Ccr1 deficiency reduces inflammatory remodelling and preserves left ventricular function after myocardial infarction

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Abstract

Myocardial necrosis triggers inflammatory changes and a complex cytokine cascade that are only incompletely understood. The chemokine receptor CCR1 mediates inflammatory recruitment in response to several ligands released by activated platelets and up-regulated after myocardial infarction (MI). Here, we assess the effect of CCR1 on remodelling after MI using Ccr1-deficient (Ccr1−/−) mice. MI was induced in Ccr1−/− or wild-type mice by proximal ligation of the left anterior descending (LAD). Mice were sacrificed and analysed at day 1, 4, 7, 14 and 21 after MI. While initial infarct areas and areas at risk did not differ between groups, infarct size increased to 20.6±8.4% of the left ventricle (LV) in wild-type mice by day 21 but remained at 11.2±1.2% of LV (P<0.05) in Ccr1−/− mice. This attenuation in infarct expansion was associated with preserved LV function, as analysed by isolated heart studies according to Langendorff. Left ventricular developed pressure was 84.5±19.8 mmHg in Ccr1−/− mice compared to 49.0±19.7 mmHg in wild-type mice (P<0.01) and coronary flow reserve was improved in Ccr1−/− mice. An altered post-infarct inflammatory pattern was observed in Ccr1−/− mice characterized by diminished neutrophil infiltration, accelerated monocyte/lymphocyte infiltration, decreased apoptosis, increased cell proliferation and earlier myofibroblast population in the infarcted tissue. In conclusion, functional impairment and structural remodelling after MI is reduced in the genetic absence of Ccr1 due to an abrogated early inflammatory recruitment of neutrophils and improved tissue healing, thus revealing a potential therapeutic target.

Keywords: myocardial infarction • myocardial remodelling • inflammation • chemokine receptor • leukocyte recruitment

Introduction

As a major cause of death in western countries, myocardial infarction (MI) due to the atherothrombotic occlusion of coronary arteries represents an irreversible necrosis of heart muscle caused by a prolonged imbalance between the oxygen supply and its metabolic demand. Myocardial necrosis is followed by an inflammatory response with complement activation and free radical generation, triggering a complex cytokine cascade and an up-regulation of chemokines [1–6]. Beyond their crucial role in coordinating leukocyte recruitment during inflammation and vascular disease [7, 8], chemokines appear to modulate the inflammatory response and to influence the myocardial healing and scar formation after MI [4, 9, 10].

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A study using gene-deficient mice and bone marrow chimeras [11] revealed that Cxcr2, a receptor for murine CXCL8 orthologs, exerts opposing effects on myocardial viability during ischaemia-reperfusion with recruitment of damaging inflammatory cells prevailing over tissue-protective effects mediated in resident myocardial cells. Besides recent findings indicating that CCL2 and its receptor Ccr2 contribute to leukocyte infiltration and left ventricular remodelling after MI [12–18], little is known about the involvement of other chemokines in the progression of tissue damage after MI, although an improved understanding may reveal promising therapeutic targets.

CCR1 is a receptor for CCL3 and CCL5, chemokines released by activated platelets and presented on the endothelium to mediate inflammatory recruitment [7, 19]. The CCR1 ligands CCL3/MIP-1α [14, 17] and CCL5/RANTES [14, 17] are potent mononuclear cell chemoattractants and markedly up-regulated after MI. Moreover, myeloid progenitor inhibitory factor-1/CCL23 induces endothelial cell migration and promotes angiogenesis via CCR1 [20, 21], while the CCR1 ligand CCL7 appears to be a potent myocardial mesenchymal stem cell homing factor [22].

The purpose of this study was to test the hypothesis that Ccr1 plays an important role in myocardial remodelling and inflammatory cell recruitment after MI using Ccr1 knockout mice.

