Regulation of monocyte subset systemic levels by distinct chemokine receptors controls post-ischaemic neovascularization

Clément Cochain1†, Mathieu P. Rodero2†, José Vilar1, Alice Récalde1, Adèle L. Richart1, Céline Loinard1, Yasmine Zouggari1, Coralie Guérin1, Micheline Duriez1, Behazine Combadière2, Lucie Poupel2, Bernard I. Lévy1, Ziad Mallat1,3, Christophe Combadière2, and Jean-Sébastien Silvestre1*

1INSERM, U970, Paris Cardiovascular Research Center—PARCC, Université Paris Descartes, UMR-S970 Paris, France; 2Laboratoire d’Immunologie et Infection, INSERM UMR-S945, Université Pierre et Marie Curie, Site Pitié-Salpétrière, Paris, France; and 3University of Cambridge, Department of Medicine, Division of Cardiovascular Medicine, Cambridge, United Kingdom

Received 12 March 2010; revised 18 May 2010; accepted 21 May 2010; online publish-ahead-of-print 25 May 2010

Time for primary review: 26 days

Aims
Monocyte systemic levels are known to be a major determinant of ischaemic tissue revascularization, but the mechanisms mediating mobilization of different monocyte subsets—Ly6C hi and Ly6C lo—to the blood and their respective role in post-ischaemic neovascularization are not clearly understood. Here, we hypothesized that distinct chemokine/chemokine receptor pathways, namely CCL2/CCR2, CX3CL1/CX3CR1, and CCL5/CCR5, differentially control monocyte subset systemic levels, and might thus impact post-ischaemic vessel growth.

Methods and results
In a model of murine hindlimb ischaemia, both Ly6C hi and Ly6C lo monocyte circulating levels were increased after femoral artery ligation. CCL2/CCR2 activation enhanced blood Ly6C hi and Ly6C lo monocyte counts, although the opposite effect was seen in mice with CCL2 or CCR2 deficiency. CX3CL1/CX3CR1 strongly impacted Ly6C lo monocyte levels, whereas CCL5/CCR5 had no role. Only CCL2/CCR2 signalling influenced neovascularization, which was increased in mice overexpressing CCL2, whereas it markedly decreased in CCL2−/− mice. Moreover, adoptive transfer of Ly6C hi—but not Ly6C lo—monocytes enhanced vessel growth and blood flow recovery.

Conclusion
Altogether, our data demonstrate that regulation of proangiogenic Ly6C hi monocytes systemic levels by CCL2/CCR2 controls post-ischaemic vessel growth, whereas Ly6C lo monocytes have no major role in this setting.

Keywords
Ischaemia • Angiogenesis • Arteriogenesis • Inflammation • Monocyte subset

1. Introduction
Arteriogenesis and angiogenesis are critical processes involved in the response of the organism to ischaemic injury, and contribute to tissue revascularization and organ preservation. Infiltration of inflammatory cells in hypoxic areas is a hallmark of tissue ischaemia, and the respective role of distinct leucocyte subsets in post-ischaemic neovascularization—CD4+ and CD8+ T cells,23 NK cells,9 regulatory T cells,3 mast cells—has been unravelled. Monocytes have also been shown to promote arteriogenesis and angiogenesis by releasing angiogenic growth factors, cytokines, and metalloproteinases.7 Mouse monocytes comprise at least two phenotypically distinct subsets: Ly6C hi 7/4+CCR2−CX3CR1lo and Ly6C lo 7/4+CCR2+CX3CR1 hi monocytes. The human counterparts are CD14+CD16− and CD14+CD16+ monocytes, respectively.9 ‘Inflammatory’ Ly6C hi monocytes rapidly enter sites of inflammation, whereas ‘Resident’ Ly6C lo monocytes enter lymphoid and non-lymphoid organs under homeostatic conditions,10 and patrol across the vascular endothelium in a CX3CR1-dependent manner.51 The specific role of each mono-}

