Homeostatic and Tissue Reparation Defaults in Mice Carrying Selective Genetic Invalidation of CXCL12/Proteoglycan Interactions.

Patricia Rueda, Adèle Richart, Alice Récalde, Pamela Gasse, José Vilar, Coralie Guérin, Hugues Lortat-Jacob, Paulo Vieira, Françoise Baleux, Fabrice Chretien, et al.

To cite this version:
Patricia Rueda, Adèle Richart, Alice Récalde, Pamela Gasse, José Vilar, et al.. Homeostatic and Tissue Reparation Defaults in Mice Carrying Selective Genetic Invalidation of CXCL12/Proteoglycan Interactions.. Circulation, American Heart Association, 2012, 126 (15), pp.1882-95. 10.1161/CIRCULATIONAHA.112.113290. pasteur-00743099

HAL Id: pasteur-00743099
https://hal-pasteur.archives-ouvertes.fr/pasteur-00743099
Submitted on 15 Apr 2013

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
HOMEOSTATIC AND TISSUE REPARATION DEFAULTS IN MICE CARRYING, SELECTIVE, GENETIC INVALIDATION OF CXCL12/PROTEOGLYCAN INTERACTIONS

Rueda et al: CXCL12/proteoglycan interactions in angiogenesis

Patricia Rueda, PhD 1*, Adèle Richart MsC 2,3*, Alice Récalle PhD 2,3*, Pamela Gasse PhD 1, José Vilar PhD 2,3, Coralie Guérin MsC 2,3, Hugues Lortat-Jacob PhD 4, Paulo Vieira PhD 5, Françoise Baleux PhD 6, Fabrice Chretien MD, PhD 7, Fernando Arenzana-Seisdedos MD 1‡, Jean-Sébastien Silvestre PhD 2,3‡

1Institut Pasteur, Unité de Pathogénie Virale, Département de Virologie, INSERM U819, Paris, France
2INSERM UMRS 970, Paris Cardiovascular Research Center, Paris, France
3Université Paris Descartes, Sorbonne Paris cité, Paris, France
4CEA, CNRS, Université Joseph Fourier – Grenoble 1, Institut de Biologie Structurale Jean-Pierre Ebel, UMR 5075, 38000 Grenoble, France
5Institut Pasteur, Unité de Lymphopoièse. INSERM U688, Paris, France
6Institut Pasteur, Unité de Chimie des Biomolécules. Centre National de la Recherche Scientifique 2128, Paris, France
7Institut Pasteur, Unité Histopathologie Humaine et Modèles Animaux, Département Infection et Epidémiologie, Paris, France; Université Versailles Saint Quentin en Yvelines, Faculté de Médecine ; Assistance Publique – Hôpitaux de Paris, Hôpital Raymond Poincaré, Paris, France

*Authors have equally contributed to this work
Authors have equally contributed to this work

\[\textsuperscript{1} \text{Correspondence should be addressed to Fernando Arenzana-Seisdedos: INSERM U819; Unité de Pathogénie Virale; Département de Virologie; Institut Pasteur; 28, Rue du Dr. Roux; 75724 Paris Cedex}

Total word count: 6994

Subject code: 129
ABSTRACT

**Background.** Interaction with heparan sulfate (HS) proteoglycans is supposed to provide chemokines with the capacity to immobilize on cell surface and extracellular matrix for accomplishing both tissue homing and signalling of attracted cells. However, the consequences of the exclusive invalidation of such interaction on the roles played by endogenous chemokines in vivo, remains unascertained.

**Methods and results.** We engineered a mouse carrying a Cxcl12 gene (Cxcl12<sup>Gagtm</sup>) mutations that preclude interactions with HS structures while they do not affect CXCR4-dependent cell-signalling of CXCL12 isoforms (α,β,γ). Cxcl12<sup>Gagtm/Gagtm</sup> mice develop normally, express normal levels of total and isoform-specific Cxcl12 mRNA and show increased counting of circulating CD34+ haematopoietic precursor cells. Following induced acute ischemia, a marked impaired capacity to support revascularization was observed in Cxcl12<sup>Gagtm/Gagtm</sup> animals associated to a reduced number of infiltrating cells in the ischemic tissue despite the massive expression of CXCL12 isoforms. Importantly, exogenous administration of CXCL12γ, which binds HS with the highest affinity ever reported for a cytokine, fully restores vascular growth, while HS-binding CXCL12γ mutants failed to promote revascularization in Cxcl12<sup>Gagtm/Gagtm</sup> animals.

**Conclusions.** These findings prove the role played by HS-interactions in the functions of CXCL12 both in homeostasis and physiopathological settings and document originally the paradigm of chemokine-immobilisation *in vivo*.

**Keywords :** Angiogenesis, Ischemia, Chemokines, Proteoglycans, CXCL12
INTRODUCTION

Chemokines control the migration of a large array of cells, thus regulating function and homeostasis of a number of tissues. Glycosaminoglycans (GAG), the glycanic moiety of proteoglycans, are considered as the critical biological structure that determines the immobilisation of chemokines on the extracellular matrix and cell surfaces. Immobilisation provides chemokines with a robust anchoring against blood flow and, by restraining their diffusion, facilitates both the formation of local gradients and the synchronous coordination of motility and cell adhesion \(^1\)\(^2\)\(^3\).

Among GAG, chemokines bind preferentially to heparan sulfate (HS) through interactions either of canonical BBXB and BBBXXB (B, basic; X, any residue) or discontinuous cationic protein epitopes, with the negatively charged sulfated residues of HS \(^4\). Although chemokine binding to GAGs has been well described from a biochemical point of view, the functional aspects of these interactions remain poorly understood and \textit{in vivo} investigations to address the importance of chemokine-GAG interactions are limited. In some cases the experimental approach, like the administration of sulfated glycans competing exclusively with HS \(^5\) or the genetic interference selectively inhibiting endothelial HS biosynthesis \(^6\), have broad effects and would affect, beyond chemokines, the interaction of GAG with many other factors. In other cases, the overlapping or spatial proximity of chemokine domains involved in receptor-mediated cell signalling and GAG-attachment has obscured interpretation and significance of the results obtained by exogenous administration of GAG-binding mutant chemokines with reduced agonist capacity \(^7\).