**Methods**

**Animals and murine model of myocardial infarction**

C57/B6 mice and Ccr1−/− [23] were intubated under general anaesthesia (100 mg/kg ketamine, 10 mg/kg xylacine, intraperitoneal) and positive pressure ventilation was maintained with oxygen and isofluran 0.2%, using a rodent respirator. Hearts were exposed by left thoracotomy and MI was produced by suture occlusion of the left anterior descending artery (LAD). The muscle layer and skin incision were closed with a silk suture. Animal experiments were approved by local authorities and complied with German animal protection law.

**Langendorff perfusion**

At the indicated time points, the mice were anaesthetized and the heart function was analysed using a Langendorff apparatus (Hugo Sachs Elektronik-Harvard Apparatus) and dedicated software (EMKA Technologies, Paris, France) under constant perfusion pressure (100 mmHg) and electrical stimulation to assure a constant heart rate (600 bpm). The coronary flow and developed pressure were measured without or with adenosine (1 μmol/l). At the end, the hearts were fixed in distension with 10% formalin and cut into 5 μm serial slices.

**Histomorphometry and assessment of myocardial infarction size**

Serial sections (10–12 per mouse, 400 μm apart, up to the mitral valve) were stained with Gomori’s 1-step trichrome stain. The infarcted area was determined in all sections using Diskus software (Hilgers) and expressed as percentage of total left ventricular volume.

**Quantitative immunohistochemistry and immunofluorescence**

Serial sections (three per mouse, 400 μm apart) were stained to analyse infarcted areas for the content of neutrophils (specific esterase, Sigma), macrophages (F4/80, Serotec), lymphocytes (CD3, Serotec), vessels (CD31, Santa Cruz) and myofibroblasts (α-smooth muscle actin, DAKO). Cells were numbered in six different fields pro-section and expressed as cells/mm². Cardiomyocytes were stained with an anti-sarcomeric myosin antibody (Sigma). Collagen content was determined by Gomori’s 1-step trichrome staining, and calculated as the positively stained area percentage of the infarcted area, using the AnalySIS software (Soft Imaging Systems). To determine apoptosis and proliferation in the infarcted area, serial sections (three per mouse, 400 μm apart) were stained with in situ cell death detection kit (Roche) or anti-Ki-67 (DAKO) respectively, and counter-stained with DAPI. Apoptotic cell or proliferation indexes were expressed as percentage of positive cells.

**Flow cytometry**

Blood samples were stained with anti-CD3e-FITC (BD Pharmingen) for lymphocytes, anti-Gr-1-PerCP-Cy5.5 (BD Pharmingen) for neutrophils and CD115-PE (eBioscience) for monocytes and analysed by FACS to determine the distribution of blood cells at different time points after MI.

**ELISA**

ELISA was also performed from serum samples using DuoSet ELISA Development System kit for mouse MIP-1α and RANTES (R&D Systems).
mRNA isolation and RT-PCR

mRNA was also isolated from the infarcted area with TRIzol Reagent (Gibco) and RT-PCR was performed using Omniscript kit (Qiagen). Following oligonucleotide primers specific were used for murine RANTES cDNA (forward primer: 5’-GTGCCAGTCAGAGGTACA-3’ and reverse primer: 5’-GGGAAGCTATACAGGTTCA-3’), murine MIP-1α cDNA (forward primer: 5’-AGATTCCCCACGCAATTCTC-3’, reverse primer: 5’-CTCAAGCCGCTGCTTACAC-3’), murine MMP2 (forward primer: 5’-CACACCGGTGAAGGTGTG-3’, reverse primer: 5’-AGGGCTCTAGGAACCTAC-3’), murine MMP9 (forward primer: 5’-CACCACCAACTGAACCAC-3’, reverse primer: 5’-CTCAAGGCGGCTACGAG-3’) and murine TIMP-1 (forward primer: 5’-ATCGAGTGGGCTTCTCT-3’, reverse primer: 5’-TGACGGGCTCTGGTAGG-3’). Murine β-actin cDNA served as housekeeping gene and was amplified in parallel with the gene of interest (forward primer: 5’-AGCCCATGTCAGTGACATCC-3’, reverse primer: 5’-CTCTCACGCTGTGGTGGAA-3’).