---

1 These authors contributed equally to this work.
2* Corresponding author: Paris Cardiovascular Research Center, INSERM U970, Hôpital Européen Georges Pompidou, Université Paris Descartes, 56 rue Leblanc, 75015 Paris, France. Tel: +33 153988060; fax: +33 153987951; Email: jean-sebastien.silvestre@inserm.fr

Published on behalf of the European Society of Cardiology. All rights reserved. © The Author 2010. For permissions please email: journals.permissions@oxfordjournals.org.
growth factor (VEGF) in the ischaemic myocardium. Accumulation of CX3CR1-expressing cells also exacerbates retinal neovascularization in a murine model of age-related macular degeneration, presumably through VEGF expression. Additionally, a population of Tie2-expressing monocytes, belonging to the Ly6C\textsuperscript{lo} subset, has been shown to promote tumour angiogenesis. In contrast, Capoccia et al.\textsuperscript{15} demonstrated that adoptive transfer of bone marrow (BM)-derived Ly6C\textsuperscript{hi} monocytes in mice with unilateral limb ischaemia improved blood flow recovery, whereas adoptive transfer of Ly6C\textsuperscript{lo} monocytes did not. Thus, specific involvement of each mono
cyte subset in post-ischaemic neovascularization requires further investigation.

Monocyte recruitment to ischaemic areas is thought to occur mainly via chemokine/chemokine receptor signalling. In ischaemic hindlimb models deficiency in CCL2 or its receptor, CCR2, reduces post-ischaemic inflammation and vessel growth.\textsuperscript{16,17} Of interest, lack of CCR2 specifically abrogates Ly6C\textsuperscript{hi} monocyte infiltration in the ischaemic myocardium.\textsuperscript{12,18} However, other chemoattractant pathways might be involved: CX3CL1/CX3CR1 and CCL5/CCR5 promote Ly6C\textsuperscript{hi} and Ly6C\textsuperscript{lo} monocyte infiltration into atherosclerotic plaques, respectively,\textsuperscript{19} and CX3CR1 governs Ly6C\textsuperscript{lo} monocyte infiltration into the ischaemic myocardium.\textsuperscript{12}

In addition to the well-established role of CCL2/CCR2 signalling in monocytes recruitment to inflamed tissues, recent reports also highlighted its involvement in monocytes mobilization from the BM.\textsuperscript{20–22} Interestingly, in two models of bacterial infection, CCR2 was involved in monocytes mobilization from the BM, but not in their recruitment towards inflamed tissues.\textsuperscript{22,23} Moreover, CX3CR1 and CCR5 have been shown to control blood monocyto
tosis in high fat diet fed hyperlipidemic mice.\textsuperscript{20} As post-ischaemic neo
vascularization is highly dependent on monocytes levels in the bloodstream,\textsuperscript{24} one can speculate that CCL2/CCR2, CX3CL1/ CX3CR1, and CCL5/CCR5 might mainly control the circulating number of monocyte subset.

Here, we studied the role of CCL2/CCR2, CX3CL1/CX3CR1, and CCL5/CCR5 in the regulation of Ly6C\textsuperscript{lo} and Ly6C\textsuperscript{hi} monocytes sys
temic levels and their subsequent roles in post-ischaemic neovascularization. We demonstrate that CCL2/CCR2 controls circulating levels of both monocyte subset, whereas CX3CL1/CX3CR1 preferentially impacts Ly6C\textsuperscript{lo} monocytes. We also show that CCL2/CCR2 signalling is required for adequate neovascularization, and that adoptive transfer of Ly6C\textsuperscript{lo}—but not Ly6C\textsuperscript{hi}—monocytes promotes functional blood flow recovery in ischaemic hindlimbs.

## 2. Methods

### 2.1 Animals

Ten-week-old males C57BL/6 (Charles River), CCL2\textsuperscript{−/−} (from Barent Rollins, Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, USA), CCR2\textsuperscript{−/−}, CX3CR1\textsuperscript{−/−}, CR5\textsuperscript{−/−} mice and mice expressing GFP under the CX3CR1 promoter (CX3CR1\textsuperscript{IgfIV}) (from Jackson Laboratories) were used.