In this regard, CXCL12 is unique among chemokines for the spatial separation between the receptor- and HS-binding sites, localized on the opposite sides of the molecule \(^8\), thus permitting the evaluation of the contribution of each domain to their biological functions.
In mice three isoforms (CXCL12α, CXCL12β and CXCL12γ), that show an exceptional degree of interspecies conservation exceeding 95% homology for their amino-acid sequences, are generated by alternative splicing. CXCL12β and CXCL12γ contain the entire amino-acid sequence (68Aa) of CXCL12α which encompasses the CXCR4-binding domain and the protein core BBXB motif (K24H25L26K27) critically required for the HS-binding. CXCL12β (72Aa) and CXCL12γ (98Aa) differs from CXCL12α by the addition of the last four and thirty Aa, respectively encoding one and four additional BBXB motifs. While CXCL12α and CXCL12β display affinities for HS or heparin in the same range of magnitude (K_D, 93 and 25nM, respectively), that of CXCL12γ is 1.5 nM and constitutes the strongest HS-affinity ever measured for a cytokine. This firm interaction with HS relies on the cooperativeness of the core and the high cationic C-ter domain of the chemokine, where the four overlapping BBXB motifs of CXCL12γ mostly accounts for the slow off-rates from immobilized HS.

We reported previously that neutralisation of the K24H25L26K27 cationic charge (K24S and K27S substitutions) is sufficient to drastically reduce binding to HS and complexing of CXCL12α either with immobilized-heparin or HS. Similarly, O’Boyle et al recently reported that combined K24S and K27S in CXCL12β, are sufficient to impede HS-binding of this isoform on the apical surface of endothelial cells and promote a dramatic blood and delayed clearance as compared to the WT counterpart. To abrogate HS-binding of CXCL12γ, the combined mutation of K24H25L26K27 and the C-ter four overlapping BBXB motifs is therefore required. Of note, while all these HS-binding CXCL12 mutants show a preserved global structure defined by Nuclear Magnetic Resonance and are full CXCR4 agonists in vitro, they have only a marginal capacity to attract leukocytes or endothelial progenitor cells in vivo and are unable to immobilise and localise properly on
GAG structures. Importantly, CXCL12 HS-binding mutants induce homologous CXCR4 desensitisation thus neutralising the chemoattractant properties of CXCL12 WT molecules. CXCL12 binds both CXCR4 and CXCR7, is the only cognate ligand of CXCR4, and plays essential and non-redundant roles in organogenesis. CXCL12 is widely expressed by mesothelial, epithelial, endothelial and osteoblasts as well as bone marrow (BM) stromal cells. It acts in post-natal life as a key chemoattractant for haematopoietic progenitor cells (HPC) and leukocytes and regulates the BM homing and retention/egress of haematopoietic cells as well as the basal trafficking and transendothelial migration of leukocytes. Aside from homeostasis, CXCL12 is involved both in tumorigenesis and cancer metastasis and is a pathogenic factor in systemic disease like rheumatoid arthritis where it shows proangiogenic and inflammatory effects. In addition, CXCL12 plays a role of paramount importance as an essential and non-redundant factor involved in tissue remodelling, in particular in vascular regeneration.

Overall, the structural characteristics and pleiotropic roles played by CXCL12 make it ideally suited for investigating how the interactions with GAG/HS modulate the biological role of a chemokine in vivo. To this aim, we engineered a transgenic mouse expressing a Cxcl12 gene where the critical sequences encoding CXCL12 domains involved in HS-binding are selectively invalidated while preserving intact the CXCR4-agonist potency and efficiency of the mutant proteins. The mutant mice show enhanced serum levels of free CXCL12 and an increased number of circulating leukocytes and CD34+ haematopoietic cells. Strikingly, and in keeping with the essential role played by CXCL12 in tissue repair, these mice display a dramatically reduced capacity to regenerate vascular growth following acute ischemia that is recovered by expression of WT CXCL12 demonstrating the importance of GAG binding for proper in vivo function. This is the first description to our knowledge of an animal model where the HS-binding capacities of an endogenous chemokine were genetically invalidated.
and the consequences of the selectively impaired function investigated both in homeostasis and physiopathological settings.
METHODS

Experiments were conducted according to the French veterinary guidelines and those formulated by the European Community for experimental animal use (L358-86/609EEC).

Chemokines: cDNA expression vectors and synthesis. Tissues were obtained by dissection of Cxcl12<sup>Gagtm/Gagtm</sup> mice or WT littermates previously sacrificed by cervical dislocation. Bone marrow was recovered from right tibiae by the flushing procedure using 5ml PBS. Total RNAs were purified using the RNAeasy Kit (Qiagen), and Cxcl12 cDNAs were synthesized using 0.5μg of the corresponding RNAs with random hexamer. The isoform-specific Cxcl12 cDNA corresponding to WT and Cxcl12<sup>Gagtm/Gagtm</sup> mice were generated as described previously 13 using the Cxcl12 primers: forward common, 5'-cacccatggaaccaaggctgtgctg-3' and the reverse, 5'-tacctttgaagctgttctcaggta-3', 5'- tcacatctggacctggttttaaage-3' and 5'-ctagttttcttttctgggcagcc-3' for Cxcl12α, Cxcl12β and Cxcl12γ. The coding CXCL12γm2 DNA was derived by mutagenesis from the WT sequence. All of them were subcloned in a CMV-promoter driven pcDNA3 expression vector (InVitrogen). HEK293 cells were transfected with CXCL12-expression vectors to generate CXCL12-containing cell supernatants. Chemically synthesized chemokines were generated and evaluated for their purity and concentration as previously described11.

Cxcl12 knock-in and Cxcl12<sup>Gagtm</sup> mouse generation. A 14.5 kb genomic DNA used to construct the targeting vector was first subcloned from a positively identified C57BL/6 BAC clone (Dr. A. Lu. iTl, Fresh Meadows, New York, USA). The AAG > TCG and AAA > TCT (aa: K > S) mutations within exon 2, as well as the stop codon TAG insertion in exon 4, that selectively prevents translation of CXCL12γ last 30Aa, were generated by 3-step PCR mutagenesis. The PCR fragments carrying mutations were then used to replace the WT
sequence using conventional subcloning methods. The long homology arm extends ~6.6 kb 5’
to the first set mutations in exon 2. The LoxP/FRT-Neo cassette was inserted ~2.2 kb
downstream of the first set mutations, and ~2.3 kb upstream of the second set mutations (stop
codon insertion in exon 4) in intron 3-4. The short homology arm extends 3.3 kb 3’ to the
second set mutations. The targeting vector (backbone, a 2.4 kb pSP72 based vector) was
validated by restriction analysis and sequencing after each modification. Ten micrograms of
the targeting vector was linearized by Cla I digestion and then transfected by electroporation
of BA1 (C57BL/6 x 129/SvEv) hybrid embryonic stem cells. After selection with G418
antibiotic, surviving clones were expanded for PCR analysis to identify recombinant ES
clones and a secondary confirmation of positive clones identified by PCR was performed by
Southern Blotting analysis. Targeted BA1 (C57BL/6 x 129/SvEv) hybrid embryonic stem
cells were microinjected into C57BL/6 blastocysts. Resulting chimeras with a high percentage
agouti coat colour were mated to wild-type C57BL/6 mice to generate F1 Cxcl12Gagtm/wt
offspring. Tail DNA was analyzed by PCR to confirm the integration of the desired
sequences. The obtained Cxcl12Gagtm/wt animals, mice were crossed with transgenic 129sv
PGK-Cre for LoxP/FRT-Neo cassette excision. Offspring that do not inherit PGK-CRE and
delete LoxP/FRT-Neo cassette were selected and the colony was amplified. Identification of
genotypes of mice encoding Cxcl12Gagtm upon excision of Neo-cassette was performed by
PCR with forward, 5’tgccagcataaaagacactcg3’ and reverse 5’cagcccttgaagtaatcactgc 3’ primers.