Transmigration assay

Neutrophils were isolated from wild-type and Ccr1−/− blood by gradient centrifugation using Lympholyte mammal (Cedarlane laboratory). After isolation, neutrophils were resuspended in assay medium (Hepes-buffered Hanks’ balanced salt solution containing 0.5% bovine serum albumin and 1 μmol/l Mg2+ and Ca2+) and allowed to transmigrate across 5 μm transwell filters toward murine RANTES (100 ng/ml, PeproTech), MIP-1α (100 ng/ml, PeproTech) or buffer control in the lower chamber for 2 hrs at 37°C. Migration was defined as chemotactic index [24, 25].

Statistical analysis

Data represent mean±S.E.M. Statistical analysis was performed with Prism 4 software (Graph Pad) using unpaired Student’s t test or 1-way ANOVA followed by Newmann-Keuls post test. P-values <0.05 were considered significant.

Results

Analysis of myocardial infarction

MI injury was induced in wild-type and Ccr1−/− mice. No differences were observed between survival rates between wild-type and Ccr1−/− mice (96% each). Initial infarct areas and areas at risk did not differ between the groups one day after MI (Fig. 1). Whereas the infarct size in wild-type mice reached 20.6±8.4% of the left ventricle (LV) at day 21 and following wound contraction decreased to 15.4±4.0% at day 28, a significant attenuation and prevention of this increase in infarct size was observed in Ccr1−/− mice, where infarct size remained at 11.2±1.2% of the LV at day 21 and only slightly further decreased to 10.3±1.7% after 28 days (P<0.05; Fig. 1). Concordantly, isolated heart studies according to Langendorff showed a decrease in LV function over time to a LV developed pressure (LVEDP) of 49.0±19.7 mmHg in wild-type mice (Fig. 2A), whereas LV function significantly preserved in Ccr1−/− mice compared to wild-type mice (84.5±19.8 mmHg, P<0.01 versus wild-type). Coronary flow analysis revealed no differences in basal flow (Fig. 2B) but a significant increase and recovery in coronary flow after adenosine administration in Ccr1−/− mice versus wild-type mice (Fig. 2C). These data indicate a reduced infarct area and improved LV function following MI in Ccr1−/− mice. Analysis of cellular parameters and scar composition demonstrated that Ccr1−/− mice display a decrease in apoptosis rates in the scar area at intermediate time points (19.4±2.9% versus 33.0±4.0% in wild-type mice at day 14, P<0.05, Fig. 3A and B). To further characterize the cell types undergoing apoptosis, double immunofluorescence staining revealed that predominantly cardiomyocytes became apoptotic at day 1–4 after MI and only few apoptotic neutrophils or myofibroblasts could be detected (Fig. 4B and C). No apoptosis in macrophages was observed at early time points (data not shown). Moreover, cell proliferation at earlier time points was increased in the infarct area of Ccr1−/− mice (17.0±0.9% versus 8.3±1.4% in wild-type mice, P<0.0001, Fig. 3C and D). Myofibroblasts were shown to proliferate at day 4 but began to undergo apoptosis at 1 week after MI (Fig. 4C and D). This was also associated with an earlier repopulation with α-actin+ myofibroblasts (3205±251 cells/mm² versus 2523±289 cells/mm² in wild-type mice at day 4, Fig. 3E and F). In addition, a significant increase in the collagen content of the infarcted areas was observed in Ccr1−/− mice (3.6±2.2% versus 10.4±0.9% in wild-type mice at day 1, P<0.05 and 13.7±0.8% versus 22.4±1.0% in wild-type mice at day 7, P<0.01, Fig. 4A).

