CC chemokine CCL5 plays a central role impacting infarct size and post-infarction heart failure in mice

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Aims
The chemokine CCL5 plays a critical role as neutrophil and macrophage activator do in atherosclerosis and myocardial infarction. Thus, we investigated whether the treatment with a neutralizing monoclonal antibody (mAb) to mouse CCL5 would provide therapeutic benefit when provoking a coronary-associated ischaemic event.

Methods and Results
C57Bl/6 mice were submitted to left coronary artery permanent ligature. Then, various parameters were monitored for up to 21 days. At 5 min and 3 days after coronary occlusion, mice received one intravenous injection of the rat anti-mouse CCL5 mAb or isotype IgG control. Infarct size was assessed histologically and by measuring serum cardiac troponin I levels. Kinetics of CCL5 tissue expression, leucocyte infiltration, matrix metalloproteinase (MMP) levels, and collagen deposition were histologically assessed. Serum chemokine levels were measured by enzyme-linked immunosorbent assay. Cardiac function and dimensions were assessed by magnetic resonance imaging (MRI). Chronic ischaemia increased both circulating and intracardiac levels of CCL5. At 24 h, treatment with the anti-CCL5 mAb resulted in a smaller infarct size and reduced circulating levels of chemokines. This effect was associated with reduction of neutrophil and macrophage infiltration within the infarcted myocardium. After 3 days of chronic ischaemia, anti-CCL5 mAb treatment reduced cardiac MMP-9. At 7 days, collagen content was significantly lower. At 21 days, neutralizing CCL5 improved mouse survival, cardiac myocyte size, and cardiac function.

Conclusion
Treatment with anti-CCL5 mAb significantly reduced both infarct size and post-infarction heart failure in a mouse model of chronic cardiac ischaemia. Cardioprotective effects were associated with the reduction of leucocyte recruitment within infarcted hearts.

Keywords
Myocardial infarction • Leucocytes • Heart failure

Introduction
Myocardial infarction is frequently complicated by pathological cardiac remodelling that diminishes contractile function and reduces stroke volume. Cardiac remodelling characterized by the alteration of ventricular mass, form, and function, may potentially lead to ventricular arrhythmias, heart failure, and ultimately increases cardiovascular mortality.1,2 Acute myocardial infarction initiates a large inflammatory response resulting in the clearance of dead cardiomyocytes, matrix debris, and replacement of the
damaged tissue with a collagen-based scar. The activation of the complement system and toll-like receptors, as well as the presence of oxidative stress induce cytokine and chemokine secretion within the injured myocardium.\(^3\)\(^-\)\(^5\) Subsequent leucocyte accumulation in the infarcted tissue is associated with potent cytotoxic effects through the release of proteolytic enzymes and degradation of collagen and extracellular matrix in cardiac tissue, resulting in ventricular dilatation and adverse remodelling. Thus, targeting chemokine-induced leucocyte recruitment during myocardial repair may represent an attractive therapeutic strategy to improve cardiac function following an acute myocardial infarct. Human and animal studies suggest a crucial role of chemokines and their cognate receptors (e.g. CCL2/MCP-1, CCL4/MIP-1\(\beta\), CXCL8/IL-8 and CCR1, CCR5, or CXCR2) in post-infarction remodelling.\(^6\)\(^-\)\(^9\) Another chemokine CCL5/RANTES (Regulated on Activation Normal T cell Expressed and Secreted) has been shown to orchestrate the recruitment to inflammatory sites of several inflammatory cell subsets, such as monocytes, neutrophils, dendritic cells, and lymphocytes through the binding to CCR1, CCR3, or CCR5.\(^10\)\(^,\)\(^11\)

Given the pathophysiological relevance of CCL5 in several immuno-inflammatory diseases such as atherosclerosis, stroke, and myocardial ischaemia/reperfusion, we investigated the potential role of CCL5/RANTES in post-infarction cardiac remodelling.\(^12\)\(^-\)\(^14\)

To better understand the inflammatory processes governing ischaemic myocardial injury and repair, we selected to block CCL5 during the early phases of chronic ischaemia. Thus, development of post-infarction heart failure as well as cardiac and systemic inflammation was assessed at different times in mice treated immediately and 3 days post-occlusion with either an anti-mouse CCL5 monoclonal antibody (mAb) or the isotype control.