The investigation conformed with 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).

All the experiments were performed in accordance with the European Community guidelines for the care and use of laboratory animals (No. 07430).

### 2.2 Hindlimb ischaemia model and plasmid electrotransfer

Mice underwent right femoral artery ligation under isoflurane anaesthesia. Right femoral artery was permanently ligated. Six hours after femoral artery ligation, 50 μg of plasmids encoding an irrelevant Ig, CCL2, CX3CL1, or CCL5 were injected into both tibial anterior and gastrocne 미us muscles of the anesthetized mouse. Then, transcutaneous electric pulses (8 square-wave electric pulses of 200 V/cm, 20 ms each, at 2 Hz) were delivered by a PS-15 electropulsator (Jouan) with two stainless steel plate electrodes placed 4.2–5.3 mm apart, at each side of the leg. The left leg was not ligated or electrotransferred and was used as an internal control.

### 2.3 Analysis of neovascularization

#### 2.3.1 Microangiography

Mice were anesthetized (pentobarbital), and longitudinal laparotomy was performed to introduce a polyethylene catheter into the abdominal aorta and inject contrast medium (barium sulfate, 1 g/mL). Angiography of hind
lims was then performed, and images (2 per animal) were acquired with the use of a high-definition digital X-ray transducer. Images were assembled to obtain a complete view of the hindlimbs. The number of pixels occupied by vessels was measured in the quantification area with the use of Primedangio software (Trophy System, Paris, France). Area of quantification was limited by placement of the ligature on the femoral artery, the knee, the edge of the femur, and the external limit of the leg. The results were then expressed as a ratio of ischaemic to non-ischaemic leg.

#### 2.3.2 Capillary density analysis

Sections (7 μm) of gastrocnemius muscles were stained using FITC-conjugated Bandeiraea simplicifolia Isolecton B4. Capillaries were counted in five randomly chosen fields of a definite area with the use of Image J (NIH) software. Analyses were performed in a blinded manner by two independent investigators. The capillary density was determined in both ischaemic and non-ischaemic legs. Results were expressed as ischaemic to non-ischaemic ratio.

#### 2.3.3 Laser Doppler perfusion imaging

Briefly, excess hairs were removed by depilatory cream from the limb and mice were placed on a heating plate at 37°C to minimize temperature variation. Foot perfusion was measured using a Moor LDI. Perfusion is expressed as a ratio of ischaemic to non-ischaemic foot paw.

### 2.4 Flow cytometry analysis

Ischaemic gastrocnemius and tibialis anterior muscles were weighed, minced, and digested in 450 U/mL Collagenase I, 125 U/mL Collagenase XI, 60 U/mL DNaseI, and 60 U/mL hyaluronidase (Sigma Aldrich) for 1 h at 37°C. Cells were also isolated from femur, venous blood, and spleen. All cell suspensions were layered on Histopaque 1083 (Sigma Aldrich) for gradient density centrifugation. The mononuclear cell fraction was counted and stained using anti-mouse CD11b–PercPCy5.5, Ly6G-PE, NK1.1-PE (BD Biosciences), 7/4-FITC or 7/4-APC (Serotec), or the corresponding isotypes, and analysed on a LSRII Flow Cytometer (Becton Dickinson) with the FacsDiva software (Becton Dickinson). Cells being CD11b\textsuperscript{lo}Ly6G\textsuperscript{−}NK1.1\textsuperscript{−} were considered to be monocytes, and subsets discrimination was made upon 7/4 expression. 7/4 expression has been shown to be equivalent to Ly6C expression on monocyte subset.\textsuperscript{20}