Staining for CD31+ endothelial cells giving rise to angiogenesis in the infarcted area by immunofluorescence revealed no differences between wild-type and...
Ccr1−/− mice (data not shown). Together, our data suggest an improved wound healing response in Ccr1−/− mice.

Inflammatory cell analysis after MI

We next analysed inflammatory cell recruitment into the infarcted area after MI. In wild-type mice, the neutrophil content in the infarcted area were transiently increased at 1 day after MI but subsequently reversed to baseline levels at day 4 (Fig. 5A). Notably, this increase was almost completely abrogated in Ccr1−/− mice (189±33 cells/mm² versus 707±244 cells/mm² in wild-type, P<0.05, Fig. 5A). In contrast, the peak in the content of both monocytes and lymphocytes in the infarcted area appeared to occur earlier in Ccr1−/− mice (day 7) as compared to wild-type mice (day 14), in line with an accelerated healing response (Fig. 5B and C). Interestingly, the peripheral blood counts of these leukocyte subsets (neutrophils, monocytes, lymphocytes) did not differ between Ccr1−/− and wild-type mice (data not shown), indicating that changes in inflammatory cell content...
Fig. 3 Scar remodelling after myocardial infarction. Apoptosis, as detected by TUNEL staining (A, B) and cell proliferation, as detected by Ki-67 staining (C, D) and myofibroblast content as determined by staining for α-actin (E, F) were determined and quantified in infarcted myocardium at different time points after injury. Ccr1−/− hearts showed lower apoptosis rates (A), earlier and higher cell proliferation rates (C) and an earlier myofibroblast population (E) in the infarcted area (n = 6–8 mice per group, mean ± S.E.M., *P<0.05 versus wild-type). Insets in the representative images (B, D) show nuclear counter-staining of the same field with DAPI. Scale bar: 50 µm.

Fig. 4 Scar remodelling after myocardial infarction. The collagen content of infarcted areas was determined in Ccr1−/− and wild-type mice after MI at the time points indicated (A). In wild-type mice, double immunofluorescence of apoptotic TUNEL+ or proliferating Ki-67+ cells with specific cell markers was performed (B–D). At 1 day after MI, cardiomyocytes (sarcomeric myosin) but not neutrophils (specific esterase) undergo apoptosis (B). After 4 days, cardiomyocytes but not myofibroblasts (α-smooth muscle actin) underwent apoptosis and myofibroblasts exhibited proliferation (C). At 7 days after MI, apoptosis can also be detected in myofibroblasts (D).
in the infarcted area were due to a defect in recruit-
ment rather than mobilization of leukocytes.

Chemokine expression and function after MI

To analyse changes in the expression and function of
chemokines accounting for the differences in inflam-
matory cell infiltration in Ccr1−/− mice, we determined
serum levels and chemotactic activities of MIP-1α and
RANTES as prominent Ccr1 ligands. As analysed by
ELISA, both chemokines rapidly increased in serum 1
day after MI in wild-type mice (Fig. 6A). This increase
was abrogated in Ccr1−/− mice for MIP-1α (27.0±2.9
versus 69.5±14.7 pg/ml in wild-type mice, P<0.05)
and for RANTES (181.3±50.7 versus 381.2±84.1 in
wild-type mice, P<0.05). To assess functional effects
of these chemokines in neutrophils, we performed in
vitro transmigration experiments using neutrophils iso-
lated from Ccr1−/− and wild-type mice (Fig. 6B).
Notably, MIP-1α rather than RANTES triggered sub-
stantial transmigration of wild-type neutrophils but not
Ccr1−/− neutrophils, indicating that neutrophil transmi-
gration was mediated by Ccr1 and that MIP-1α was
more important than RANTES in this process (Fig.
6B). As analysed by RT-PCR, the expression of MIP-
1α transcripts was up-regulated but did not differ
between wild-type and Ccr1−/− hearts 1 day after MI
(Fig. 6C). Notably, expression of RANTES mRNA was
markedly enhanced in Ccr1−/− compared to wild-type
mice 4 days after MI (Fig. 6C), whereas the expression
of MCP-1 mRNA was unaltered (Fig. 6D), implying that
RANTES rather than MCP-1 is responsible for the
more prominent, that is accelerated monocyte and
lymphocyte infiltration in Ccr1−/− mice at this time point.
In contrast, the rapid decrease in monocyte recruit-
ment might be related to the reduction in MCP-1 expres-
sion after 1 week in Ccr1−/− compared with wild-
type mice (Fig. 6D). No differences in the expression
levels of MMP2, MMP9 or TIMP-1 were observed after
MI in Ccr1−/− compared with wild-type mice (Fig. 6D).