### Methods

#### Antibodies

The rat anti-mouse CCL5 IgG2a MAB478 was purified using protein G affinity chromatography from supernatants of hybridoma (R&D Systems Inc, Minneapolis, MN, USA). In an in vitro chemotaxis assay, MAB478 specifically neutralizes migration of CCR5 expressing cells induced by 36 ng/mL of mouse CCL5 with an IC50 of 0.2 \(\mu\)g/mL (data not shown). MAB478 half-life time \(\text{in vivo}\) in adult male C57Bl/6 mice is 211 h. The rat anti-bovine calf acetylcholine receptor alpha subunit IgG2a (clone mAb64) was used as an isotype control. All antibodies were tested using a Limulus Amoebocyte Lysate-based assay and endotoxin levels were found undetectable (<0.005 EU/mL, from Charles River Laboratories International, Wilmington, MA, USA).

**In vivo cardiac chronic ischaemia and ischaemia/reperfusion protocols**

Male C57Bl/6 mice (8–12 weeks of age) were obtained from the University Medical Centre (CMU) animal facility, Medical Faculty, University of Geneva, Geneva, Switzerland. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and has been approved by local authorities.

In both protocols, mice were initially anaesthetized with 4% isoflurane and intubated. After starting mechanical ventilation (tidal volume of 150 \(\mu\)L, 120 breaths/min) by supplementation with 100% oxygen, anaesthesia was maintained with 2% isoflurane. A thoracotomy was performed in the left third intercostal space and the pericardium was removed. Ligation of the left anterior coronary artery at the inferior edge of the left atrium was performed using an 8-0 Prolene suture. In ischaemia/reperfusion protocol, a small piece of polyethylene tubing was used to secure the ligature without damaging the artery. After 30 min of ischaemia, the occlusion of the left anterior coronary artery occlusion was released and reperfusion occurred. Reperfusion was confirmed by visible restoration of colour to the ischaemic tissue (Figure 1A). In chronic ischaemia protocol, ligation was maintained till animal sacrifice (Figure 1B). In both protocols, after 5 min of ischaemia onset, either the rat anti-mouse CCL5 mAb (at doses ranging 0.1–1.0 mg/mouse) or the isotype control (1 mg/mouse) was administered intravenously to neutralize serum and cardiac CCL5 bioactivity \(\text{in vivo}\). Next, the chest was closed and the ventilator removed to restore normal respiration. Sham-operated animals were submitted to the same surgical protocol as described but without arterial occlusion. In the chronic ischaemia protocol, a second intravenous injection of the anti-CCL5 mAb (1 mg/mouse) or the isotype control (1 mg/mouse) was administered after 3 days of chronic ischaemia (Figure 1A). At different time points of chronic ischaemia (from 8 h to 21 days) or reperfusion (1 day), animals were sacrificed for infarct size determination or immunohistochemical analysis.

**Area at risk (AAR) and infarct size (I) assessment**

Area at risk and infarct size were assessed as described in the Supplementary material online.

**Serum cardiac troponin I and chemoattractant level detection**

Levels of serum cardiac Troponin I (cTnI) and chemokines were measured as described in detail in the Supplementary material online.

**Immunostaining**

Immunostaining were performed as described in detail in the Supplementary material online.
Sirius Red staining for collagen content
Sirius Red staining was performed as described in detail in the Supplementary material online.

Magnetic resonance imaging (MRI)
MRI was performed at days 1 and 21 of chronic myocardial ischaemia in the same mice, as described in the Supplementary material online.

Statistical analysis
Statistical analysis was performed as described in detail in the Supplementary material online.