Characterisation of Cxcl12 mRNA and protein expression in normal and ischemic
tissues. For quantitative real time PC, cerebellum, brain, thymus, heart and skeletal muscle
tissues were obtained by dissection of Cxcl12Gagtm/Gagtm mice or WT littermates. Bone marrow
was recovered from right tibiae by the flushing procedure using 5ml PBS. Total RNAs were
purified using the Rneasy Kit (Qiagen) according to the manufacturer instructions, and
cDNAs were synthesized using 0.5µg of the corresponding RNAs with random hexamer. Quantitative real time-PCR was performed in a Mix3005Tm QPCR System with a MxPro QPCR Software 3.00 (Stratagene, La Jolla, CA) and SYBR Green detection system, using the forward common primer 5'tgccttcagattgtgcac3’ and the reverse primers 5'ccaaggtgctagccttc3’, 5'cttgagccttctgtaagct3’, or 5'gtagcttacagccagcagagcactgeg3’ for Cxcl12α, Cxcl12β or Cxcl12γ, respectively. Mouse HPRT and GAPDH genes were used as controls. Quantitative analysis was then performed using the Livak method.

For evaluating CXCL12 accumulation and localisation in muscle, frozen sections (7 µm) were incubated with CXCL12 mAb K15C (10µg/ml) antibodies, FITC-Griffonia Simplicifolia Agglutinin isolectin B4 (1:100, Sigma) and rabbit anti-mouse alpha smooth muscle actin (1:100, Abcam) at room temperature. Finally, sections were incubated with DAPI (1:10,000, Sigma). Alexa fluor 594 (Invitrogen, 1:100) and Cy5-labeled secondary antibodies (1:200, Jackson Immunoresearch) were then used to reveal CXCL12 and alpha actin-positive stainings, respectively.

Quantification of CXCL12 in mice blood serum was carried out using the DuoSet ELISA Development kit for mouse CXCL12 (R&D Systems, MN, USA). Blood was collected by cardiac puncture.

**Cell surface binding potential and chemotaxis assay.** Evaluation of synthetic CXCL12 chemokines adsorption on cell surfaces of CHO GAG-expressing cells and chemotaxis assay (human CD4+ T lymphocytes or human endothelial progenitor cells (EPC), were performed as described previously11 13. EPC were isolated from human umbilical cord blood and differentiated ex vivo, as previously described33.
Peripheral blood analysis. WT and Cxcl12\textsuperscript{Gagtm/Gagtm} mice (n=10) were killed and blood collected as described above and transferred to EDTA pre-treated tubes (Sarstedt). Then, 20\mu l of sample were collected using the disposable diluting pipette system of the BD Unopette procedure (BD, NJ, USA). Cells were stained using the Gr1-FITC, CD4-PB and CD34-PE antibodies or the matching control isotypes and analyzed in a BD FACS Canto Flow Cytometer (BD Bioscience). Eythrocytes and Thrombocytes were quantified by flow cytometry (Siemens ADVIA 120).

Experimental model of surgically-induced hindlimb ischemia.

Induction of ischemia and plasmids electrotransfert. Cxcl12\textsuperscript{Gagtm/Gagtm} mice and their wild-type C57BL/6 littermate underwent surgical ligation of the proximal part of the right femoral artery, above the origin of the circumflexa femoris lateralis. 50\mu g of expression plasmids encoding for CXCL12\textsubscript{α}, CXCL12\textsubscript{γ} or CXCL12\textsubscript{γm2} were then injected into both tibial anterior and gastrocnemius muscles of the anesthetized mouse, as previously described\textsuperscript{34}. 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) using two stainless steel plate electrodes placed 4.2 to 5.3 mm apart, at each side of the leg. The left leg was not ligated neither electrotransfered and was used as an internal control. In additional set of experiments, C57BL/6 mice with surgically-induced hindlimb ischemia and treated with expression plasmids also received intravenous injection of 1.10\textsuperscript{5} human EPC.

Analysis of neovascularization. Post-ischemic neovascularisation was evaluated by three different methods, as previously described\textsuperscript{34}.

Microangiography. Mice were anesthetized (pentobarbital) and longitudinal laparotomy was performed to introduce a polyethylene catheter into the abdominal aorta and inject contrast medium (Barium sulphate, 1 g/mL). Angiography of hindlimbs was then performed and
images (two per animals) were acquired using a high-definition digital X-ray transducer. Images were assembled to obtain a complete view of the hindlimbs.

*Capillary and arteriole density analysis.* Frozen tissue sections (7 μm) from calf muscle were incubated with rabbit polyclonal antibody directed against total fibronectin (dilution 1:50, Abcys) to identify capillaries and rabbit anti-mouse alpha smooth muscle actin (dilution 1/100, abcam) to identify arterioles. The capillary-to-myocytes ratio was determined in both ischemic and non ischemic legs.

*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. Nevertheless, to account for variables, including ambient light, temperature, and experimental procedures, perfusion was calculated in the foot and expressed as a ratio of ischemic to non ischemic legs.

**Analysis of cell infiltration in the ischemic tissue.**

To evaluate the number of infiltrating CD45.1-positive cells, $10^7$ mononuclear cells isolated from the bone marrow of CD45.1 mice were intravenously injected to Cxcl12<sup>Gagtm/Gagtm</sup> mice and their wild-type C57BL/6 littermate, 1 day after femoral artery ligation. Five days after the injection, the ischemic gastrocnemius muscles were harvested, weighed, minced and digested in 450 U/ml Collagenase I, 125 U/ml Collagenase XI, 60 U/ml DNAse I and 60 U/ml hyaluronidase (Sigma Aldrich) for 1 hour at 37°C. After Ficoll separation, infiltrating cells were stained with CD45.1-PerCPCy5.5, CD34-APC and CXCR4-PE and analyzed using a FACS Aria (BD).