Discussion

Here, we show that genetic deficiency in Ccr1 pro-
tects against myocardial re-modelling and inflamma-
tory changes and improves myocardial function after
MI. This appeared to be due to an attenuation of neu-
trophil infiltration and associated with reduced apo-
tosis in Ccr1−/− compared with control mice. Similarly,
treatment with a blocking anti-IL8 antibody targeting
neutrophil transmigration or genetic deletion of Cxcr2
in neutrophils impairing their recruitment inhibited
myocardial ischaemia-reperfusion injury [11, 26].
Since CXCR2 has been described to mainly act as an
arrest receptor [27, 28], it is not inconceivable that
Ccr1 can complement CXCR2 functions during neu-
trophil transmigration. Indeed, Ccr1 can exert non-
redundant functions in haematopoiesis, host defence
and inflammation [29], and blocking Ccr1 has been
found to significantly reduce the inflammation in
models of sepsis [30] or lung injury [31]. Moreover, a
function of Ccr1 in neutrophil recruitment has recently
been supported by a study employing a model of
ischaemia-reperfusion, where Ccr1−/− mice display an
impairment in early neutrophil arrest and transmigra-
tion in the cremasteric microcirculation [32]. On the
other hand, the reduction in apoptosis encountered in
the scar area of Ccr1−/− mice was mainly evident
between day 7 and 14 and not at day 1 and 4, when
cardiomyocytes apoptosis is most abundant, and
thus appears to be attributable to effects on recruited
leukocytes and fibroblasts, rather than early effects
on cardiomyocytes.

In accordance with our findings, a rapid and tran-
sient up-regulation of CCR1 and MIP-1α transcripts
has been observed after myocardial ischaemia-
reperfusion injury in a study by Dewald et al., which
differed from a more prolonged up-regulation of
CCR2. Moreover, the genetic deficiency in CCR2 or
its ligand CCL2 attenuated LV remodelling after
experimental MI, resulting in decreased and/or
delayed macrophage infiltration, reduced gelatinolyt-
ic activity, delayed phagocytic clearance of injured
cardiomyocytes and diminished granulation and
myofibroblast accumulation, but did not affect neu-
trophil recruitment [16, 17]. However, in contrast to
our findings in Ccr1-deficient mice, these mice did
not display a reduction in infarct size, suggesting that
an abrogation of Ccr1 effects on neutrophil recruit-
ment may more markedly limit infarct size with sub-
sequent benefits for LV function after MI.

Unlike in mice, MIP1-α appears to produce only
negligible levels of calcium flux and chemotaxis in
human neutrophils [33]. However, CCR1 expression in
these cells can be increased by granulocyte-
macrophage colony stimulating factor [34], which is
elevated in serum after MI [14, 35, 36], or by interferon-γ [37]; which is up-regulated in myocardium after MI [38]. This may result in relevant effects of CCR1 in human disease. Indeed, blocking human CCR1 by a specific antagonist CP-481,715 decreased cell infiltration and inflammatory responses in human CCR1-transgenic mice [39]. Moreover, the human CCR1 antagonist BX471 reduced heart and renal transplant rejection in rats, which may also involve a component of initial reperfusion damage [40, 41]. It is further notable that Ccr1 deficiency resulted in a prevention of acute chemokine secretion as evident by reduced serum levels after MI. This may be related to local attenuation inflammatory cell recruitment and their interactions with platelets and microvascular endothelium.

