

CCL2/Monocyte Chemoattractant Protein-1 Regulates Inflammatory Responses Critical to Healing Myocardial Infarcts

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Abstract—The CC chemokine Monocyte Chemoattractant Protein (MCP)-1/CCL2 has potent mononuclear cell chemoattractant properties, modulates fibroblast and endothelial cell phenotype and may play an important role in wound healing. In order to examine whether MCP-1 critically regulates myocardial infarct healing, we studied the effects of MCP-1 gene disruption and antibody neutralization in a closed-chest model of reperfused murine myocardial infarction. MCP-1^{-/-} mice had decreased and delayed macrophage infiltration in the healing infarct and demonstrated delayed replacement of injured cardiomyocytes with granulation tissue. In contrast, the time course and density of neutrophil infiltration was similar in MCP-1 null and wild-type animals. MCP-1^{-/-} infarcts had decreased mRNA expression of the cytokines TNF- α , IL-1 β , TGF- β ₂, - β ₃, and IL-10 and demonstrated defective macrophage differentiation evidenced by decreased Osteopontin-1 expression. MCP-1 deficiency diminished myofibroblast accumulation but did not significantly affect infarct angiogenesis. Despite showing delayed phagocytotic removal of dead cardiomyocytes, MCP-1^{-/-} mice had attenuated left ventricular remodeling, but similar infarct size when compared with wild-type animals. MCP-1 antibody inhibition resulted in defects comparable with the pathological findings noted in infarcted MCP-1^{-/-} animals without an effect on macrophage recruitment. MCP-1 has important effects on macrophage recruitment and activation, cytokine synthesis and myofibroblast accumulation in healing infarcts. Absence of MCP-1 results in attenuated post-infarction left ventricular remodeling, at the expense of a prolonged inflammatory phase and delayed replacement of injured cardiomyocytes with granulation tissue. (*Circ Res.* 2005;96:881-889.)

Key Words: monocyte chemoattractant protein-1 ■ myocardial infarction ■ myocardial inflammation ■ pathology ■ cytokines

Myocardial infarct healing is a dynamic biological process initiated by induction of acute inflammation, followed by formation of granulation tissue and deposition of extracellular matrix.¹⁻³ Chemokine expression is a prominent feature of the postinfarction inflammatory response and may play an important role in inflammatory leukocyte recruitment.^{2,4,5} Marginated leukocytes contribute to removal of dead tissue through the release of proteolytic enzymes and produce fibrogenic and angiogenic mediators, leading to the proliferative phase of infarct healing. In addition, chemokines exert important effects on nonhematopoietic cells, such as fibroblasts, smooth muscle, and endothelial cells and may modulate fibrous tissue deposition and wound angiogenesis.⁶ Recent investigations using experimental models of myocardial infarction demonstrated marked induction of the CC chemokines MCP-1, MIP-1 α , and MIP-1 β and the CXC chemokines CXCL8/IL-8 and CXCL10/IP-10 in the infarcted hearts supporting a role for these chemokines in leukocyte recruitment, infarct angiogenesis, and healing.^{5,7}

One of the best-studied CC chemokines, MCP-1/CCL2, is a potent chemoattractant for monocytes, T cells, and NK cells and has been implicated in diseases characterized by monocyte-rich infiltrates.⁸ Its expression and functional significance have been documented in a wide variety of disease processes, such as atherosclerosis,⁹ multiple sclerosis, rheumatoid arthritis, stroke, and nephritis.⁸ MCP-1 upregulation has been demonstrated in canine,¹⁰ rat,¹¹ and murine¹² models of experimental myocardial infarction. In the canine model of reperfused infarction, induction of MCP-1 mRNA occurred only in ischemic segments within the first hour of reperfusion, peaked at 3 hours, and was localized by immunostaining on the venular endothelium.¹⁰

MCP-1-deficient mice demonstrate defective mononuclear cell recruitment in a model of peritoneal inflammation.¹³ In addition, recent investigations indicated that MCP-1 may have important effects on the healing process beyond its mononuclear cell recruiting properties. MCP-1 has direct

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angiogenic effects and human endothelial cells express the MCP-1 receptor CCR2.¹⁴ Furthermore, MCP-1 may directly modulate fibroblast phenotype and activity by increasing collagen expression¹⁵ and by modulating matrix metalloproteinase expression.¹⁶ Hence, MCP-1 may exert diverse effects on different cell types involved in the postinfarction inflammatory response.

Our study examines the functional role of MCP-1 in myocardial infarct healing using two distinct models of defective MCP-1 signaling: MCP-1^{-/-} mice and inhibition with a neutralizing antibody. We examined the effects of MCP-1 deficiency on inflammatory leukocyte recruitment, cytokine expression, fibrous tissue deposition, and wound angiogenesis in the healing infarct and investigated the role of the chemokine in cardiac repair and postinfarction remodeling. Our findings suggest an important role for MCP-1 in macrophage recruitment and activation, cytokine synthesis, and formation of granulation tissue in the healing infarct. Absence of MCP-1 results in attenuated postinfarction left ventricular remodeling, at the expense of a prolonged inflammatory phase and delayed replacement of injured cardiomyocytes with granulation tissue.

Materials and Methods

Murine Ischemia/Reperfusion Protocols

All animal protocols were approved by the Baylor College of Medicine Institutional Review Board. Wild-type (WT) C57/BL/6 mice (purchased from Charles River, Wilmington, MA) and MCP-1^{-/-} mice¹³ (bred by BJ Rollins) were used for myocardial infarction experiments. MCP-1^{-/-} animals were genotyped using PCR as previously described.¹³ Male and female WT and MCP-1 knockout (KO) C57BL/6 mice, 8 to 12 weeks of age, were anesthetized by an intraperitoneal injection of sodium pentobarbital (10 μ g/g). A closed-chest mouse model of reperfused myocardial infarction was utilized as previously described,^{12,17} in order to avoid the confounding effects of surgical trauma and inflammation, which may influence the baseline levels of chemokines and cytokines. The left anterior descending (LAD) coronary artery was occluded for 1 hour then reperfused for 6 hours to 7 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 were prepared identically without undergoing coronary occlusion/reperfusion. Animals used for histology underwent 24-hour, 72-hour, and 7-day reperfusion protocols (8 animals per group). Mice used for RNA extraction underwent 6, 24, and 72 hours of reperfusion (8 animals per group). Additional animals (KO, n=11; WT, n=12) were used for perfusion-fixation after 7 days of reperfusion in order to assess remodeling-associated parameters.