### 2.5 RNA extraction and quantitative reverse transcription polymerase chain reaction

Total RNA from tibialis anterior muscle was extracted with Trizol reagent according to the manufacturer’s instructions (Invitrogen, Paris, France). RNA was extracted from sorted monocytes with RNeasy microkits.
(Qiagen) according to the manufacturer’s instructions. cDNA synthesis was performed with QuantiTect Reverse Transcription Kit (Qiagen). Polymerase chain reaction was performed on an ABI Prizm 7700 with the use of Power SYBR Green PCR Master Mix (Applied Biosystems). Mouse GAPDH was used to normalize sample amplification. The following oligonucleotides (Applied Biosystems, Courtaboeuf, France) served as primers: GAPDH forward: 5′-CGTCCCGTAGACAAAATGGTGA-3′, reverse: 5′-GCGGTGAGTGAGTCTACTGGAACA-3′; CCL2 forward: 5′-CTCCACTACCT GCTGCT A-3′, reverse: 5′-TTACGGTG TCAACTCAGATTCAAA-3′; CCL5 forward: 5′-GCTGCCCTCACCAT CATCCTCACT 3′, reverse: 5′-GSCACACACTTTGCGGTTCTTC-3′; CX3CL1 forward: F5′-GTGGCTTTGCTCATCCGCTATCAG-3′, reverse: 5′-CACATTGTCCACCCGCTCTCA-3′; MMP9 Forward: 5′-CGGAAGGCTCTGCTGTTCA-3′, Reverse: 5′-GGAAACTCACACGCTTTCCTTCA-3′.

**2.6 Chemokine plasma levels**

Plasma CCL2, CX3CL1, and CCL5 were measured using Quantikine Elisa Kits (R&D Systems) according to the manufacturer’s instructions.

**2.7 Monocyte sorting and adoptive transfer experiments**

For RT–PCR experiments, 7/4hi and 7/4lo monocytes were sorted from digested ischaemic muscles of blood and 3 days after femoral artery ligation, CX3CR1+ mice. For adoptive transfer experiments, spleens

---

**Figure 1** (A) Representative fluorescence activated cell sorting analysis of monocyte. Analysis of circulating leucocytes is shown. Left: representative gating of CD11b^hi^Ly6G^−^NK1.1^−^ monocytic cells. Right: histograms showing 7/4 expression on gated cells at D0 and D3 after ischaemia. Monocyte counts in the (B) BM and (C) spleen at D0, D1, D2, and D3 after ischaemia. Counts of 7/4hi (full line) and 7/4lo (dotted line) monocytes in the (D) blood, and (E) ischaemic muscles at D0, D1, D2, D3, D7, and D14 after ischaemia. *P < 0.05; ***P < 0.001 vs. D0; n = 4–10/time point.
from 8 weeks old C57BL/6 mice were mechanically dissociated on 40 μm cell strainer. Ly6G<sup>−</sup>CD11b<sup>+</sup> cells were magnetically selected with Ly6G and CD11b microbeads (Miltenyi), according to the manufacturers' instructions, and stained with CD11b-PerCPCy5.5, Ly6G-PE, NK1.1-PE, and 7/4-FITC. CD11b<sup>+</sup>Ly6G<sup>−</sup>NK1.1<sup>−</sup>7/4<sup>hi</sup> and CD11b<sup>+</sup>Ly6G<sup>−</sup>NK1.1<sup>−</sup>7/4<sup>lo</sup> were then selected using a FACS Aria (BD). Purity for both subset was >99%. Before injection, cells were counted by trypan blue exclusion. 10<sup>5</sup> 7/4<sup>hi</sup> monocytes or 10<sup>5</sup> 7/4<sup>lo</sup> monocytes were then intravenously injected to C57BL/6 recipients, 6 h after femoral artery ligation.

2.8 Statistical analysis

Results were expressed as mean ± SEM. Kruskal–Wallis analysis of variance was used to compare each parameter. Post hoc Mann–Whitney U test with Bonferroni correction were then performed to identify which group differences account for the significant overall Kruskal–Wallis. A value of \( P < 0.05 \) was considered significant.