To measure the number of infiltrating EPC, the ischemic gastrocnemius muscles were digested as described above. The number of cells being positive for β2-microglobuline anti-human FITC (Biolegend, 1:200) and DAPI was then evaluated on a LSRII Flow Cytometer.
(Becton Dickinson). In a third set of experiment, 10-week wild-type C57BL/6 mice underwent medullar aplasia by total body irradiation (9.5 gray). Bone marrow cells were then isolated from femurs and tibias of GFP C57BL/6 mice and intravenously injected in irradiated animals. After 8 weeks, mice underwent surgical ligation of the proximal part of the right femoral artery, as described above. The gastrocnemius muscles were then harvested 2 days after ischemia. Infiltrating circulating BM-derived GFP-positive cells were detected by fluorescent microscopy and their endothelial cell phenotype revealed by costaining with rhodamine Griffonia Simplicifolia Agglutinin isolectin B4 (1:100, Sigma) and their inflammatory phenotype by co-staining with rat anti-mouse Mac-3 (BD pharmingen, 1:300) and donkey anti-rat RITC (Jackson ImmunoReaserch, 1:200).

**Statistical Analysis.** Statistical analysis were performed using StatView. Kruskall Wallis analysis of variance was used to compare each parameter. Bonferroni-corrected Mann-Whitney tests were then performed to identify which group differences account for the significant overall Kruskall Wallis. Results were expressed as mean ± SEM. A value of p<0.05 was considered significant.
RESULTS
We have engineered mice encoding a Cxcl12 gene carrying mutations (Figure 1A and S1) that drastically reduce CXCL12/HS interactions as deduced from biochemical and biophysical measurements and functional assays in vitro and in vivo. K24S and K25S point mutations were introduced in the critical HS-binding domain (encoded in the second exon) shared by the three CXCL12 isoforms (Figure S2(A)). Furthermore, a nonsense amber mutation was introduced in the fourth exon to prevent the translation of the distinctive, thirty-last Aa of the highly cationic C-ter domain of CXCL12γ (Figure S1(A)). We opted for this strategy to avoid unpredictable consequences in the expression of a heavily CXCL12γ mutated protein carrying multiple substitutions in the overlapping C-ter BBXB motifs required for abrogating HS-binding of this isoform.

The relative amounts of each mRNA isoform in Cxcl12Gagtm/Gagtm were comparable to that of WT littermates (Figure 1C and S2(C)) while serum CXCL12 levels were markedly increased in mutant animals, likely as a consequence of the mutant chemokines to immobilise on HS structures (Figure 1D). Expression and functional analysis of Cxcl12Gagtm/Gagtm cDNA isoforms confirmed their preserved CXCR4-agonist capacities (Figure S3(D)).

Cxcl12Gagtm/Gagtm mice were both viable and fertile. Extensive macroscopic and histologic analysis did not reveal any detectable anatomic abnormalities in the Cxcl12Gagtm/Gagtm mice. Micro photos corresponding to tissue preparations of WT and Cxcl12Gagtm/Gagtm BM and cerebellum, which development is affected in Cxcl12 and Cxcr4 knock-out animals, as well as skeletal muscle, are shown in Figure 1B.

No significant differences in the composition of blood, BM and CD34+ HPC were observed between WT and mutant animals (data not shown). Interestingly, whereas the number of BM haematopoietic cell subpopulations was similar in WT and Cxcl12Gagtm/Gagtm mice, mutant animals displayed higher numbers of circulating total leukocytes, granulocytes and CD34+
cells (Figure 1E) and no significant differences for both T and B lymphocytes. The number of erythrocytes (10.72±0.33/mm\(^3\) versus 10.32±0.31/mm\(^3\), n=6) and that of platelets (944±132/mm\(^3\) versus 1029±197/mm\(^3\), n=6) in WT and Cxcl12\(^{Gagt/m\text{m}/Gagt/m}\) mice, respectively, were similar.

CXCL12 regulates both the tissue homing and survival of circulating tissue-specific progenitors, HPC and BM-stromal stem cells\(^{32\text{ 35}}\), triggers inflammatory cells infiltration and is required for the peri-endothelial retention of circulating, BM-derived myeloid cells\(^{36\text{ 32\text{ 35}}}\).

These findings prompted us to assess the role of CXCL12/GAG interactions in the regenerative process following acute regional ischemia.

We observed that in both WT and Cxcl12\(^{Gagt/m\text{m}/Gagt/m}\) mice, hindlimb-ischemia induced massive transcription and expression of Cxcl12 isoforms (Figure 2A). Kinetic analysis of mRNA levels showed that Cxcl12 isoforms expression enhanced by day 4 post-ischemia and returned to basal levels by day 21 (Figure 2A). The expression ratio between the Cxcl12 isoforms varies between different tissues (Figure S2(D)). Of interest, CXCL12\(^{\gamma}\) was the most abundant isoform in both the normal and ischemic muscle (Figure 2B). We also determined that CXCL12 was expressed in ischemic capillary structure and, in both WT and Cxcl12\(^{Gagt/m\text{m}/Gagt/m}\) mice, is co-localized with isolectin B4 staining, a specific marker of endothelial cells (Figure 2C). In addition, CXCL12 expression was also detected in number of arterioles, as revealed by the co-staining between CXCL12 and alpha-smooth muscle actin (Figure 2D).

It is of note that Cxcl12\(^{Gagt/m\text{m}/Gagt/m}\) mice displayed a markedly impaired capacity to support efficient post-ischemic revascularization in a model of hindlimb ischemia. Indeed, laser Doppler imaging showed a reduced paw perfusion in ischemic hind limb of Cxcl12\(^{Gagt/m\text{m}/Gagt/m}\) as compared to WT animals, as early as day 14 after the onset of ischemia (Figure 3A). Angiographic scores obtained by microangiographic analysis revealed that vessel density was hampered by 30% in ischemic muscle of Cxcl12\(^{Gagt/m\text{m}/Gagt/m}\) mice in reference to that of WT
animals (Figure 3B). Furthermore, analysis of post-ischemic frozen calf muscle samples showed that regeneration of both capillary and arteriole vessels were reduced in Cxcl12\textsuperscript{Gagtm/Gagtm} animals by up to 44\% and 55\%, respectively as compared to WT animals (Figure 3C and 3D).

As previously mentioned, CXCL12/CXCR4 interactions are involved in the recruitment and/or the retention of circulating cells in the ischemic tissue. To gain further insights into the cellular and molecular mechanisms associated with the reduction of post-ischemic vessel growth in Cxcl12\textsuperscript{Gagtm/Gagtm} mice, we analyzed the ability of circulating cells to home to ischemic tissues in Cxcl12\textsuperscript{Gagtm/Gagtm} animals. CD45.1-positive cells were intravenously injected one day after the onset of ischemia, and their number and fate were analysed in the blood and in the ischemic tissue, 6 days after ischemia, a time point associated with a marked upregulation of transcription and expression of Cxcl12 isoforms. The number of CD45.1+, CD45.1+/CD34+ and CD45.1+/CXCR4+ was higher in the blood of Cxcl12\textsuperscript{Gagtm/Gagtm} animals compared to that of WT mice. In contrast, the percentage of CD45.1+/CD34+ and CD45.1+/CXCR4+ was decreased in the ischemic muscle of Cxcl12\textsuperscript{Gagtm/Gagtm} animals compared to that of WT mice (Figure 4A, B). As a consequence, the post-ischemic inflammatory response, a key component of ischemic tissue remodelling is affected in our experimental conditions and we showed that the number of Mac3-positive cells was reduced in ischemic leg of Cxcl12\textsuperscript{Gagtm/Gagtm} mice compared to WT littermates (Figure 4C).

