer (Beckman Coulter). Cell permeable DRAQ5 dye (Alexis Biochemicals) was used to define the gate for nucleated cells. Data analysis was performed using FlowJo (Tree Star, Inc).

7. Perfusion fixation and assessment of ventricular volumes

For assessment of post-infarction remodeling, infarcted hearts after 7 days of reperfusion were used for perfusion-fixation as previously described. A cardioplegic solution was perfused through the jugular vein to promote relaxation. After excision and rinsing in cold cardioplegic solution, the aorta was cannulated and a PE-50 catheter was pushed across the mitral valve into the left ventricle and secured in place. Hearts were fixed for 10 minutes with 10% zinc buffered formalin by aortic perfusion. The entire heart was cross-sectioned from base to apex at 250µm intervals. Ten serial 10µm sections were obtained at each interval, corresponding to an additional 50µm segment. The first section from each interval was stained with hematoxylin/eosin. For each section the left ventricular chamber area (LVCA) and the infarct area (IA) were measured using Image Pro software using methodology established in our laboratory. The size of the scar was expressed as a percentage of the left ventricular volume. Assessment of cardiac end-diastolic volumes was performed by calculating the sum of the volumes of all 300µm partitions. Anterior wall (AW), posterior wall (PW) and mid-septal thickness was measured at the papillary muscle level for each heart. Because histological processing results in shrinkage of the processed tissue, the values were corrected by a linear factor of 1.25.

8. Echocardiography

Short axis M-mode echocardiographic studies were performed prior to instrumentation and after 7, 14 and 28 days of reperfusion (WT: n=10, IP-10 -/-: n=12) using a 25 MHz probe (Vevo 770; Visualsonics. Toronto ON). Left Ventricular End-Diastolic Diameter (LVEDD) and
Left Ventricular End-systolic Dimension (LVESD) were measured as indicators of cardiac remodeling and Left Ventricular Fractional Shortening (LVFS) was calculated as an indicator of systolic function.

9. **RNA extraction and Ribonuclease protection assay**

RNA extraction from sham and infarcted whole hearts was performed using standard protocols as previously described[^3]. In order to examine IP-10 expression in infarcted vs. non-infarcted myocardium, hearts from C57/BL6 mice undergoing 1h ischemia/6h reperfusion (n=7) and 1h ischemia/24h reperfusion (n=8) protocols were separated into infarcted and non-infarcted segments on the basis of visual inspection. The mRNA expression level of the chemokines Macrophage Inflammatory Protein (MIP)-1α, MIP-1β, MIP-2, and MCP-1, of the cytokines Tumor Necrosis Factor (TNF)-α, Interleukin (IL)-1β, IL-6, IL-10, and of the growth factors TGF-β1, 2, and 3, Fibroblast Growth Factor (FGF)-1, FGF-2 and Macrophage–Colony Stimulating Factor (M-CSF), the Matrix Metalloproteinases (MMP)-2, -3, -8, and -9 and the Tissue Inhibitors of Metalloproteinases (TIMP)-1 and -2 was determined using a ribonuclease protection assay (RiboQuant; Pharmingen) according to the manufacturer’s protocol. Gene expression was normalized to the ribosomal protein L32 mRNA.