In order to examine the effects of MCP-1 antibody inhibition on myocardial infarcts, WT mice undergoing coronary occlusion/reperfusion received daily intraperitoneal injections (20 μ g/d, the first dose was administered immediately after reperfusion) with a goat anti-mouse neutralizing antibody (R&D Systems) or goat IgG on the first three days of reperfusion. At the end of the experiment the hearts were fixed in zinc-formalin (Z-fix; Anatech) and embedded in paraffin for histological studies. Animals used for histology underwent 24 hours, 72 hours, and 7 days of reperfusion (8 animals per group).

Immunohistochemistry and Quantitative Histology

Sections were cut at 3 μ m and stained immunohistochemically with the following antibodies: anti- α -smooth muscle actin (α -SMA) (Sigma), rat anti-mouse macrophage antibody clone F4/80 (Research Diagnostics Inc), goat anti-osteopontin (OPN)-1 (Santa Cruz Bio-

technology), rat anti-neutrophil antibody (Serotec), and rat anti-mouse CD31 antibody (Pharmingen). Staining was performed using a peroxidase-based technique with the Vectastain ELITE rat or goat kit (Vector Labs). The Mouse on Mouse (MOM) kit (Vector) was used for α -SMA immunohistochemistry. For CD31 staining, sections were pretreated with trypsin, and staining was performed using the Tyramide Signal Amplification (TSA) kit (Perkin Elmer). Collagen staining was performed using picrosirius red. Quantitative assessment of macrophage density was performed by counting the number of F4/80-positive cells in the infarcted area.¹⁸ Myofibroblasts were identified as extravascular α -SMA-positive cells and counted in the infarcted myocardium. Collagen percent staining was quantitatively assessed in infarcts after 7 days of reperfusion using Sirius red-stained sections. Macrophage, neutrophil, and myofibroblast density was expressed as cells/mm². Infarct microvascular density was assessed by counting the number of CD31 stained vascular profiles in infarcted murine hearts. In addition, arteriolar density was measured by counting the number of mature vessels with a muscular coat.

Perfusion Fixation and Assessment of Ventricular Volumes

For assessment of postinfarction remodeling, infarcted hearts after 7 days of reperfusion were used for perfusion-fixation (n=12 for WT and n=11 for MCP-1 KO animals) as previously described.¹⁹ Briefly, a cardioplegic solution¹⁹ 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 from base to apex was cross-sectioned at 250 μ m intervals. Ten serial 5 μ m sections were obtained at each interval. The left ventricular end-diastolic volume (LVEDV) and left ventricular end-diastolic diameter (LVEDd) were assessed with ImagePro software using methods developed in our laboratory.¹⁹ The size of the infarct was expressed as a percentage of the left ventricular volume.

RNA Extraction and Ribonuclease Protection Assay

The mRNA expression level of the chemokines MIP-1 α , MIP-1 β , MIP-2, and IP-10, the cytokines TNF- α , IL-1 β , and IL-6, leukemia inhibitory factor (LIF) and IL-10, the growth factors TGF- β ₁, - β ₂, and - β ₃, stem cell factor (SCF), GM-CSF and M-CSF, and the chemokine receptors CCR1, CCR2, CCR5, CCR6, CCR7, CCR8, CCR9, CXCR2, and CXCR4 was determined using a ribonuclease protection assay (RiboQuant; Pharmingen) according to the manufacturer's protocol.¹² Phosphorimaging of the gels was performed (Storm 860; Molecular Dynamics), and signals were quantified using Image QuaNT software and normalized to the ribosomal protein L32 mRNA.

Statistical Analysis

Statistical analysis was performed using ANOVA followed by *t* test corrected for multiple comparisons (Student-Newman-Keuls). Data were expressed as mean \pm SEM. Statistical significance was set at 0.05.

Results

Chemokine Receptor Expression in Mouse Infarcts

We have recently demonstrated that reperfused mouse infarcts exhibit a marked but transient upregulation of chemokine mRNA levels.¹² The CC chemokines MCP-1, MIP-1 α , MIP-1 β , and the CXC chemokines MIP-2 and IP-10 are induced in the infarct, peaking after 6 hours of reperfusion.¹² Of all chemokines examined, MCP-1 showed the highest levels of mRNA expression.¹² Chemokine receptors are also significantly induced after myocardial infarction. CCR1 (the receptor for RANTES and MIP-1 α) is transiently upregulated