The predominant expression of CXCL12\gamma in muscle and in particular its massive accumulation in ischemic tissues, suggests that this isoform might play a critical role in neovascularisation through an HS-binding dependent mechanism. Thus, if the angiogenic default observed in Cxcl12\textsuperscript{Gagtm/Gagtm} was due to the selective invalidation of HS-binding capacity of Cxcl12 products, exogenous administration of wild type CXCL12 proteins with
full HS-binding activity, should restore vascular regeneration in ischemic tissues with the highest efficiency as compared.

To assess this hypothesis we first compared the capacity of both WT CXCL12α and CXCL12γ, which differ notably for their affinity capacity to bind cell surface in a GAG-dependent manner, to induce post-ischemic vascular regeneration in control animals. Electrotransfered expression of DNA plasmids encoding for CXCL12α or CXCL12γ (Figure 5A), revealed a dramatically increased potency of CXCL12γ to promote neovascularisation as evaluated by tissue perfusion, angiographic score, capillary density and attraction of inflammatory macrophages in the ischemic tissue (Figure 5B, 5C, 5D and 5E). To bring direct and formal evidence that the superior efficiency of CXCL12γ was actually related to the interaction of the chemokine with HS, we evaluated the pro-angiogenic capacities of CXCL12γ and mutant CXCL12γm2 which carries the K24S and K27S substitutions combined with the neutralisation of nine of the seventeen positively charged residues encompassed in the C-ter domain of this isoform (Figure S3(A)). CXCL12γm2 displays no detectable HS-binding to cell surface HS whereas it activates CXCR4 with preserved efficiency and even with increased potency as compared to the WT control (Figure S3 (B,C)) and as we reported previously. Our findings unambiguously proved that while CXCL12α and CXCL12γ showed pro-angiogenic efficiency correlating with their affinities for HS, CXCL12γm2 failed to induce post-ischemic vessel growth beyond baseline levels (Figure 5B, C and E).

To further investigate the mechanism of the increased pro-angiogenic effect of CXCL12γ, we compared the capacity of each isoform to regulate both the number of cells infiltrating the ischemic tissue and their fate. To this aim, hindlimb ischemia was induced in mice that has been lethally irradiated and reconstituted with BM-derived cells isolated from GFP+ mice. Electrotransfer of CXCL12γ plasmid enhanced by 300% and 150% the number of GFP+ cells
infiltrating the ischemic muscle compared to mice treated with empty plasmid or CXCL12α, respectively (Figure 6A). The failure of CXCL12γm2 to induce a significant effect further proved the involvement of an HS-dependent mechanism in the robust neovascularisation induced by CXCL12γ. Of note, the percentage of GFP+/BS1-lectin+ cells of the endothelial lineage, and that of GFP+/Mac3+ revealing the macrophage phenotype, were similar in mice treated with CXCL12γ or CXCL12α. Further experiments using human endothelial progenitor cells (EPC) labelled with CFSE revealed that CXCL12γ, but not CXCL12γm2, increased the number of EPC infiltrating ischemic hindlimb muscle (day 4 post-ischemia) by 150% and subsequently vessel growth by roughly 50% (day 14 post-ischemia) when compared to mice treated with CXCL12α (Figure S4).

Collectively, these results suggest that CXCL12γ regulates the accumulation of BM-derived cells in ischemic tissues with the highest efficiency, in comparison to CXCL12α, and in a strictly HS-dependent manner, while it does not differ from CXCL12α regarding induction of cell differentiation. The increased number of EPC homing to the injured muscle early after ischemia permits to ascribe its prominent pro-angiogenic effect to a primary attraction of endothelial cell precursors.

Finally, to demonstrate that the deficient neoangiogenesis observed in Cxcl12Gagtm/Gagtm animals was related to the expression of CXCL12 HS-binding mutants and their inability to promote efficient vascular reparation, we electrotransfered a plasmid encoding WT CXCL12γ plasmid in the ischemic hindlimb. Expression of this isoform virtually normalized vascular regeneration in ischemic tissues as proved by the significant increase in both vessel density and infiltration of inflammatory macrophages (Figure 7). This finding conclusively links the default of tissue reparation observed in these animals to the reduced capacity of mutant CXCL12 proteins to form complexes with HS proteoglycans.
DISCUSSION

The animal model we have engineered allows the selective characterization of proteoglycan-chemokine interactions in vivo, separately from interactions of a defined chemokine to its specific receptor. Cxcl12\textsuperscript{Gagtm/Gagtm} mice exhibit normal expression of isoform-specific Cxcl12 mRNAs encoding for CXCR4-agonist chemokines. No detectable developmental anomalies were observed in Cxcl12\textsuperscript{Gagtm/Gagtm} animals. This is in sharp contrast to Cxcl12- and Cxcr4-deficient mice, which show in both cases abnormal haematopoiesis and multiple organogenesis default associated to perinatal mortality\textsuperscript{20} 21 37, and to Cxcr7- knock-out mice, which show disrupted cardiac development\textsuperscript{38}. Manifold reasons could explain the lack of detectable anatomic anomalies in the mutant animals. First, the preserved expression levels of total and isoform specific Cxcl12 mRNA in Cxcl12\textsuperscript{Gagtm/Gagtm} mice tissues. Second, the intact cell signaling capacities of mutant chemokines leading to orientated cell migration, as previously shown for mutated CCL19 and CCL21 \textsuperscript{1}. Third, the oligosaccharidic HS structure recognized by CXCL12, which remains unknown, could not feature during embryogenesis the high affinity CXCL12 binding site, thus explaining the lack of observed effects. In this line, it has been proved a binding specificity switch of brain HS proteoglycans from FGF-2 to FGF-1 \textsuperscript{39}, demonstrating that indeed the binding capacity of embryonic HS for a given protein is not necessarily present, and is acquired during development.

The Cxcl12\textsuperscript{Gagtm/Gagtm} mice nevertheless display a subtle anomaly revealing an increased number of circulating white blood cells, including CD34+ HPC. This phenomenon could be explained by a reduced attraction/retention in lymphoid tissues and is probably related to impaired HS-dependent tissue adhesiveness of the CXCL12 mutant proteins \textsuperscript{15} 5. Given the crucial role played by CXCL12 and CXCR4 in both the homing of haematopoietic cells to the BM\textsuperscript{25} 26, the inability of the HS-binding CXCL12 mutants to adhere to the cell surface and the surrounding extracellular matrix of CXCL12-producing stromal cells, forming CXCL12-BM
niches could facilitate their egress from the BM. In this regard, the accumulation of circulating CD34+ cells, which normally reside in BM, strongly argues in favour of reduced retention of these cells in BM. The BM egress of the mature leukocytes could also be due to the same mechanism. Alternatively, the increased number of these cells in the periphery could be ascribed to the gradient generated by the blood accumulation of free, HS-binding disabled CXCL12 proteins and/or their failure to firmly immobilize on the apical surface of sinus and post-capillary endothelial cells that would inhibit the return of leukocytes to the BM.