10. **Cardiac fibroblast isolation and transwell migration assay**

Mouse cardiac fibroblasts were isolated by enzymatic digestion with a collagenase buffer as previously described[^8]. Cardiac fibroblast migration was studied using the colorimetric transwell system QCM™ (Millipore Corporation, Billerica, MA), which allows cells to migrate through an 8-µm pore size polycarbonate membrane. Fibroblasts used in the assay were serum starved for 24h prior to the experiment. Cells were harvested using TrypLe express (GIBCO Invitrogen Corporation, Carlsbad, CA), counted and reconstituted with serum free DMEM/F12 (GIBCO Invitrogen Corporation, Carlsbad, CA) to bring the cells to concentration of $5 \times 10^5$
cells/ml. Cell suspensions were stimulated with vehicle or recombinant mouse IP-10 (R&D systems, Mineapolis, MN) (100 ng/ml) and incubated for 1h. Subsequently, 300 µl of IP-10-treated or control cell suspensions were added to the upper chamber of each insert. 500 µl of serum free DMEM/F12, 1% Fetal Calf Serum (FCS) DMEM/F12 or 20 ng/ml bFGF DMEM/F12 were added to the lower chamber and incubated for 4 h at 37°C in 5% CO₂. After incubation remaining cell suspensions from upper wells were removed and inserts were placed in cell stain solution for 20 min at room temperature. Subsequently, inserts were collected and rinsed several times in distilled water. Migrated cells were visualized and photographed using an inverted microscope. Optical density of dye extract was measured at 560 nm using NanoDrop spectrophotometer. Cell migration in each individual experiment was normalized to mean migration in chamber without chemoattractant (control).

11. Assessment of contraction in collagen lattices populated with cardiac fibroblasts

Cardiac fibroblasts at passage 3 were overnight serum starved. Prior to the experiment, cell suspensions were incubated at 37°C with vehicle or 100 ng/ml IP-10 for 1h. Collagen matrix was prepared on ice by diluting a stock solution of rat 3,48 mg/ml collagen I (GIBCO Invitrogen Corporation, Carlsbad, CA) with 2x DMEM and distilled water for a final concentration of 1 mg/ml collagen. IP-10-stimulated and control cell suspensions in 1x DMEM were mixed with collagen solution to achieve the final 3*10⁵ cells/ml concentration. Subsequently, 500 µl of this suspension was aliquoted to a 24-well culture plate (BD Falcon, San Jose, CA) and allowed to polymerize at 37°C for 15 min. Following polymerization pads were released from wells, transferred to 6-well culture plate (BD Falcon, San Jose, CA) and cultured in 0% FCS DMEM, 10% FCS DMEM or 20 ng/ml bFGF DMEM in the presence or absence of 100 ng/ml IP-10. At 0 h and after 24 h the pictures of the plates were taken in flatbed scanner and the area of each pad was measured using Image Pro software.
12. Cell proliferation assay

Proliferation of cardiac fibroblasts was assessed using a colorimetric BrdU Cell Proliferation ELISA kit (Roche Applied Biosciences, Indianapolis, IN) as previously described. Briefly, cells at passage 3 were rendered quiescent by overnight incubation in serum-deprived medium. The following conditions were studied to examine the effect of IP-10 on fibroblast proliferation: 0% FCS with either vehicle or 10, 100, 250 ng/ml IP-100, 1% FCS with either vehicle or 10, 100, 250 ng/ml IP-10, 5% FCS with either vehicle or 10, 100, 250 ng/ml IP-10, and 0.2-2 mM bFGF in 0% FCS with either vehicle or 250 ng/ml IP-10. Each condition was run in triplicate in six independent experiments. Cell proliferation in each individual experiment was normalized to the mean proliferation in presence of 5% FCS.

13. Assessment of cardiac fibroblast apoptosis

Apoptosis in isolated cardiac fibroblasts was assessed using the Cell Death Detection ELISA PLUS (Roche Applied Biosciences, Indianapolis, IN) according to the manufacturer’s manual. The basis of this assay is quantitative detection of cytoplasmic histone-associated DNA fragments that occur as cells undergo apoptosis. Cells were stimulated with 10-250 ng/ml IP-10 for 24h in serum-free environment; stimulation with cycloheximide (CHX - Sigma) was used as a positive control. Each condition was duplicated in 6 independent experiments. Following incubation, lysis buffer was added to each well and microplates were centrifuged at 200 g for 10 min. 20 µl of supernatant was transferred carefully into streptavidin-coated microplates and combined with the mixture of biotynlated anti-histone and peroxidase-conjugated anti-DNA antibodies. Photometric analysis was performed at 370 nm.