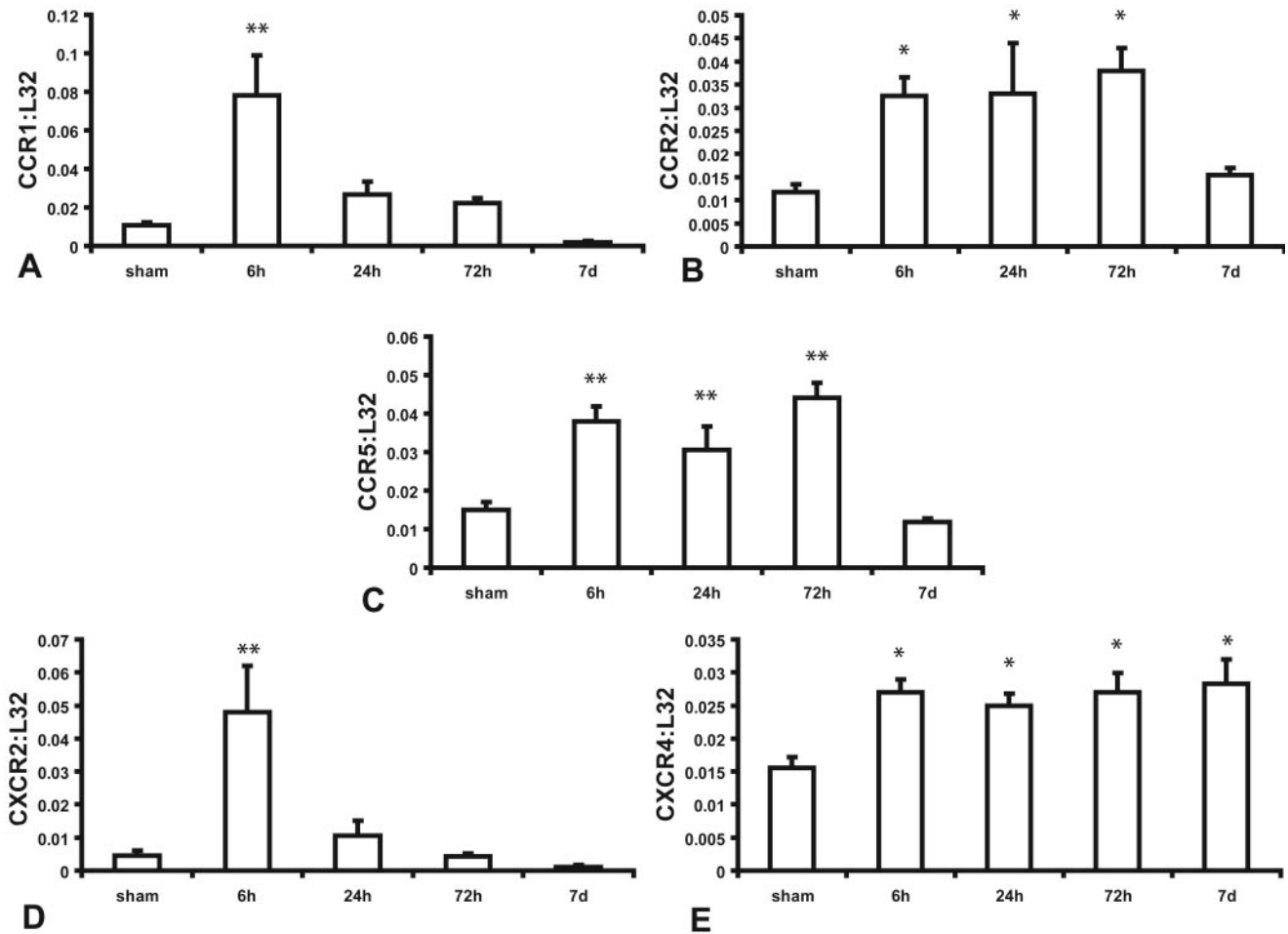


Figure 1. Chemokine receptor upregulation in reperfused mouse infarcts. RPA assay demonstrates rapid but transient induction of CCR1 mRNA in the infarcted murine myocardium (A). In contrast, CCR2 (B) and CCR5 (C) exhibit more prolonged upregulation. CXC chemokine receptors CXCR2 (D) and CXCR4 (E) are also induced in mouse infarcts (* $P < 0.05$ vs sham; ** $P < 0.01$ vs sham).

after 6 hours of reperfusion (Figure 1A), whereas CCR2 (the MCP-1 receptor) and CCR5 (the main receptor for MIP-1 β) show a more prolonged induction (Figure 1B and 1C). Expression of the CC chemokine receptors CCR6, CCR7, CCR8, and CCR9 was very low in both infarcted and sham-operated animals (not shown). Expression of CXC chemokine receptors is also induced in mouse infarcts. CXCR2 (the receptor for the CXC chemokines MIP-2, LIX, and KC) mRNA is upregulated after 6 hours of reperfusion (Figure 1D), whereas CXCR4 (the SDF-1 receptor) shows a more modest and prolonged upregulation for 7 days after reperfusion (Figure 1E).

MCP-1 Deficiency Delays Macrophage Recruitment and Cardiomyocyte Replacement With Granulation Tissue in Murine Infarcts

Control MCP-1^{-/-} and WT hearts have similar morphological characteristics. In the absence of injury, both MCP-1 deficient and WT hearts have a well-developed microvasculature and contain a very small resident macrophage population. WT and MCP-1^{-/-} mice have similar mortality rates after myocardial infarction (WT, 8% versus MCP-1^{-/-}, 8.1%). Myocardial infarction results in rapid infiltration of the injured myocardium with macrophages and neutrophils. Neutrophil density in the infarcted myocardium peaks after 24

hours of reperfusion and decreases significantly after 3 days. MCP-1 null and WT infarcts have a similar time course of neutrophil infiltration and comparable neutrophil density at all time points examined (Figure 2A). In contrast, macrophage recruitment in MCP-1 null mice is suppressed and delayed in comparison to WT animals. After 24 hours of reperfusion, $-/-$ mice have a significantly lower number of macrophages in the infarcted heart than their WT littermates (WT: 1562.3 ± 126.8 versus KO 922 ± 107.7 ; $P < 0.05$) (Figure 2B). After 3 to 7 days of reperfusion, macrophage density in $-/-$ infarcts is not significantly different than in WT infarcts. Thus, WT infarcts have a rapid increase in macrophage density that peaks after 24 hours of reperfusion (Figure 2B and 2C), whereas macrophage density in MCP-1-deficient animals shows a delayed peak after 72 hours of reperfusion (Figure 2B and 2F). In WT mice, leukocyte infiltration is followed by debridement of the infarcted myocardium, resulting in almost complete replacement of dead cardiomyocytes with granulation tissue after 72 hours of reperfusion (Figure 2J). In contrast, at the same time point, MCP-1 KO animals exhibit persistent presence of dead cardiomyocytes in the infarct and delayed replacement with granulation tissue, suggesting defective phagocytosis of injured cells (Figure 2K).