Results
Circulating and cardiac levels of CCL5 are up-regulated during chronic ischaemia
In order to investigate the possible role of CCL5 during chronic myocardial ischaemia, we assessed CCL5 expression within the myocardium and in the serum of sham-operated and infarcted untreated mice (permanent ligation protocol). Chronic ischaemia significantly increased the cardiac expression of CCL5 after 3 and 7 days as compared to sham-operated mice (Figures 2A and B). On days 1 and 3 of chronic ischaemia, serum levels of CCL5 were significantly increased as compared to sham-operated mice (Figure 2C). At 1 day, CCL5 expression was detected in ventricular regions rich in infiltrating inflammatory cells (Supplementary material online Figure S1A), indicating that these cells have to be considered as the major candidates mediating the release of CCL5 in infarcted hearts. At 3 days of chronic ischaemia, CCL5 staining was particularly detected in macrophage-rich areas (Supplementary material online Figure S1B).

Blocking CCL5 reduces infarct size at 24 h in I/R and chronic ischaemia protocols
In order to confirm and further validate the potential therapeutic benefit of CCL5 neutralization in myocardial infarction, we first assessed treatment with an anti-CCL5 mAb in a mouse model of...
myocardial ischaemia/reperfusion. Similar to our results obtained with another CCL5 antagonist (44AANA47-RANTES), the single administration of the anti-CCL5 mAb reduced the infarct size \( (n = 10, \text{average} + \text{SEM}, \text{I/area at risk: } 24.06\% + 1.28) \) as compared to controls \( (n = 10, \text{average} + \text{SEM}, \text{I/area at risk: } 50.76\% + 6.1, P = 0.0005) \). The benefit of the anti-CCL5 mAb treatment was also associated with a decrease in the serum cTnI levels at 24 h of reperfusion \( (n = 7 \text{ per group, average} + \text{SEM, anti-CCL5 mAb vs. isotype control: } 15.67 + 6.63 \text{ vs. } 46.34 + 5.76 \text{ ng/ml, } P = 0.004) \). After in vivo validation of the anti-CCL5 mAb neutralizing activity, we investigated the role of CCL5 neutralization in the mouse model of chronic myocardial ischaemia. The anti-CCL5 mAb treatment was also associated with a decrease in the serum cTnI levels at 24 h of reperfusion \( (n = 7 \text{ per group, average} + \text{SEM, anti-CCL5 mAb vs. isotype control: } 15.67 + 6.63 \text{ vs. } 46.34 + 5.76 \text{ ng/ml, } P = 0.004) \). After in vivo validation of the anti-CCL5 mAb neutralizing activity, we investigated the role of CCL5 neutralization in the mouse model of chronic myocardial ischaemia. The anti-CCL5 mAb or isotype control was administered via a single intravenous administration 5 min after the ischaemic period onset and infarct size was assessed after 24 h. In the chronic ischaemia protocol, area at risk (AAR) was similar in all treatment groups, indicating that ligation was reproducibly performed at the same level of the left anterior coronary artery (Figure 3A). Treatment with the anti-CCL5 mAb dose-dependently reduced the infarct as compared to the isotype control (Figures 3B and C). The anti-CCL5 mAb treatment significantly reduced serum cTnI levels (a marker of myocardial necrosis) levels as compared to isotype-treated mice after 8 h and 24 h of reperfusion (Figures 3D and E).

**Blocking CCL5 reduces neutrophil and macrophage recruitment in infarcted hearts during chronic ischaemia**

To investigate whether neutralization of CCL5 modified leucocyte recruitment during chronic ischaemia, neutrophil, and macrophage infiltration was analysed at different time points (from 8 h through 21 days). Considering the kinetics of the serum levels and cardiac expression of CCL5 during chronic ischaemia (Figure 2C) and half-life time (211 h), we administered two injections of the anti-CCL5 mAb or isotype control, i.e. at 5 min and 3 days after onset of chronic ischaemia. The anti-CCL5 mAb treatment significantly reduced neutrophil infiltration on days 1 and 3 as compared to isotype controls (Figures 4A and B). The anti-CCL5 mAb also reduced macrophage (CD68+ cell) recruitment at 3 days of chronic ischaemia (Figures 4C and D).
Blocking CCL5 reduces serum levels of neutrophil and monocyte chemoattractants during chronic myocardial ischaemia