3. Results

3.1 Ischaemia induces monocyte mobilization from the BM to the bloodstream and recruitment to ischaemic muscles

CD11b<sup>+</sup>Ly6G<sup>−</sup>NK1.1<sup>−</sup> cells were considered to be monocytes (Figure 1A), and 7/4 expression levels were used to discriminate between monocytes subsets (Figure 1A).

In the BM, monocyte count decreased between D0 and D1, and returned to basal levels thereafter (Figure 1B). In a model of myocardial infarction, monocytes have been shown to be mobilized from the spleen.\(^{18}\) However, we did not observe any decrease in spleen monocyte content in our experimental conditions. In contrast, spleen 7/4<sup>hi</sup> and 7/4<sup>lo</sup> monocyte numbers remained stable between D0 and D1, and increased thereafter (Figure 1C).

Figure 2 Left: mRNA levels of (A) CCL2, (B) CX3CL1, and (C) CCL5 in the ischaemic tibialis anterior muscle at 0, 1, 3, 7, and 14 days after induction of limb ischaemia, expressed as percentage of mRNA levels at D0. ***\(< P < 0.001 \) vs. D0. Right: Plasma levels of (D) CCL2, (E) CX3CL1, and (F) CCL5 measured at 0, 1, 3, 7, and 14 days after ischaemia. *\(< P < 0.05 \) **\(< P < 0.01 \) vs. D0. n = 4–10/time point.
In the blood, 7/4<sup>hi</sup> monocyte count raised between D0 and D1, peaked at D3 and returned to basal levels at D14. 7/4<sup>lo</sup> monocyte levels were increased at D2 and D7 after the onset of ischaemia (Figure 1D).

In the ischaemic muscle, 7/4<sup>hi</sup> monocytes were the main subset of infiltrating monocytes, and peaked at day 3 after ischaemia. 7/4<sup>lo</sup> monocytes levels were upregulated at D3, and remained stable from D3 to D14 (Figure 1E).

### 3.2 Ischaemia upregulates CCL2, CX3CL1, and CCL5 mRNA and protein levels

CCL2 mRNA was upregulated as early as D1 after ischaemia and peaked at D3 (Figure 2A, P < 0.001). CX3CL1 and CCL5 mRNAs were raised at D3 (Figure 2B and C, P < 0.001). CCL2, CX3CL1, and CCL5 mRNAs contents returned to basal levels at D7 (Figure 2A, B, and C).

Plasma CCL2 was strongly increased by 74 and 92% at D1 and D3, respectively (P < 0.05 vs. D0), and returned to basal levels thereafter (Figure 2D). A significant (P < 0.05) increase in plasma CX3CL1 levels was observed at D7 after ischaemia, only (Figure 2E). Plasma CCL5 was slightly increased at D1 after ischaemia, but this did not reach statistical significance (P = 0.067). However, it significantly decreased at D7 and D14 (Figure 2F). Those results show that ischaemia markedly upregulates inflammatory chemokines expression and secretion.

### 3.3 Differential role of chemokine/chemokine receptor pathways in the control of circulating monocyte levels after ischaemia

In CCR2<sup>−/−</sup> mice, circulating 7/4<sup>hi</sup> and 7/4<sup>lo</sup> monocyte levels were reduced when compared with wild-type mice (Figure 3A and B, P < 0.05 to P < 0.001), and were not upregulated after femoral artery ligation. Similar results were observed in CCL2<sup>−/−</sup> mice.
Figure 4 Infiltration of (A) 7/4hi and (B) 7/4lo monocytes in the ischaemic muscles of WT, CCR2−/−, CX3CR1−/−, and CCR5−/− mice D3. *P < 0.05; **P < 0.01; ***P < 0.001. Infiltration of (C) 7/4hi and (D) 7/4lo monocyte in the ischaemic muscles of mice electrotransfered with a plasmid expressing an irrelevant IG (pIG), CCL2 (pCCL2), CX3CL1 (pCX3CL1), or CCL5 (pCCL5) at D3 after ischaemia. *P < 0.05; **P < 0.01 vs. pIG. n = 4–8 mice/group. (E) Correlation between circulating and infiltrating 7/4 hi monocyte at D3. All genotypes (WT, CCR2−/−, CCL2−/−, CX3CR1−/−, CCR5−/−) and plasmid-treated groups (pIG, pCCL2, pCX3CL1, pCCL5) are represented, R = 0.678; P < 0.0001; n = 53.
Interestingly, CX3CR1 deficiency mainly reduced 7/4lo monocytes levels and hampered the ischaemia-induced 7/4lo monocyte counts upregulation. CCR5 deficiency had no obvious effects. Those results are consistent with additional data showing that overexpression of CCL2 by plasmid electrotransfer markedly increased circulating 7/4hi (Figure 3C, \( P < 0.01 \)) and 7/4lo (Figure 3D, \( P < 0.001 \)) monocyte counts at D3. In addition, CX3CL1 overexpression raised 7/4lo monocytes, only (Figure 3D, \( P < 0.01 \)), whereas CCL5 overexpression had no effect (Figure 3C and D).