Whereas basal coronary flow were impaired in Cxcr4(+/−) mice, paralleled by reduced angiogenesis and myocardial vessel density, we did not detect changes in the basal number of capillary and arterioles in skeletal muscle of Cxcl12Gagtm/Gagtm animals. In contrast, Cxcl12Gagtm/Gagtm animals show a marked default in angiogenesis and neovascularisation of ischemic tissues, revealing a biological role for CXCL12/GAG interactions in tissue repair after ischemia. It has been previously reported that recruitment of CXCR4-positive progenitor cells to regenerating tissues is mediated by hypoxic gradients via HIF-1-induced expression of CXCL12. Furthermore, CXCL12 attracts EPC, induces angiogenesis and increases the number of newly formed vessels and blood flow in models of hindlimb ischemia when overexpressed, either alone or combined with cell therapy. Recruitment and retention of BM-derived circulating inflammatory cells mediated by CXCL12 participates to vascular remodelling and arteriole growth. In addition, CXCL12 seems to have a direct effect on migration of smooth muscle cells and smooth muscle progenitor cells.

However, all previous studies focussed on deciphering the capacity of CXCL12α to promote angiogenesis, without dissecting the respective contribution of receptor activation and GAG-binding. Furthermore, only the therapeutic capacity of exogenous WT chemokines was investigated, while the role played by endogenous CXCL12/GAG interactions in the spontaneous post-ischemic recovery remained unknown.
The defective post-ischemic neovascularisation observed in $\text{Cxx}12^{\text{Gagtm/Gagtm}}$ animals thus brings a new light to the original evidence that, beyond $\text{CXCL12}$-induced receptor cell signalling, the endogenous $\text{CXCL12}$/$\text{GAG}$ interactions play a critical role in tissue regeneration and in particular in vascular growth. Indeed, the efficiency of $\text{CXCL12}^\gamma$ to rescue the default in $\text{Cxx}12^{\text{Gagtm/Gagtm}}$ animals contrasts with the lack of effect of $\text{CXCL12}^\gamma\text{m}2$ and reveals the key role of this mechanism in the reparative process. This finding is in keeping with the efficiency of $\text{CXCL12}^\gamma$ and the inability of $\text{CXCL12}^\gamma\text{m}2$ to promote homing of BM-derived circulating inflammatory cells and of circulating EPC into ischemic tissues, and modulate vessel growth in this setting.

Bound to HS-structures on the apical surface of endothelial cells $^{47}$, $\text{CXCL12}$ expressed by autocrine or juxtacrine mechanisms could be determinant for vascular targeting of circulating progenitor and inflammatory cells and induction, in an integrin- and CXCR4-dependent manner, of both firm adhesion and transendothelial migration of infiltrating BM-derived cells $^{48}$. Among $\text{CXCL12}$ isoforms, $\text{CXCL12}^\gamma$ binds to microvascular endothelial cell surface with the highest efficiency $^{13,47}$ and is ideally suited to accomplish this role. Abundantly expressed and induced in injured tissues by hypoxia, $\text{CXCL12}^\gamma$ could delimit a static field of chemokine keeping cells motile (haptokinesis) in a restrained tissue compartment. This may contribute to the reparative effect of EPC by enhancing cell-adhesiveness and survival $^{49}$. It could be hypothesized that, released progressively upon cleavage of its C-ter domain the pool of immobilized $\text{CXCL12}^\gamma$ could provide a gradient of active, diffusible chemokines that would promote long-range chemotaxis toward the injured tissue. An analogous mechanism contributes to the regulation by CCL21 of lymphocyte T cell homing in secondary lymphoid organs $^1$.

Regarding the potential of $\text{CXCL12}$ in regenerative medicine, the continuous delivery or administration in an immobilized form of $\text{CXCL12}^\alpha$ is required to provide therapeutic effect.
in post-ischemic treatment\textsuperscript{50}. CXCL12\textgamma could overcome this limitation thus activating efficiently the pro-angiogenic mechanisms.

Collectively, our findings unravel the contribution of endogenous CXCL12/HS complexes to the biological functions of this chemokine. The prevalence of CXCL12\textgamma expression in hypoxic conditions and its superior capacity to promote neovascularisation further highlights the importance of HS-binding mechanisms in the tissue reparative activity of CXCL12.

The findings of this work pave the way for investigating the contribution of CXCL12/HS interactions to other homeostatic and physio-pathological functions played by this unique chemokine and could have broad relevance eventually for other chemokine systems.

**FUNDING SOURCES**

This work was supported by grants from Agence Nationale de la Recherche (ANR; Chemoglycan, NT05-4_41967; Chemrepair 2010-BLAN112702), INSERM, Institut Pasteur PTR-395, Mizutani Fundation (Japan) and by institutional funding from INSERM. J.S.S is a recipient of a Contrat d’Interface from Assistance Publique-Hôpitaux de Paris and supported by “Fondation pour la Recherche Médicale”. P.R and P.G are recipients of ANR fellowships. A.Récalde is a recipient of fellowships from Fondation pour la Recherche Médicale and from Région Ile de France. The Funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**DISCLOSURES**

None
REFERENCES


42. Martin C, Burdon PC, Bridger G, Gutierrez-Ramos JC, Williams TJ, Rankin SM. Chemokines acting via cxcr2 and cxcr4 control the release of neutrophils from the bone marrow and their return following senescence. *Immunity*. 2003;19:583-593


FIGURE LEGENDS

Figure 1. Characterization of \textit{Cxcl12}^{Gagtm/Gagtm} mice. (A) Schematic representation of \textit{Cxcl12} genomic locus, targeting vector and recombined \textit{Cxcl12} locus (\textit{Cxcl12}^{Gagtm}). Exons 1-4 are represented by darked boxes. Mutations incorporating residue substitutions (2\textsuperscript{nd} exon) and stop codon (4\textsuperscript{th} exon) are indicated by vertical arrows and stars. The LoxP-FRT-Neo cassette is inserted in an opposite orientation regarding to the target gene in intron 3-4. Long (LA) and short (SA) homology arm positions and length are indicated. (B) Hematoxylin and eosin staining of bone marrow (left panels), cerebellum (middle panels) and skeletal muscle (right panels) tissue sections from \textit{Cxcl12}^{Gagtm/Gagtm} (MUT) and wild-type littermate (WT) animals. Arrows show megakaryocytes; 1 display white substance; 2, granular layer; 3, molecular layer. (C) Real-time PCR analysis of \textit{Cxcl12}. Relative RNA expression levels of \textit{Cxcl12} isoforms normalized to HPRT in cerebellum, bone marrow and skeletal muscle tissues. \textit{Cxcl12}α, \textit{Cxcl12}β and \textit{Cxcl12}γ ARN levels on MUT animals are expressed relative to the WT animals level, arbitrary set to 1 (n=12 per group). (D). CXCL12 levels in blood were measured by ELISA in MUT and WT animals. (E) Cell populations in peripheral blood collected from MUT and WT animals. Results are mean±SEM. **p<0.01, ***p<0.001 versus WT.