CCL5 has been shown to induce neutrophil and monocyte chemotaxis in vivo and in vitro. In order to investigate if CCL5 neutralization might influence neutrophil and macrophage recruitment within infarcted hearts, we analysed the possible reduction of serum levels of other chemoattractants (i.e. CXCL1, CXCL2, and CCL2). These chemokines were markedly up-regulated in mouse serum of isotype-treated mice after 8 h and 1 day of chronic ischaemia as compared to sham-operated mice (Figures 5A–C). The anti-CCL5 mAb treatment significantly reduced CXCL1 and CXCL2 serum levels at 8 h of chronic ischaemia as compared to isotype-treated mice (Figure 5A and B). The anti-CCL5 mAb also reduced CCL2 serum levels at 1 and 3 days of chronic ischaemia as compared to isotype-treated mice (Figure 5C).

Blocking CCL5 transiently reduced MMP-9 and collagen amounts, but not MMP-8 in infarcted hearts during chronic ischaemia

In order to investigate if the anti-CCL5 mAb treatment could modulate some molecular mediators of post-infarction cardiac remodelling
during chronic ischaemia, we assessed the cardiac content of MMP-8, MMP-9, and different collagen types. Treatment with the anti-CCL5 mAb did not affect MMP-8 amounts at time points measured (from 1 to 21 days) as compared to isotype-treated mice (Figure 6A and data not shown). In contrast, MMP-9 content was significantly reduced in infarcted hearts after 3 days of chronic ischaemia as compared to the isotype-treated mice (Figure 6B) and remained low. In addition, the anti-CCL5 mAb treatment also initially impaired total collagen production after 7 days of chronic ischaemia as compared to isotype-treated mice (Figures 7A and D). This effect was mainly associated with the reduction of collagen type III (Figure 7C), as collagen type I was not affected (Figure 7B).

**Blocking CCL5 improves survival and cardiac function in infarcted mice during chronic ischaemia**

Mice treated with the anti-CCL5 mAb exhibited an overall survival rate of 63.2% (24/38) during 21 days of chronic ischaemia compared with mice treated with isotype IgG (survival rate 36.8%, 14/38, P = 0.02, Figure 8A). During chronic ischaemia, neither the anti-CCL5 mAb- nor the isotype IgG-treated mice presented a decrease in body weight (Figure 8B). No significant benefit in cardiac function (left ventricle ejection fraction [EF%]) was observed after 1 day of chronic ischaemia (Figures 9A and B). At 21 days following chronic ischaemia, the EF% was markedly improved in the anti-CCL5 mAb- as compared to the isotype IgG-treated mice (Figure 9A and C). Furthermore, cardiac end-systolic volume (ESV) and end-diastolic volume were improved by the anti-CCL5 mAb treatment, although without reaching statistical significance (Figure 9C, isotype-treated group [n = 5] vs. anti-CCL5 mAb-treated group [n = 14], average ± SEM. ESV: 133.97 ± 19.66 vs. 105.49 ± 9.69 μl, P = 0.171; EDV: 165.34 ± 18.25 vs. 147.57 ± 11.14 μl, P = 0.422). Post-infarction adverse cardiac remodelling was also assessed by mouse heart weight, body weight, MRI determination of left ventricular dimensions and wall thickness, and histological assessment of cardiac myocyte size. After 21 days of chronic ischaemia, no difference between anti-CCL5 mAb- and isotype IgG-treated mice was observed in all parameters assessed (Table 1 and Supplementary material online Table S1), except for cardiac myocyte size that was increased in anti-CCL5 mAb-treated mice as compared to controls (Figure 9D and E). Accordingly, at 21 days of chronic ischaemia, cardiac myocyte size positively

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**Figure 5** Treatment with anti-CCL5 mAb reduces neutrophil and macrophage chemoattractant serum levels during chronic ischaemia. Serum levels of CXCL1, CXCL2 (neutrophil chemoattractants) and CCL2 (monocyte/macrophage chemoattractant) were measured in sham-operated (Sham, sacrificed at 24 h), isotype IgG (1 mg/mouse)- or anti-CCL5 mAb (1 mg/mouse)-treated mice, respectively. Data are expressed as mean ± SEM (n = 7–12). (A) CXCL1; (B) CXCL2; and (C) CCL2.
correlated with left ventricle EF% (r = 0.776, P = 0.001). These data suggest that, after 21 days of chronic ischaemia, selective treatment neutralizing CCL5 improves infarct size and post-infarction with a marginal effect on left ventricular adverse remodelling.