3.4 Monocyte recruitment to ischaemic tissues depends on their circulating levels

7/4hi (Figure 4A) and 7/4lo (Figure 4B) monocyte infiltration was almost abolished in the ischaemic muscles of CCR2−/− mice. 7/4lo monocyte subset infiltration was reduced by 62%, and that of 7/4hi by 35% only, in CX3CR1-deficient mice (Figure 4A and B). CCR5 deficiency did not alter monocyte numbers in ischaemic muscles (Figure 4A and B).

In line with these results, overexpression of CCL2 increased infiltration of 7/4hi (Figure 4C, \( P < 0.05 \)) and 7/4lo (Figure 4D, \( P < 0.01 \)) monocytes. CX3CL1 upregulation raised 7/4lo monocytes only (\( P < 0.05 \)). CCL5 plasmid had no significant effect. Taken together, our results showed that, CCL2/CCR2 signalling affected infiltration of both monocyte subset, whereas CX3CL1/CX3CR1 preferentially acted on the 7/4lo population. Even though adoptively transferred CCR2−/− monocytes had a reduced ability to infiltrate ischaemic tissues (data not shown), the rate of infiltrating monocytes was highly dependent on their circulating numbers (Figure 4E, \( r = 0.678; P < 0.0001 \)).

3.5 CCL2/CCR2, but not CX3CL1/CX3CR1 or CCL5/CCR5, controls post-ischaemic neovascularization

Next, we sought to evaluate the impact of the three signalling pathways on post-ischaemic vessel growth. Twenty-one days after the onset of...
ischaemia, foot perfusion ($P < 0.01$), angiographic score ($P < 0.001$), and capillary density ($P < 0.001$) were reduced in CCL2$^{-/-}$ mice. In contrast, post-ischaemic vessel growth was unchanged in CX3CR1$^{-/-}$ and CCR5$^{-/-}$ mice (Figure 5A and C). In this line, electrotransfer of CCL2 expressing plasmid increased foot perfusion ($P < 0.001$), angiographic score ($P < 0.001$), and capillary density ($P < 0.001$) when compared with control mice. Neither CX3CL1 nor CCL5 overexpression induced significant effects (Figure 5D, E, and F). Altogether, these results show that CCL2/CCL2 signalling, but not CX3CL1/CX3CR1 or CCL5/CCR5, controls post-ischaemic neovascularization.

3.6 7/4$^{hi}$ monocytes promote post-ischaemic neovascularization

Because 7/4$^{lo}$ monocytes are scarce in the BM and difficult to collect in sufficient amounts from blood, we used splenic 7/4$^{hi}$ and 7/4$^{lo}$ monocytes as surrogates for circulating monocyte subset. In addition, a recent report has shown that blood and spleen monocyte subset are almost identical in transcriptome and function. Six hours after femoral artery ligation, mice received intravenous injection of PBS, $10^5$ 7/4$^{hi}$ monocytes or $10^5$ 7/4$^{lo}$ monocytes. 7/4$^{hi}$ monocytes transfer