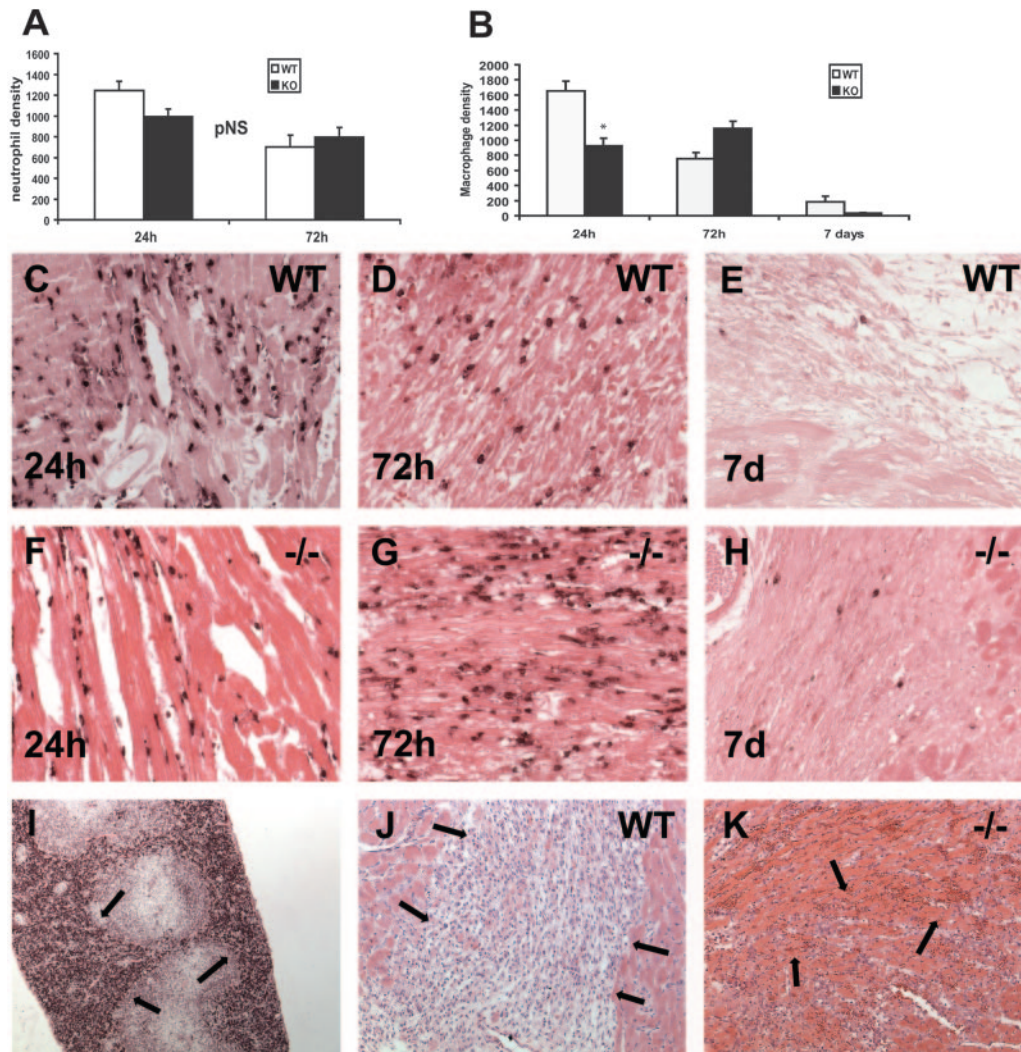


Figure 2. A, WT and MCP-1^{-/-} mice had similar time course of neutrophil infiltration in the infarcted myocardium. B, MCP-1-deficient animals have significantly lower macrophage density after 24 hours of reperfusion ($*P < 0.05$ vs WT) and demonstrate a delayed peak in macrophage density after 72 hours of reperfusion. C through E, Immunohistochemical staining with F4/80 identifies macrophages in WT mice after 24 hours (C), 72 hours (D), and 7 days (E) of reperfusion. F through H, MCP-1^{-/-} mice demonstrate delayed macrophage recruitment that peaks after 72 hours of reperfusion (G). Representative images from MCP-1^{-/-} infarcts after 24 hours (F) and 7 days of reperfusion (H) are also shown. I, Sections from the mouse spleen serve as a positive control for F4/80 immunohistochemistry labeling macrophages in the red pulp (arrows). J through K, Delayed macrophage recruitment in MCP-1^{-/-} infarcts is associated with defective phagocytosis of injured cardiomyocytes. After 72 hours of reperfusion, WT animals demonstrate almost complete replacement of dead cardiomyocytes with granulation tissue (J, arrows). In contrast, at the same time point, MCP-1 deficient mice show delayed granulation tissue formation and persistent presence of injured cardiomyocytes in the infarct (K, arrows).

Myofibroblast Infiltration and Infarct Angiogenesis in MCP-1-Deficient Animals

During the proliferative phase of infarct healing, granulation tissue composed of macrophages, myofibroblasts, and neovessels is formed. As the wound matures, myofibroblasts undergo apoptotic death, a dense collagen network is created, and the infarct capillaries regress, while stable pericyte-coated vessels are formed. MCP-1 deficient mice show decreased myofibroblast infiltration after 3 days of reperfusion compared with their WT littermates (Figure 3) (KO, 268 ± 42.1 versus WT, 599.4 ± 88.3). After 7 days of reperfusion, both MCP-1 KO and WT mice exhibit scars with a relatively low cellular content and significant deposition of matrix (Figure 4A and 4B). Collagen content is similar in MCP-1^{-/-} and WT infarcts (collagen % staining: WT,

$40.1\% \pm 1.1$ versus $-/-$, $39.9\% \pm 1.7$; $P = \text{NS}$). Infarct angiogenesis appears not to be significantly affected by the absence of MCP-1. WT and MCP-1^{-/-} animals show comparable infarct capillary density (Figure 4C). However, MCP-1-deficient animals demonstrated a trend toward a lower density of mature pericyte-coated vessels (Figure 4D) ($P = 0.09$).