**Discussion**

In the present study, we demonstrate that CCL5 is up-regulated at both systemic (blood stream) and local (within the infarcted myocardium) sites in animals during chronic ischaemia. In particular, a marked peak in CCL5 production was observed in the period between 3 and 7 days after myocardial infarction. Significant elevation of CCL5 serum levels have been reported in patients after acute myocardial infarction complicated by heart failure and severe left ventricular dysfunction. Increased CCL5 serum levels were also observed in patients with severe congestive heart failure (NYHA Class IV), independently of the cause of the disease. At a local level, we have recently documented an increase in CCL5 production within a few minutes of the initiating reperfusion in another model of myocardial ischaemia/reperfusion using ApoE−/− mice. Thus, our results, that confirm the observations of others, imply that CCL5 would be an appropriate protein to target to modulate the inflammation underlying adverse cardiac remodelling. Due to the published mechanism of action for CCL5, we hypothesized that the selective neutralization of CCL5 during the early phases (e.g. in the first 3 days) of permanent ischaemia would directly reduce neutrophil and monocyte recruitment to myocardial infarcted areas. Indeed, we observed a potent inhibitory effect of the anti-CCL5 mAb was observed at days 1 and 3 for neutrophils and at 3 days for macrophages. However, our data also suggest either an additional or an alternative mechanism, which could indirectly inhibit leucocyte recruitment within the infarcted hearts. The anti-CCL5 mAb treatment significantly reduced serum levels of other neutrophil (CXCL1 and CXCL2) and monocyte/macrophage (CCL2) chemotactants as compared to the isotype-treated control group. These data indicate that the neutralization of CCL5 can be associated with the reduction of serum levels of other classical leucocyte chemotactants, thus impairing leucocyte migration via other receptors. Such a precedent has been recently reported by Mei and colleagues, who demonstrated that removing the chemokine CXCL5 from the system leads to a decrease in other chemokines, such as CXCL1 and CXCL2, thereby altering their gradient. Administration of the anti-CCL5 mAb not only reduced leucocyte recruitment, but also infarct size. Thus, we have been able to reinforce our previous results using a different CCL5 antagonist in the murine model of cardiac I/R. In this study, the beneficial effect of CCL5 neutralization was also shown at 24 h in the permanent ligation protocol. Taking all results together then indicate a potential use of anti-CCL5 mAb to reduce infarct size, also mechanical or pharmacological myocardial reperfusion therapies are not performed.

Interestingly, we also showed that the anti-CCL5 mAb treatment improved post-infarction ‘clinical’ outcomes, such as survival.
and cardiac function. Since the mice did not modify their body weight during permanent myocardial ischaemia, their death was most probably associated with the severity of cardiac heart failure or possibly with other post-infarction complications, such as cardiac arrhythmias. These long-term positive effects of anti-CCL5 mAb treatment were also observed on molecular mediators (such as MMP-9 and collagen) of cardiac remodelling during chronic ischaemia. These effects might actively contribute to the anti-CCL5 mAb-mediated improvement on cardiac function. The reduction in cardiac collagen content we observed was a surprising result. In fact, these data are not in accordance with the classical paradigm showing an inverse association between collagen and its degrading enzymes MMPs. Furthermore, reduction in cardiac collagen content during post-infarction remodelling is not widely considered as a beneficial event, mainly because of the increased risk in cardiac rupture. Despite being potentially adverse, the effect of anti-CCL5 mAb on collagen was relatively weak and certainly transient. In addition, it could be explained as a consequence of the reduced infarct size and corresponding scar surface in the anti-CCL5 mAb-treated mice.