---

**Figure 6** Left: quantitative evaluation and right: representative photomicrographs of (A) cutaneous blood flow, (B) capillary density (scale bar = 50 micrometers), and (C) angiographic score 14 days after ischaemia in animals intravenously injected with PBS, $10^5$ 7/4$^{hi}$ monocytes or $10^5$ 7/4$^{lo}$ monocyte. **P < 0.05; ***P < 0.01; ****P < 0.001** vs. PBS injected mice. n = 4–6 mice/group. (D) Representative dot plot of gated CD11b$^+$Ly6G$^-$NK1.1$^-$ monocytes obtained from digested ischaemic muscles of CX3CR1$^{GFP^{+}}$ mice. Representative gating used for monocyte subset sorting is shown. (E) MMP-9 mRNA expression in sorted peripheral blood monocyte subset (A.U., arbitrary units; N.D., not detected). (F) MMP-9 mRNA expression in sorted infiltrating monocyte subset.
improved foot perfusion (Figure 6A, P < 0.05), capillary density (Figure 6B, P < 0.001), and angiographic score (Figure 6C, P < 0.01) compared with PBS-treated mice. In contrast, injection of 7/4 hi monocytes only enhanced angiographic score by 25% (Figure 6C, P < 0.001 vs. PBS). To start to investigate the mechanism implicated in promotion of vessel growth by the two distinct populations, we analysed MMP-9 and VEGF-A levels. At 3 days after ischaemia, monocyte subset were sorted from peripheral blood and ischaemic muscles of mice expressing GFP under the CX3CR1 promoter (CX3CR1gfp/+ mice) and RT–PCR analysis was performed. MMP-9 mRNA was detected in circulating 7/4 hiGFPlo but not in 7/4 loGFPhi monocytes. MMP-9 levels were increased by 1.5-fold in infiltrating 7/4 hiGFPlo monocytes compared with 7/4 loGFPpho monocytes, although this did not reach statistical significance (Figure 6D, P = 0.12). In this line, MMP-9 levels were reduced in the ischaemic muscles of CCR2−/− mice, at D3 (data not shown). VEGF expression did not differ between monocyte subset (data not shown).

4. Discussion

Our results emphasize the major role of CCL2/CCR2 signalling in the control of monocyte subset circulating levels and subsequently in ischaemia-induced neovascularization.

In mice with femoral artery ligation, circulating 7/4 hi monocyte levels were increased as early as day 1 after ischaemia. Similarly, blood monocytosis has been shown in patients with critical limb ischaemia.25 In mice with myocardial infarction, mobilized monocytes originated from the spleen.19 However, splenic counts of each monocyte subset did not decrease after femoral artery ligation, suggesting that monocytes are not mobilized from the spleen in this setting. In contrast, we noted a significant decrease of BM 7/4 hi monocytes at day 1 suggesting that monocytes mobilized in the bloodstream following ischaemia originate from the BM.

CCL2/CCR2 controls monocytes recruitment to ischaemic tissues.12–17 However, we demonstrate here that this signalling pathway is mainly involved in the regulation of their circulating levels following ischaemia. Blood monocytosis number is a critical determinant of post-ischaemic vessel growth.24 Our results clearly show that disruption of the CCL2/CCR2 pathway leads to a drastic reduction of monocytes mobilization to the bloodstream and hampers post-ischaemic vessel growth. Similarly, overexpression of CCL2 strongly increased both 7/4 hi and 7/4 lo monocytes circulating numbers and activated neovascularization. Altogether, our results suggest that, even though recruitment of adoptively transferred monocytes was dampened by CCR2 deficiency, infiltration of 7/4 hi monocytes into ischaemic tissues was mainly dependent on their circulating levels.