Infarcted MCP-1 KO Hearts Have Decreased Cytokine Expression

Sham MCP-1^{-/-} and WT hearts have similar cytokine, chemokine, chemokine receptor, and growth factor mRNA expression profiles (not shown). Both KO and WT sham hearts show minimal expression of the chemokines MIP-1 α , MIP-1 β , IP-10, and MIP-2, and the cytokines TNF- α , IL-1 β , IL-6, LIF, and IL-10. Comparable low-level expression of

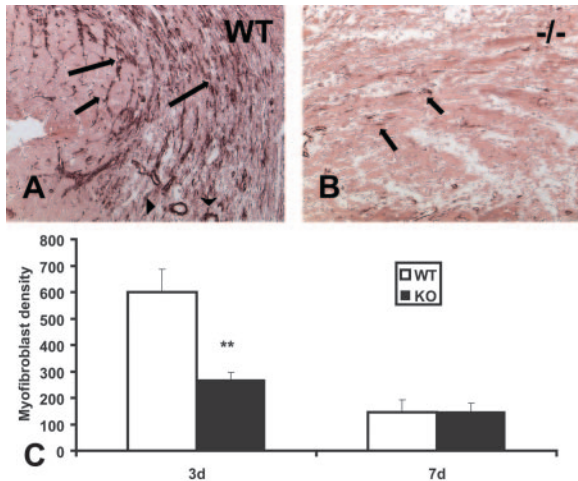


Figure 3. Decreased myofibroblast accumulation in MCP-1-deficient infarcts. α -SMA immunohistochemistry labels vascular mural cells (arrowheads) and myofibroblasts (extramural spindle shaped cells, arrows) in WT (A) and MCP-1^{-/-} (B) infarcts after 72 hours of reperfusion. WT mice have significantly higher peak myofibroblast density that KO mice (C) (** $P < 0.01$ vs WT).

ICAM-1 and E-selectin mRNA is noted in WT and KO sham hearts. The growth factors M-CSF, TGF- β_1 , - β_2 , - β_3 , VEGF, FGF1, and FGF2 are constitutively expressed in both WT and MCP-1^{-/-} sham hearts; however, only VEGF mRNA levels are higher in MCP-1 KO animals (VEGF:L32 ratio: WT, 0.22 ± 0.01 versus MCP-1^{-/-}, 0.39 ± 0.03 ; $P < 0.01$). As we have previously demonstrated,¹² reperfused infarcts show marked induction of the proinflammatory cytokines IL-1 β and TNF- α , the gp130-related cytokines IL-6 and LIF, and the chemokines MIP-1 α , MIP-1 β , MIP-2, and IP-10 that peak after 3 to 6 hours of reperfusion. MCP-1 KO mice have significantly lower TNF- α , IL-1 β , IL-6 (Figure 5) and LIF mRNA expression after 6 hours of reperfusion compared with WT infarcts. Expression of the inhibitory cytokine IL-10 is

rapidly and persistently induced in WT infarcts. In contrast, MCP-1^{-/-} mice demonstrate markedly reduced IL-10 mRNA levels after 6 and 24 hours of reperfusion, with a delayed peak after 72 hours of reperfusion (Figure 5). Expression of the chemokines MIP-1 α , MIP-1 β , MIP-2, and IP-10 is similarly induced in KO and WT animals. In addition, MCP-1 KO mice demonstrate selectively decreased mRNA expression of TGF- β_2 and - β_3 (not shown). In contrast, TGF- β_1 , VEGF, and FGF-1 and -2 expression is similar in WT and KO infarcts.

MCP-1-Deficient Mice Exhibit Decreased Expression of the Matricellular Protein OPN-1, a Marker of Macrophage Maturation

The matricellular protein OPN-1, a gene highly upregulated during monocyte to macrophage differentiation,²⁰ is markedly induced in reperfused infarcts (Figure 6A). Although sham KO and WT animals have negligible expression of OPN-1 mRNA, KO mice demonstrate markedly decreased OPN-1 induction after 24 hours of reperfusion, compared with WT animals. OPN-1 expression in MCP-1^{-/-} infarcts shows a delayed peak after 72 hours of reperfusion. Immunohistochemical staining identified a significant number of OPN-1 expressing macrophages in WT infarcts after 24 to 72 hours of reperfusion (Figure 6B). In contrast, at the same time point, most infarct macrophages in MCP-1-deficient animals are OPN-1 negative (Figure 6D).

Postinfarction Remodeling in MCP-1 KO Mice

Although MCP-1 null and WT mice exhibit similar scar size (WT, $8.9\% \pm 1.14$, $n = 11$, versus KO, $8.6\% \pm 1.44$, $n = 11$; $P = \text{NS}$) after 7 days of reperfusion, infarcted $-/-$ hearts have significantly lower LVEDD (KO, 3.74 ± 0.15 mm, $n = 11$, versus WT, 4.26 ± 0.2 mm, $n = 11$; $P = 0.05$) and show a trend toward decreased LVEDV (WT, 68.7 ± 7.1 mm³ versus KO, 50.2 ± 7 mm³; $P = 0.09$) when compared with their WT littermates (Figure 7).

MCP-1 Antibody Neutralization in Mouse Infarcts

Much like MCP-1-deficient animals, mice treated with neutralizing MCP-1 antibody demonstrate delayed replacement of injured cardiomyocytes with granulation tissue (Figures 8A and 8B). However in contrast with MCP-1 gene disruption, antibody inhibition does not affect macrophage recruitment in the infarct ($P = \text{NS}$ versus IgG-treated controls; Figure 8C). Furthermore, antibody-treated mice have diminished accumulation of myofibroblasts after 72 hours of reperfusion. Although MCP-1 inhibition has no effect on macrophage density in the infarct, it significantly decreases the number of OPN-1-positive cells (Figure 8D and 8E).

Discussion

MCP-1 induces monocyte migration in vitro and plays a critical role in mononuclear cell recruitment in an in vivo model of peritoneal inflammation.¹³ In addition, recent investigations have indicated significant effects of MCP-1 on nonhematopoietic cells, such as endothelial cells and fibroblasts, and suggested a potential role for this chemokine in cutaneous wound angiogenesis.²¹ MCP-1 is markedly induced in healing myocardial infarcts; however, its effects on

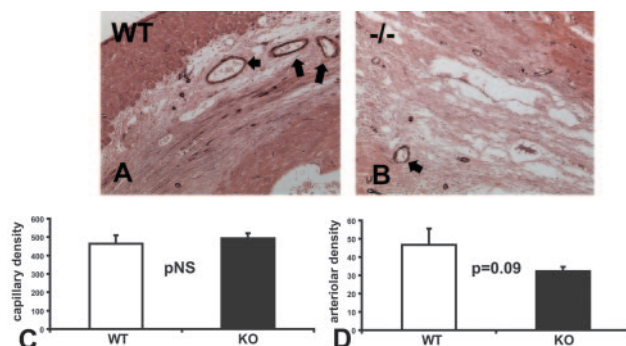


Figure 4. As the infarct matures, many wound microvessels acquire a muscular coat, which serves to stabilize the neovasculature. These infarct arterioles were identified with α -SMA immunohistochemistry (arrows), in WT (A) and MCP-1 KO mice (B) after 7 days of reperfusion. At this time point, the mature scar contains an organized matrix network and relatively few myofibroblasts. C, Number of capillaries (identified by CD31 staining and expressed as vascular profiles/mm²) in the infarct was not significantly affected by the absence of MCP-1. However, there was a trend toward a lower number of mature coated vessels (infarct arterioles) in MCP-1 KO infarcts (D, $P = 0.09$).