**Figure 7** Treatment with anti-CCL5 mAb transiently reduces collagen in infarcted hearts during chronic myocardial ischaemia. Quantifications of total collagen (A), collagen I (B), and collagen III (C) in frozen sections of sham-operated (Sham, sacrificed at 7 days) and infarcted hearts at different time points of chronic ischaemia (7–21 days). Data are expressed as mean ± SEM (n = 7–9 per group). (D) Representative microphotographs of infarcted hearts showing bright-field sections without polarization (figures on the left) and corresponding sections under polarized light illumination (figures on the right).
instead of an altered collagen deposition in scar tissue. This specula-
tion was also suggested by the absence of post-infarction myo-
cardial rupture events observed in the entire animal study
cohort during autopsy. Interestingly, treatment with anti-CCL5
mAb did not induce any significant improvement in certain
cardiac remodelling parameters (such as the measurement of
heart weight and by MRI determination of ventricular dimensions
and diastolic wall thickness at mid-papillary muscle level) at
21 days of chronic ischaemia. On the other hand, in the same
mice submitted to MRI protocol, cardiac myocyte size was
increased in anti-CCL5 mAb-treated mice as compared to controls
and it positively correlated with left ventricle EF% ($r = 0.776$, $P =
0.001$). Although treatment with anti-CCL5 mAb induced only
marginal effects on regional adverse cardiac remodelling (assessed
by MRI), the increase of viable cardiomyocyte surface at 21 days in
the same mice should be considered as a crucial determinant of
the improvement in cardiac function. To summarize, our results
suggest that anti-CCL5 mAb treatment was enough to reduce
the infarct size and leucocyte infiltration in the first few days post-
myocardial infarction. These beneficial effects resulted in a delayed
increase in cardiomyocyte size and left ventricular function at
21 days. Conversely, acute anti-CCL5 treatment was not sufficient
to significantly modify cardiac adverse remodelling. Since infarct
size and adverse cardiac remodelling have been shown as strongly
associated with the development of post-infarction heart failure,
our results might appear as apparently discordant. However,
our treatment approach was planned to selectively neutralize
CCL5-mediated leucocyte inflammation in the first few days post-
myocardial infarction, thus potentially interfering with early cardiac
repair and healing.

Figure 8  Treatment with anti-CCL5 mAb improves survival in mice after acute myocardial infarction followed by chronic myocardial ischae-
mia. (A) Kaplan–Meier survival curve following myocardial infarction and permanent ischaemia in isotype IgG- and anti-CCL5 mAb-treated mice
($n = 38$ per group). (B) Body weight of isotype IgG- and anti-CCL5 mAb-treated mice during permanent ischaemia (from the day of surgery
[time 0] till 21 days of chronic ischaemia). Data are expressed as mean ± SEM ($n = 11$ in isotype-treated mouse group and $n = 23$ in anti-CCL5
mAb-treated mouse group). Differences in body weight at any time point investigated and also between treatment groups were not statistically
significant.

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Number of mice at risk:

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In conclusion, we have shown that CCL5 is highly expressed during chronic ischaemia following myocardial infarction. Administration of a neutralizing anti-CCL5 mAb during early phases of ischaemia reduced infarct size in vivo in two mouse models (myocardial ischaemia/reperfusion and permanent ischaemia). The cardioprotective effects of CCL5 neutralization during early phases of chronic ischaemia were associated with the decrease of neutrophil and macrophage recruitment in the infarcted myocardium. Furthermore, treatment with anti-CCL5 mAb induced a reduction in MMP-9 levels yet, only a transient impairment of collagen content and an increase in cardiac myocyte size. These partial changes on well-accepted molecular mediators of cardiac remodelling resulted in marginal effects on regional adverse cardiac remodelling (as assessed by MRI). However, anti-CCL5 mAb-mediated increase in cardiomyocyte size at 21 days was strongly associated with the improvement of left ventricle ejection fraction. Indeed, after 21 days, treatment with the anti-CCL5 mAb also improved the survival rate. Therefore, neutralization of CCL5 bioactivity...
during the early phase of chronic ischaemia following myocardial infarction could represent a promising therapy to reduce infarct size, post-infarction heart failure, and related mortality.

**Supplementary material**

Supplementary material is available at European Heart Journal online.

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**Conflict of interest:** None to be declared.

**References**