Statistical analysis was performed using ANOVA followed by t-test corrected for multiple comparisons (Student-Newman-Keuls). Paired t-test was used to compare
echocardiographic endpoints before and after infarction. Data were expressed as mean ± SEM. Statistical significance was set at 0.05.

RESULTS:

1. In the absence of injury IP-10 null and WT mice have comparable structure and function

In the absence of injury, IP-10 null and WT mice had comparable structural and functional characteristics suggesting that IP-10 does not play a role in cardiac homeostasis. Echocardiographic assessment demonstrated that cardiac dimensions and function were comparable between WT and IP-10 null hearts prior to myocardial infarction (Online Table I). Flow cytometry of single cell suspensions harvested from control WT and IP-10 null hearts showed comparably low numbers of CD45+ leukocytes, CD3+ T cells, and α-SMA positive cells (Table 1). In addition, CD31 immunohistochemistry showed comparable microvascular density in IP-10 -/- and WT hearts (microvascular density: WT 3547.2 ± 445.5 vessels/mm² vs. IP-10 -/- 3334.1 ± 291.0 vessels/mm², pNS).

2. Echocardiographic assessment of remodeling-associated parameters in infarcted WT and IP-10 null hearts

IP-10 absence was associated with accentuated early remodeling following myocardial infarction. After 7 days of reperfusion echocardiographically-derived LVEDD (Online Table I) were significantly higher in infarcted IP-10 null hearts when compared with WT animals. Although there was a trend towards a lower LVFS in IP-10 null hearts, the difference in systolic function between IP-10 -/- and WT animals did not reach statistical significance at this timepoint. Serial echocardiographic imaging of infarcted hearts demonstrated that, although both IP-10 null and WT hearts continued to dilate, and cardiac dimensions remained higher in IP-10 null animals after 28 days of reperfusion, the difference was no longer statistically
significant. However, after 28 days of reperfusion IP-10 null hearts had significantly lower LVFS, indicating accentuated systolic dysfunction (Online Table I). Development of late systolic impairment in IP-10 null hearts was associated with progressive thinning of the left ventricular walls (Figure 2D). Thus, in comparison to WT hearts, IP-10 -/- hearts had markedly increased dilative remodeling over the first week following infarction associated with a more extensive fibrotic area. As the scar matured, the differences in chamber dilation between WT and IP-10 null hearts were attenuated; however, IP-10 -/- mice developed rapid wall thinning and accentuated systolic dysfunction (Online Table I).

3. Cytokine and chemokine mRNA expression in WT and IP-10 -/- infarcts

mRNA expression of the cytokines TNF-α, IL-1β, IL-6, M-CSF, and IL-10 (Online Table II) and of the chemokines MIP-1α and MIP-1β (Online Figure IA, B) was comparable between WT and IP-10 null infarcts at all timepoints examined. In contrast, peak MIP-2 expression was reduced in IP-10 null infarcts, whereas MCP-1 levels were elevated in comparison to WT animals (Online Figure IC, D).

4. Comparison of FGF, MMP and TIMP mRNA levels between WT and IP-10 -/- infarcts.

FGF-1 and FGF-2 mRNA levels were comparable between WT and IP-10 -/- infarcts after 72h of reperfusion (FGF1/L32 ratio: WT, 0.77±0.06 vs. IP-10, -/- 0.87±0.07, pNS; FGF2/L32 ratio: WT, 0.24±0.02 vs. -/-, 0.24±0.01, pNS). In addition, assessment of MMP and TIMP mRNA expression after 24h and 72h of reperfusion showed only subtle differences between IP-10 null and WT hearts. MMP-2, MMP-3, MMP-8, MMP-9 and TIMP-1 mRNA expression levels were comparable between groups, whereas TIMP-2 levels were higher in IP-10 -/-infarcts after 24h of reperfusion (Online Table III).
Online Table I: Echocardiographic indicators of adverse remodeling in WT and IP-10 null infarcted hearts