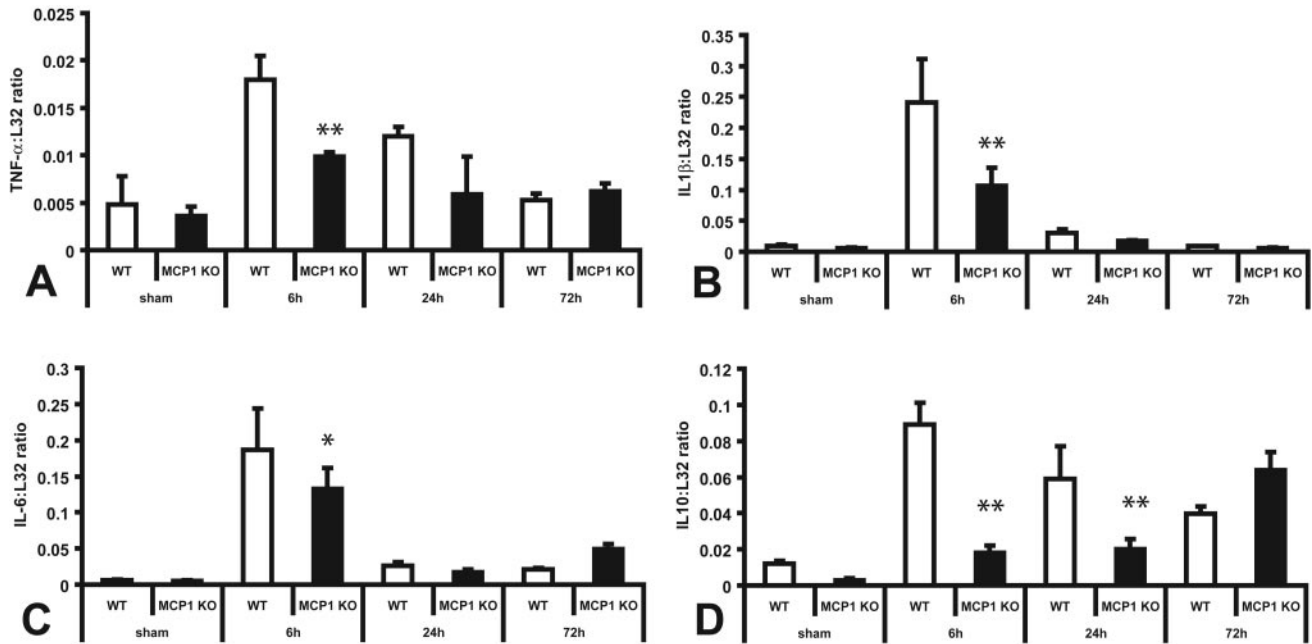


Figure 5. RPA demonstrates decreased cytokine mRNA expression in MCP-1 KO infarcts. TNF- α (A), IL-1 β (B), and IL-6 (C) mRNA levels were significantly lower in the absence of MCP-1 after 6 hours of reperfusion. In addition, expression of the antiinflammatory cytokine IL-10 was markedly lower in MCP-1 KO animals (D) (* P <0.05 vs WT; ** P <0.01 vs WT).

scar formation and cardiac repair remain poorly understood. Our study demonstrates that MCP-1 plays a critical role in regulating early recruitment of macrophages in healing myocardial infarcts. Animals with MCP-1 gene disruption showed decreased macrophage infiltration in the infarct, associated with delayed replacement of injured cardiomyocytes with granulation tissue. In addition, MCP-1 KO infarcts showed decreased expression of proinflammatory cytokines and diminished accumulation of myofibroblasts compared with their WT littermates.

Leukocyte Recruitment in the Absence of MCP-1

Reperfused murine infarcts exhibit a rapid release of leukocyte chemoattractants in the ischemic area. Complement activation, free radical generation, and chemokine induction is followed by extensive infiltration of the myocardium with neutrophils and mononuclear cells. However, the relative significance of various leukocyte chemotactic signals in inflammatory leukocyte recruitment remains unclear. CCL2/MCP-1 is a potent chemoattractant for monocytes in vitro and critically regulates mononuclear cell recruitment in animal models of peritoneal inflammation¹³ and nephritis.²² MCP-1 upregulation in the infarct is followed by prolonged induction of the MCP-1 receptor, CCR2, which peaks after 72 hours of reperfusion (Figure 1), while macrophage density is decreasing. This may reflect CCR2 expression in endothelial cells¹⁴ and myofibroblasts,²³ which are abundant in the infarct granulation tissue.

Although naïve neutrophils do not express CCR2 and do not respond to MCP-1, recent investigations indicated that MCP-1 may play an active role in neutrophil recruitment in inflammatory sites. CCR2 KO mice have decreased neutrophil infiltration in a model of pulmonary inflammation,²⁴ suggesting an interdependence between monocyte and neu-

trophil recruitment.²⁵ In addition, in a model of preexisting inflammation CCL2/MCP-1 induced neutrophil chemotaxis, indicating that chronic inflammatory states may alter leukocyte recruitment profiles by inducing CCR2 surface expression in neutrophils.²⁶ We found that MCP-1 gene disruption results in decreased and delayed recruitment of macrophages, but does not affect the time course and density of neutrophil infiltration in healing myocardial infarcts (Figure 2). Macrophage density in WT mice peaked after 24 hours of reperfusion, in contrast to MCP-1 null mice that demonstrate a late peak after 72 hours of reperfusion. WT and $-/-$ mice demonstrated similar expression of the chemokines MIP-1 α , MIP-1 β , and IP-10, suggesting that, in the absence of MCP-1, compensatory upregulation of other chemokines is not responsible for the delayed influx of macrophages in MCP-1-deficient animals.