Another important mechanism contributing to the preserved LV function and the improved tissue healing with higher content of collagen and myofibroblasts could be the earlier monocyte and lymphocyte infiltration of the infarcted area, likely due to the increased RANTES expression in infarcted myocardium. The exact role of monocyte and lymphocytes in the healing scar has not been fully investigated; however they might have a pivotal role in the transition between inflammation and repair. They create an appropriate environment to promote neovessel formation, fibroblast proliferation and extracellular matrix metabolism and serve as a major source of cytokines and growth factors [42]. In particular, a Gr-1<sub>low</sub> (or in the human system CD14<sup>+</sup>CD16<sup>+</sup>) monocyte

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**Fig. 5** Myocardial infiltration with inflammatory blood cells after infarction. Neutrophils, monocytes and lymphocytes were analysed in infarcted myocardium by immunostaining for specific esterase, F4/80 or CD3. Compared to wild-type hearts, Ccr<sup>+/−</sup> hearts displayed a significantly reduced infiltration with neutrophils (A), whereas infiltration with monocytes (B) and lymphocyte (C) occurred earlier and more prominent in Ccr<sup>+/−</sup> myocardium (n = 6–8 mice per group, mean ±S.E.M., *P<0.05 versus wild-type).
subset with high expression of Ccr5 [43] has recently been found to display angiogenic properties [44]. Therefore, an earlier recruitment of a distinct monocyte subset in the infarcted area could be one mechanism for an improved cardiac function in Ccr1−/− compared to wild-type mice. This accumulation could be favoured by the marked up-regulation of the Ccr5 ligand in the infarcted myocardium of Ccr1−/− mice.

Monocytes/macrophages also produce transforming growth factor-β1 (TGF-β1), a major fibrogenic cytokine, which can mediate the differentiation of fibroblasts into myofibroblasts [45]. As a versatile cell type, myofibroblasts can assume different functions in extracellular matrix metabolism and contractile activity [3, 46, 47] and contribute to scar formation after MI. Our results indicate that the appearance and proliferation of myofibroblasts occurs earlier in Ccr1−/− than in wild-type mice. This might be related to the early RANTES-induced recruitment of specific monocyte subsets releasing TGF-β1 or of fibroblast progenitors and their differentiation into myofibroblasts in Ccr1−/− mice. In addition, the attenuation of neutrophil recruitment in Ccr1−/− mice associated with a reduced release of proteolytic enzymes or reactive oxygen species might contribute to an auspicious and less inflammatory environment permitting other cells, such as fibroblast to proliferate and further differentiate in response to ischaemic injury. The exact functions of these cell types, however, warrants further investigation and refinement.

Angiogenesis, that is the formation of new vessels in the area of ischaemia is also important to preserve and improve cardiac function [1, 3, 5, 48] and Ccr1 has been reported to mediate endothelial cell migration and to promote angiogenesis through CCL23, which besides MIP-1α and RANTES is another ligand for Ccr1 [20, 21]. However, we did not find differences in CD31 staining between Ccr1−/− and wild-type mice. This mechanism is therefore unlikely to explain the improvement of tissue healing and LV function after MI in our model.

In summary, our study provides evidence that Ccr1 deficiency preserves cardiac function and precipitates wound healing processes by attenuating the neutrophil-induced myocardial injury and necrosis and by supporting the early infiltration with monocytes, collagen synthesis and myofibroblast accumulation. Given the availability of effective small molecular antagonists [30, 31, 42, 43], we therefore propose that targeting CCR1 may represent a promising and
accessible strategy to optimize cardiac repair and to prevent ventricular remodelling after myocardial injury.

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