We also evidenced a role of CX3CL1/CX3CR1, but not of CCL5/CCR5, in the control of monocytes circulating numbers. Monocytes are short-lived cells, and CX3CR1 signalling has been shown to promote their survival:26 endogenous CX3CR1 signalling might be required for the survival of newly mobilized BM-originating monocytes. This is supported by our observation of a lower number of 7/4 lo monocytes in CX3CR1−/− mice. CCL5/CCR5 is involved in the regulation of monocyte circulating counts in high fat diet fed ApoE−/− mice, which represent a context of chronic inflammation. In the early phase of limb ischaemia, which might be considered as a context of acute inflammation, we were unable to evidence a clear role of CCL5/CCR5 in the regulation of circulating or infiltrating monocytes. Similarly, treatment with the CCR5 antagonist Met-Rantes only affects monocyte number in hypercholesterolemic and chronically inflammed ApoE−/− mice, but not in ApoE+/+ controls.20

Following the increase of their levels in the bloodstream, both subsets of monocytes, 7/4 hi (Ly6C+2) and 7/4 lo (Ly6C−), were recruited to ischaemic muscles. Inflammatory 7/4 hi monocytes were preponderant at early time points, whereas only 7/4 lo monocytes were present after day 7 onward, as previously reported.12 Variations in monocytes levels were associated with changes in the chemokines expression pattern: CCL2 was increased at day 1 in the ischaemic tissues and blood, whereas CX3CL1 was upregulated at day 3 and 7, only.

The proangiogenic potential of the distinct monocyte subset in the context of tissue ischaemia has been unclear so far.12,15 In this study, we highlighted a major role of 7/4 hi monocytes in post-ischaemic vessel growth. Indeed, abrogation of ischaemia-induced 7/4 hi monocyte infiltration in CCL2−/− mice fully inhibited post-ischaemic vessel growth. In contrast, a drastic reduction of infiltrating 7/4 lo monocytes number associated with CX3CR1 deficiency did not hamper arteriogenesis or angiogenesis. Similarly, intravenous administration of 7/4 hi (Ly6C+) enhanced arteriogenesis and angiogenesis, leading to functional blood flow recovery, whereas that of 7/4 lo (Ly6C−) monocytes only promoted arteriogenesis and did not affect tissue perfusion. This distinct proangiogenic potential might result from the differential expression of angiogenic factors. Sorted 7/4 hi monocytes expressed more MMP-9 than 7/4 lo monocytes, which is consistent with their high proteolytic activity observed in a model of myocardial infarction.12 The role of MMP-9 in angiogenesis has been well documented: MMP-9 has a crucial role in newly formed capillaries branching after tissue ischaemia.8 MMP-9 also triggers the angiogenic switch in tumours through release of extracellular matrix bound VEGF,27 and myeloid cells-induced tumour angiogenesis is dependent on MMP-9.28

Interestingly, activation or disruption of CCL2/CCR2 signalling impacted circulating and infiltrating levels of 7/4 hi monocytes, which barely express CCR2: 7/4 hi monocytes might trigger the secondary recruitment of 7/4 lo monocytes. 7/4 hi monocytes might also switch to a 7/4 lo phenotype, as proposed previously.29

In conclusion, we showed that ischaemia induces the mobilization of proangiogenic and proarteriogenic monocytes to the bloodstream through CCL2/CCR2, with an accessory role of CX3CL1/CX3CR1. This chemokine-dependent regulation of monocyte systemic levels dictates the amplitude of their recruitment into ischaemic tissues, where they promote revascularization and functional recovery of blood flow.

Conflict of interest: none declared.

Funding

This work was supported by grants from Inserm, ANR «Cardiovasculaire, obésité et diabète» (AO5088DS), ANR 2008 «Neurologiques et Maladies Psychiatriques», European Grant Innochem 518167 European community (ENDOSTEM No. 241440) and Assistance Public-Hôpitaux de Paris/Institut Pasteur. M.P.R. is supported by European Grant Innochem 518167 and L.P. by Assistance Public-Hôpitaux de Paris.

References

3. Stabile E, Kinnaird T, la Sala A, Hanson SK, Watkins C, Campia U et al. CD8+ T lymphocytes regulate the arteriogenic response to ischemia by infiltrating the site of