Beyond its mononuclear cell chemotactic effects, MCP-1 also modulates monocyte phenotype and activity. In vitro, MCP-1 stimulates the respiratory burst²⁷ and induces expression of the proinflammatory cytokines IL-6 and IL-1 in isolated mononuclear cells.²⁸ Our studies demonstrated that macrophages infiltrating MCP-1 KO infarcts exhibit significant phenotypic alterations compared with WT mice. In the absence of MCP-1, OPN-1 expression by infarct macrophages was markedly decreased (Figure 6). OPN-1 is a marker of monocyte-to-macrophage differentiation,²⁰ highly expressed in mature macrophages of the healing infarct (Figure 6).²⁹ Furthermore, a critical effect of MCP-1 on macrophage activation was suggested by the antibody neutralization experiments. Although antibody inhibition did not significantly affect macrophage recruitment in the healing infarct (Figure 8), suggesting that antibody injection may have resulted in incomplete neutralization of MCP-1 activity, it delayed replacement of cardiomyocytes with granulation

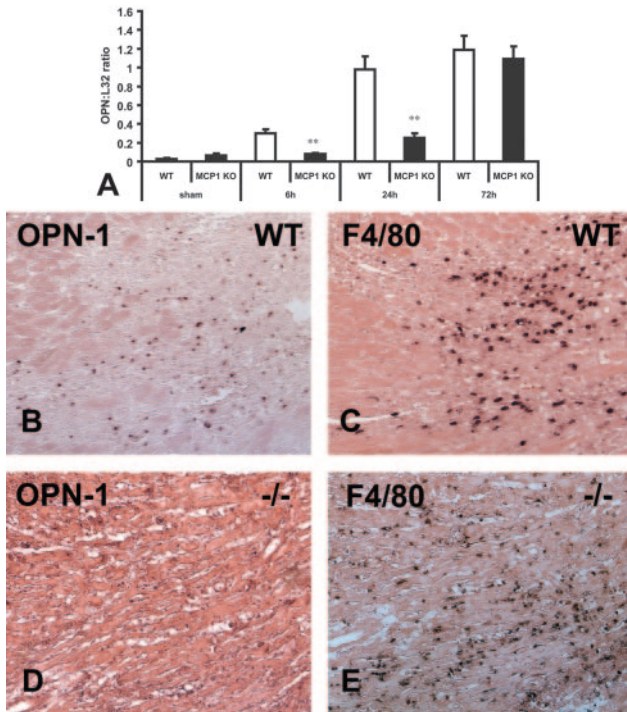


Figure 6. Matricellular protein OPN-1, a marker of macrophage maturation, is significantly induced in healing infarcts. A, Upregulation of OPN-1 mRNA was markedly suppressed in the absence of MCP-1 (** $P < 0.01$ vs WT). B, A significant number of OPN-1-positive cells was found in WT infarcts after 72 hours of reperfusion. Distribution and morphology of these cells were similar to macrophages, identified by F4/80 immunohistochemistry (C). In contrast, MCP-1^{-/-} infarcts had weak OPN-1 staining (D), despite the presence of a large number of F4/80-positive macrophages in the infarcted myocardium (E).

tissue. Macrophage OPN-1 expression was significantly lower in antibody-treated mice when compared with the respective IgG-treated controls. Thus, some of the consequences of impaired MCP-1 signaling in the infarct are not explained by decreased recruitment of mononuclear cells and are related to modulatory effects on monocyte phenotype.

Recent studies demonstrated that MCP-1 plays a crucial role in T helper cell polarization.³⁰ MCP-1-deficient animals have markedly impaired Th2 response, and lymph node cells from immunized MCP-1^{-/-} mice show very low IL-10 mRNA expression.³⁰ Our experiments indicated that MCP-1 KO infarcts exhibit markedly decreased IL-10 mRNA levels compared with their WT littermates (Figure 5). The disproportionate suppression of IL-10 synthesis in MCP-1 KO infarcts may reflect defective Th2 polarization of infiltrating lymphocytes in the absence of MCP-1.

Role of MCP-1 in Fibrous Tissue Deposition and Infarct Angiogenesis

MCP-1-deficient infarcts demonstrated decreased infiltration with myofibroblasts after 72 hours of reperfusion (Figure 3) compared with their WT littermates. Two distinct mechanisms may be responsible for this effect. First, decreased infiltration of macrophages and defective monocyte function and cytokine expression may result in suppressed expression of growth factors with an important role in fibrous tissue

formation. TGF- β_2 and - β_3 mRNA levels are significantly diminished in MCP-1 KO infarcts. Second, MCP-1 may have direct effects of MCP-1 on fibroblast phenotype. MCP-1 stimulates collagen expression via endogenous upregulation of TGF- β^{15} and enhances matrix metalloproteinase expression in stimulated fibroblasts.¹⁶ However, both WT and MCP-1 KO mice formed collagen-rich scars after 7 days of reperfusion, suggesting that the decrease in myofibroblast density was not associated with a significant healing defect.

Recent investigations suggested that MCP-1 may also play a role in angiogenesis. Human endothelial cells express the MCP-1 receptor CCR2, and recombinant MCP-1 induces endothelial cell chemotaxis and angiogenesis in the chick chorioallantoic membrane and the matrigel plug assays.¹⁴ The in vivo significance of these effects is less clearly established. MCP-1 signaling appears to be important for arteriogenesis and formation of collateral vessels in a model of femoral artery occlusion.^{31,32} Our experiments did not indicate a significant effect of MCP-1 deficiency in infarct microvascular density. However, MCP-1 null mice demonstrated a trend toward a decreased number of mature vessels with a muscular coat in the infarct (Figure 4). Formation of these arterioles is part of the maturation process of the vasculature, which results in capillary regression and creation of stable vascular structures in the mature scar.³³ MCP-1 may be