Figure 2. Expression of CXCL12 isoforms in ischemic tissue. (A) Quantitative evaluation and relative levels of CXCL12α, CXCL12β and CXCL12γ mRNA contents 1, 4, 6 and 21 days after ischemia in ischemic and non ischemic legs of \textit{Cxcl12}^{Gagtm/Gagtm} (MUT) and wild-type littermate (WT) animals. Values are mean ± SEM. n=10/group, representative of 2 independent experiments. 28 possible comparisons for Bonferroni correction, a value of p<0.0018 was considered significant, **p<0.0003 versus N Isch at day 1. (B) mRNA levels of each \textit{Cxcl12} isoform were normalized to that of \textit{Cxcl12}α in skeletal muscle of non ischemic...
tissue. 6 possible comparisons for Bonferroni correction, a value of p<0.008 was considered significant, n=10 per group. (C-D) Histological sections from ischemic skeletal muscles of WT and MUT animals showing CXCL12 expression in capillary (c) and arteriolar (d) structures. Red staining, alpha smooth muscle actin (alpha smooth muscle cells); green staining, Griffonia Simplicifolia Agglutinin isolectin B4 (endothelial cells); purple staining, CXCL12. Nuclei were stained with DAPI (blue staining). Bars: 10µm. One representative experiment out of six is shown.

**Figure 3. Postischemic revascularization in Cxcl12Gagtm/Gagtm animals.** Quantitative evaluation and representative photomicrographs of foot perfusion at different time points (A), and at day 21, for angiographic score (B, vessels in white), capillary density (C, capillaries in green, arrows show fibronectin–labelled capillaries), arteriole density (D, arterioles in green, arrows show α-actin–labelled arterioles) in Cxcl12Gagtm/Gagtm (MUT) and wild-type littermate (WT) animals. Results are shown in ischemic (Isc) and non-ischemic (N.Isc) legs. D indicates day. Values are mean ± SEM. n = 15 per group, representative of 3 independent experiments. 

A) 15 possible comparisons for Bonferroni correction, a value of p<0.003 was considered significant, *p<0.03 and **p<0.0006 versus WT. B) 1 possible comparisons for Bonferroni correction, a value of p<0.05 was considered significant, ***p<0.001 versus WT. n = 15 per group. C-D) 6 possible comparisons for Bonferroni correction, a value of p<0.008 was considered significant, **p<0.0016 versus Isc WT. n = 15 per group.

**Figure 4. Quantification of infiltrating circulating cells in Cxcl12Gagtm/Gagtm animals.** Percentage of CD45.1+, CD45.1+/CD34+ and CD45.1+/CXCR4+ in blood (A) and ischemic muscle (B), 6 days after the onset of ischemia. Representative FACS dot plots obtained from WT and MUT digested muscle at 6 days after ischemia. Cells were gated on CXCR4
expressing CD45.1-positive cells. n = 7 per group. A-B) 1 possible comparisons for Bonferroni correction, a value of p<0.05 was considered significant. *p<0.05 versus WT. (C) Quantitative evaluation and representative photomicrographs of Mac3-positive cells (macrophages in brown, arrows show Mac-3 labelled macrophages) in Cxcl12<sup>Gagtm/Gagtm</sup> (MUT) and wild-type littermate (WT) animals. Results are shown in ischemic (Isc) and non-ischemic (N.Isc) legs. Values are mean ± SEM. n = 15 per group, representative of 3 independent experiments. 6 possible comparisons for Bonferroni correction, a value of p<0.008 was considered significant, **p<0.0016 versus Isc WT.

Figure 5. Effect of CXCL12 isoforms on post-ischemic revascularization. Quantitative evaluation of foot perfusion (A), microangiography (B), capillary density (C), and arteriolar density (D) and macrophages infiltration (E), 21 days after ischemia in C57BL/6 mice transfected with empty DNA plasmid (CONT) or DNA expression vectors encoding CXCL12α, CXCL12γ or CXCL12γm2. Values are mean ± SEM. n = 15 per group, representative of 3 independent experiments. A-B) 6 possible comparisons for Bonferroni correction, a value of p<0.008 was considered significant, *p<0.008, **p<0.0016 versus PBS, † p<0.008 versus CXCL12α, #p<0.008, ##p<0.0016 versus CXCL12γ. C-E) 28 possible comparisons for Bonferroni correction, a value of p<0.0017 was considered significant, *p<0.0017 versus Isc CONT, †p<0.0017 versus Isc CXCL12α, #p<0.0017 versus Isc CXCL12γ. Isc indicates ischemic leg and N.Isc, non ischemic leg.

Figure 6. CXCL12γ controls BM-derived cells infiltration in ischemic tissues. Quantitative evaluation and representative photomicrographs of the percentage of GFP-expressing cells (A), GFP-expressing and BS1-labelled cells (B) and GFP-expressing and Mac3-labelled cells (C) in the ischemic leg of irradiated wild-type mice reconstituted with
bone marrow of WT GFP+ animals and treated with empty DNA plasmid (CONT), or DNA expression vectors encoding for CXCL12α, CXCL12γ or HS-binding mutant CXCL12γm2. Value quantification are represented in histograms. Values are mean ± SEM. n = 10 per group, representative of 2 independent experiments. A-C) 28 possible comparisons for Bonferroni correction, a value of p<0.0017 was considered significant, *p<0.0017 versus Isc CONT, †p<0.0017 versus Isc CXCL12α, #p<0.0017 versus Isc CXCL12γ. Isc indicates ischemic leg and N.Isc, non ischemic leg.