<table>
<thead>
<tr>
<th></th>
<th>Pre</th>
<th>7d</th>
<th>14d</th>
<th>28d</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
<td>WT</td>
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<tr>
<td>LVEDD</td>
<td>4.21±0.10</td>
<td>4.37±0.10</td>
<td>4.60±0.19</td>
<td>5.15±0.10* (p=0.019)</td>
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<td>LVESD</td>
<td>3.00±0.09</td>
<td>3.00±0.10</td>
<td>3.97±0.27</td>
<td>4.66±0.20 (p=0.06)</td>
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<td>LVFS</td>
<td>0.29±0.08</td>
<td>0.32±0.02</td>
<td>0.15±0.03</td>
<td>0.11±0.02</td>
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LVEDD, Left Ventricular End-Diastolic Diameter; LVESD, Left Ventricular End-Systolic Diameter; LVFS, Left Ventricular Fractional Shortening.
Online Table II: Cytokine expression in infarcted WT and IP-10 -/- hearts

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sham</th>
<th>6h</th>
<th>24h</th>
<th>72h</th>
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<tr>
<td></td>
<td>(Ratio:L32)*100</td>
<td>WT</td>
<td>-/-</td>
<td>WT</td>
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<tr>
<td>TNF-α</td>
<td>0.3+0.1</td>
<td>0.2+0.1</td>
<td>1.0+0.04**</td>
<td>0.9+0.06**</td>
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<tr>
<td>IL-1β</td>
<td>0.6+0.1</td>
<td>0.5+0.1</td>
<td>12.3+0.9**</td>
<td>13.0+2.5**</td>
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<tr>
<td>IL-6</td>
<td>0.6+0.1</td>
<td>0.5+0.1</td>
<td>16.9+3.2**</td>
<td>15.3+3.5**</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.5+0.1</td>
<td>0.4+0.1</td>
<td>4.3+0.4**</td>
<td>3.6+0.5**</td>
</tr>
<tr>
<td>M-CSF</td>
<td>9.0+0.6</td>
<td>10.0+0.5</td>
<td>25.6+1.8**</td>
<td>25.4+1.6**</td>
</tr>
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</table>

*p<0.05, **p<0.01 vs. corresponding sham; the differences in cytokine mRNA expression between WT and IP-10 null infarcts were not statistically significant at all timepoints examined.
Online Table III: MMP and TIMP mRNA levels in the infarcted heart

<table>
<thead>
<tr>
<th>Ratio to L32</th>
<th>1h/24h</th>
<th>1h/72h</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
<td>IP-10 -/-</td>
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<tr>
<td>MMP-2</td>
<td>0.11±0.04</td>
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<tr>
<td>MMP-3</td>
<td>0.03±0.01</td>
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<td>MMP-8</td>
<td>0.05±0.04</td>
<td>0.02±0.01</td>
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<td>MMP-9</td>
<td>0.06±0.04</td>
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</tr>
<tr>
<td>TIMP-1</td>
<td>0.29±0.09</td>
<td>0.20±0.07</td>
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<tr>
<td>TIMP-2</td>
<td>0.19±0.03</td>
<td>0.31±0.05**</td>
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**p<0.01 vs. corresponding WT
Online Figure I
Online Figure I: Chemokine mRNA expression in infarcted WT and IP-10 null hearts. Infarcted IP-10 KO and WT hearts had comparable MIP-1α (A) and MIP-1β (B) mRNA expression levels at all timepoints examined. IP-10 null mice exhibited higher MCP-1 (C) and lower MIP-2 (D) expression levels than their wildtype littermates. Expression of pro-inflammatory and inhibitory cytokines was comparable between WT and IP-10 null mice at all timepoints examined (Online Table II).
REFERENCES