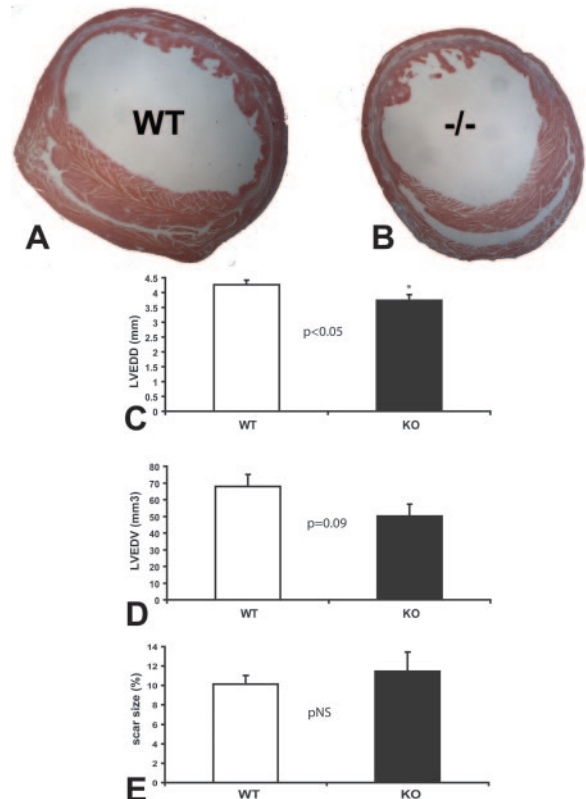


Figure 7. MCP-1^{-/-} mice demonstrate attenuated postinfarction remodeling after myocardial infarction. Infarcted WT (A) and KO (B) hearts were perfusion-fixed after 7 days of reperfusion. LVEDD was significantly lower in KO animals compared with their WT littermates (C) (* $P < 0.05$). In addition, infarcted MCP-1 null mice showed a trend toward decreased LVEDV ($P = 0.09$) (D). Infarct size was comparable between the groups (E).

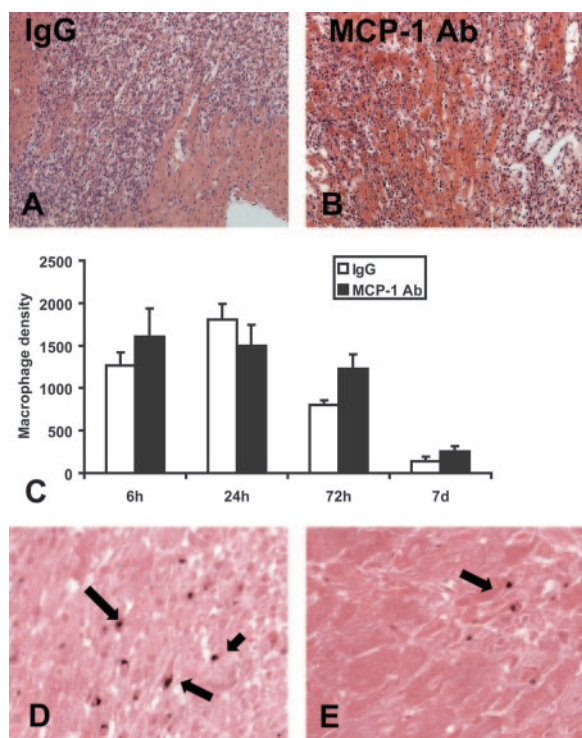


Figure 8. MCP-1 inhibition with a neutralizing antibody resulted in pathological changes similar to the defects noted in MCP-1 KO infarcts, in the absence of a significant effect on macrophage recruitment. Delayed replacement of injured cardiomyocytes with granulation tissue was noted in antibody-treated mice (B), in comparison with goat IgG-injected controls (A). However, the time course of macrophage infiltration was similar in antibody-treated and control animals (C). D, Numerous OPN-1-positive macrophages (arrows) were noted in IgG-treated animals. In contrast, rare OPN-1-expressing cells were found in MCP-1 antibody-treated mice after 72 hours of reperfusion.

important in the selective formation of vessels with arteriolar morphology.

MCP-1 and Postinfarction Remodeling

MCP-1 KO mice had attenuated postinfarction remodeling, demonstrating lower LVEDD and a trend toward a decrease in LVEDV but similar infarct size, when compared with WT animals (Figure 7). Suppression of inflammatory cytokine synthesis, decreased macrophage activation, and diminished myofibroblast infiltration may be important mechanisms in attenuating left ventricular remodeling in the absence of MCP-1. Decreased postinfarction remodeling was noted in mice receiving anti-MCP1 gene therapy³⁴ and in CCR2^{-/-} animals.³⁵

Role of MCP-1 in the Pathology of Myocardial Infarction: A Synthetic Approach

MCP-1 has a critical role in early recruitment of macrophages in the healing infarct. MCP-1-deficient animals exhibit reduced and delayed infiltration of the infarcted heart with mononuclear cells, resulting in impaired replacement of injured cardiomyocytes with granulation tissue and have decreased cytokine levels, impaired macrophage differentiation, and diminished myofibroblast infiltration. The role of MCP-1 extends beyond its monocyte chemoattractant effects:

MCP-1 inhibition with a neutralizing antibody results in defects comparable with the pathological findings noted in infarcted MCP-1^{-/-} animals in the absence of an impairment in monocyte recruitment. In the absence of MCP-1, suppressed inflammatory mediator expression and decreased fibrosis result in attenuated left ventricular remodeling. However, this does not necessarily imply a salutary effect for anti-MCP-1 strategies in healing infarcts. Defective MCP-1 signaling results in decreased macrophage activation, defective phagocytosis of injured cardiomyocytes, and delayed granulation tissue formation. Persistent presence of “mummified,” nonphagocytized cardiac myocytes in the infarct has been previously noted in animals receiving high-dose corticosteroids.³⁶ Furthermore, glucocorticoid treatment proved catastrophic in patients with acute myocardial infarction.³⁷ Although in MCP-1-deficient mice the delayed replacement of the infarcted myocardium with granulation tissue is associated with attenuated left ventricular remodeling, the clinical consequences of MCP-1 inhibition in the early phase of healing may be detrimental. It is possible that in patients with acute myocardial infarction, delayed phagocytosis of injured cardiomyocytes may increase the arrhythmogenic potential or predispose to mechanical complications, such as rupture or ventricular aneurysm formation. Because these events rarely occur in our model of reperfused murine myocardial infarction, the effects of MCP-1 inhibition in large mammalian models of infarction should be carefully studied before identifying MCP-1 as a potential target for therapeutic intervention.

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