Figure 7. CXCL12γ restores the defective angiogenic phenotype in Cxcl12Gagtm/Gagtm animals. Quantitative evaluation of foot perfusion (A), capillary density (B), arteriole density (C) and Mac3-positive cells (D) in Cxcl12Gagtm/Gagtm (MUT) and wild-type littermate (WT) animals treated with or without DNA expression vector encoding CXCL12γ. Results are shown in ischemic (Isc) and non-ischemic (N.Isc) legs. Values are mean ± SEM. n = 10 per group. A) 3 possible comparisons for Bonferroni correction, a value of p<0.016 was considered significant *p<0.016 versus WT. B-D) 15 possible comparisons for Bonferroni correction, a value of p<0.003 was considered significant. **p<0.0006 versus Isc WT, #p<0.003, ##p<0.0006 versus Isc MUT.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Supplemental Material
Supplementary Figure 1. Characterization of Cxcl12<sup>Gagm</sup><sup>Gagm</sup> animals. (A) Schematic diagram of Cxcl12 isoforms structures and sequence alignment of the corresponding mature proteins. Mutations introduced in Cxcl12<sup>Gagm</sup> are indicated (stars). In the amino-acid sequence the substituted residues corresponding to mutations are underlined. The incorporation of the non-sense mutation in the open reading frame of Cxcl12<sub>y</sub> generates a truncated protein of 68Aa-length identical to Cxcl12<sub>a</sub>, which last aminoacid (K68) is marked by a star. (B) PCR amplification on genomic DNA from wild type (WT), Cxcl12<sup>Gagm</sup><sup>Wt</sup> (Het) and Cxcl12<sup>Gagm</sup><sup>Gagm</sup> (Mut) animals. (C-D) Real-time PCR analysis of Cxcl12 products. (C) RNA expression levels of each isoform normalized to HPRT in brain and thymus tissues from WT and Cxcl12<sup>Gagm</sup><sup>Gagm</sup> animals. (D) mRNA levels of each Cxcl12 isoform were normalized to that of Cxcl12<sub>a</sub>.
Supplementary Figure 2. Functional properties of WT and HS-binding mutant CXCL12 used for post-ischemic revascularization treatment or expressed from Cxcl12<sup>G<sup>gt</sup>im</sup> animals. (a) Sequence alignment of CXCL12 WT and mutant chemokines used in experiments described in b and c. In bold, HS-binding motifs; underlined, substituted amino acids. (b) Cell surface (CHO cells) binding activity and (c) chemotactic properties of synthetic WT and HS-binding mutants CXCL12αm and CXCL12γm2 assessed in CD4<sup>+</sup> T lymphocytes. (d) Quantitative evaluation of EPC migration through transwell membrane in response to treatment with supernatant of HEK293 T transfected cells with cDNA coding either for WT or mutant isoforms isolated from Cxcl12<sup>G<sup>gt</sup>im</sup> animals. CXCL12 content in supernatants was measured by ELISA and for each WT/mutant couple equal amounts (ranging from 0.5 to 1nM) were used. Open bars: synthetic CXCL12α, 1nM. AMD3100 (5μM) is a low molecular, synthetic CXCR4 antagonist. PBS, Phosphate buffered saline, **p<0.01 versus PBS, † p<0.05 versus conditions without AMD3100. n = 10 per group, representative of 2 independent experiments.
Supplementary Figure 3. CXCL12γ controls EPC infiltration in ischemic tissues. (a) Quantitative evaluation of EPC migration through transwell membrane in response to VEGF, CXCL12α or CXCL12γ, in presence or absence of the synthetic CXCR4 antagonist, AMD3100 (5μM). n = 6 to 8 per group, representative of 2 independent experiments. *p<0.05, **p<0.01, ***p<0.001 versus PBS, ††† p<0.001 versus CXCL12α, ## p<0.01 versus CXCL12γ. (b) Number of CFSE-stained human EPC (EPC) in the ischemic leg of C57BL/6 mice treated with intramuscular administration of cDNA expression vector encoding CXCL12α, CXCL12γ or CXCL12γm2, 4 days after the onset of ischemia. Quantitative evaluation of foot perfusion (c), microangiography (d) and capillary density (e), 14 days after ischemia in C57BL/6 mice treated with intravenous administration of EPC only or along with intramuscular cDNA expression vector encoding CXCL12α, CXCL12γ or CXCL12γm2. Empty DNA plasmid (CONT). Values are mean ± SEM. n = 10 per group, representative of 2 independent experiments. 10 possible comparisons for Bonferroni correction, a value of p<0.005 was considered significant. *p<0.05, **p<0.01, versus CONT, † p<0.005, †† p<0.001 versus CXCL12α+EPC, ##p<0.001 versus CXCL12γ + EPC.
Supplementary Figure 4. Construction and sequencing validation of point mutation targeting vector of Cxcl12. (a) Point mutations in 2nd and 4th exons were generated by 3-step PCR mutagenesis using PT1-PT4 and PT5-PT8 primers. The PCR fragments carrying mutations were then used to replace the correspondent wild type sequence using conventional sub cloning methods. (b) The boundaries of the two homology arms (LA, SA) were confirmed by sequencing with P6 and T7 primers that read through both sides of the backbone vector. The floxed Neo cassette was confirmed by sequencing with N1 and N2 primers that read from the 5'- and 3'-ends of the Neo cassette, respectively, into the genomic sequences. The mutations were confirmed by sequencing with PT1, SQ1 and PT8 primers. The sequencing results also proved that no other mutations were introduced into exon 2 and exon 4 before the natural stop codon.

Primers used to generate the point mutations (mutations are in bold and underlined)
PT1 (Aec651): 5’- ACGTGGTACCCAACCTTCTCTAACATTCCTGC -3’
PT2: 5’- AGACAGATGCGAGACGTGGGCTGTGGGATGTGGGCTC -3’
PT3: 5’- ACATCGCCAGAGCACCACGTCCTCGCATCTGTCTATCCTCAACACTCCAAACTGTG -3’
PT4 (Aec651): 5’- ACCTGGTACCTCTTGAGCAAGTGAGTGCAAGTG -3’
PT5 (Aec651): 5’- ACCTGGTACCCTCTCTAAGAAATCTCCAGGGTCACCC -3’
PT6: 5’- CTACCTAGAAAAATTAGTAGGAACC -3’
PT7: 5’- GTTCCTACTAAATTTTATCTAAGGTTAGGGGCGAGAGGAAGAAAGTGG -3’
PT8 (Aec651): 5’- ACATGGTACCAGAGTTATACCGAGGGTGTGAGC -3’

Sequencing Primer Sequences
Primer N1 5’- TGCGAGCGAGACAGGCACCACCTTCTGAGTGC -3’
Primer N2 5’- TTTCCCTGGCTTACGTTACCGTATCG - 3’
Primer P6 5’- GAGTGGCAACATATTGGACATATTGTC - 3’
Primer T7 5’- TAAATGCAGTTAACCCTGGCTTACCGTATCG - 3’
Primer PT1 5’- ACCTGGTACCCTTCTCTAACATTCCTGC - 3’
Primer SQ1 5’ - ACAGGACACATCTCCAGCATGTCGTC - 3’
Primer PT8 5’- ACCTGGTACCAGAGTTATACCGAGGGTGTGAGC - 3’