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Cyclophilin A modulates bone marrow-derived CD117$^+$ cells and enhances ischemia-induced angiogenesis *via* the SDF-1/CXCR4 axis

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ABSTRACT

Background: Critical limb ischemia (CLI) is a major health problem with no adequate treatment. Since CLI is characterized by insufficient tissue vascularization, efforts have focused on the discovery of novel angiogenic factors. Cyclophilin A (CyPA) is an immunophilin that has been shown to promote angiogenesis *in vitro* and to enhance bone marrow (BM) cell mobilization *in vivo*. However, its potential as an angiogenic factor in CLI is still unknown. Thus, this study aimed to evaluate whether CyPA might induce neo-angiogenesis in ischemic tissues.

Methods and results: Wild-type C57Bl/6j mice underwent acute hind-limb ischemia (HLI) and received a single intramuscular administration of recombinant CyPA or saline. Limb perfusion, capillary density and arteriole number in adductor muscles were significantly increased after CyPA treatment. Interestingly, BM-derived CD117$^+$ cell recruitment was significantly higher in ischemic adductor tissue of mice treated with CyPA *versus* saline. Therefore, the effect of CyPA on isolated BM-derived CD117$^+$ cells *in vitro* was evaluated. Low concentrations of CyPA stimulated CD117$^+$ cell proliferation while high concentrations promoted cell death. Moreover, CyPA enhanced CD117$^+$ cell adhesion and migration in a dose-dependent manner. Mechanistic studies revealed that CyPA up-regulated CXCR4 in CD117$^+$ cells and in adductor muscles after ischemia. Additionally, SDF-1/CXCR4 axis inhibition by the CXCR4 antagonist AMD3100 decreased CyPA-mediated CD117$^+$ cell recruitment in the ischemic limb.

Conclusion: CyPA induces neo-angiogenesis by recruiting BM-derived CD117$^+$ cell into ischemic tissues, at least in part, through SDF-1/CXCR4 axis.

1. Introduction

Critical limb ischemia (CLI) represents a social and medical problem with no effective pharmacologic therapy available [1]. Since it is characterized by impaired vascularization of the lower extremities, therapeutic angiogenesis has been proposed as a novel treatment strategy [2–5].

Emerging data suggest the therapeutic potential of bone marrow (BM)-derived stem/progenitor cells in limb ischemia [6,7]. In particular, a BM-derived cell population expressing the stem cell factor receptor CD117 has been reported to be recruited into the ischemic limb tissue to promote neovascularization [8,9]. Different mechanisms by which these cells contribute to the revascularization of the ischemic tissue have been proposed including paracrine actions [10–14] and/or ability to differentiate into endothelial cells [15].

Recently, it has been reported that the chemokine stromal cell-derived factor-1α (SDF-1) and its specific receptor CXC chemokine receptor 4 (CXCR4), which is expressed at high levels on CD117$^+$ cells, play critical roles in the recruitment of these cells in a mouse model of hind-limb ischemia (HLI) [16–18].

An important mediator of the SDF-1/CXCR4 axis is Cyclophilin A (CyPA), a ubiquitously distributed protein belonging to the immunophilin family. Like other cyclophilin family members, CyPA has peptidyl–prolyl *cis–trans* isomerase activity, which regulates protein folding and trafficking [19]. In particular, intracellular CyPA plays a key role in CXCR4-mediated signalling such as nuclear export of heterogeneous nuclear ribonucleoprotein A2, activation and nuclear translocation of extracellular-signal-regulated kinase 1/2 (ERK1/2), and chemotactic cell migration [20]. Notably, we found that CyPA induces

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the recruitment of BM-derived cells in the diseased aorta [21] and hypertrophic hearts [22] of ApoE$^{-/-}$ mice. In addition, CyPA was found to be upregulated in murine adductor muscles after HLI [23].

Although CyPA was initially believed to function primarily as an intracellular protein, recent studies revealed that it can be secreted by cells in response to inflammatory stimuli [24–26]. Extracellular CyPA is a potent leukocyte chemoattractant for human monocytes, neutrophils, eosinophils, and T cells [27,28]. We have shown that extracellular CyPA stimulates pro-inflammatory signals in endothelial cells [21,29] and others have reported that CyPA increases endothelial cell proliferation, migration, invasive capacity, and tubulogenesis *in vitro* [30]. Importantly, plasma CyPA levels were found to be significantly increased in patients with inflammatory diseases including peripheral artery disease [31–33].

We speculated that CyPA may modulate ischemia-induced neoangiogenesis, based on experimental evidence underlying the importance of CyPA in the SDF-1/CXCR4 axis, the enhanced expression in HLI, the ability to promote BM cell mobilization or recruitment *in vivo* and to modulate angiogenesis *in vitro*.

Here, we provide new insight into the mechanisms by which extracellular CyPA regulates BM-derived CD117$^+$ cell functions in the process of neovascularization of ischemic limb tissues and we show that CyPA might offer a new strategy to treat limb ischemia.

2. Materials and methods

2.1. Animal procedures

All animal studies were conducted in conformity with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes or the NIH guidelines, and in accordance with experimental protocols approved by the University Committee on Animal Resources at the University of Milan. Animals were housed under a 12 h light/dark regimen. All mice were genotyped by PCR of tail clip samples.

2.2. In vivo procedures

Wild-type (WT) male mice, 8-week-old on a C57Bl/6j background were anaesthetized with an intraperitoneal injection of a mixture containing medetomidine 0.5 mg/kg and ketamine 100 mg/kg, and hind-limb trichotomy was performed. A surgical skin incision was made in the left inguinal region, starting from the groin and finishing at the level of the bifurcation of saphenous and popliteal arteries. After skin incision, the subcutaneous fat pad of the hind-limb was removed and the femoral artery was isolated from the saphenous nerve and vein, both of them were kept intact. Arterial ischemia was induced by electrocoagulation of two femoral artery sites: a proximal site, below the branch of external iliac artery, and a distal site, in the bifurcation of saphenous and popliteal arteries. This artery segment was completely removed. Contralateral non-ischemic hind-limbs were used as control. At the time of surgery, 10 ng of recombinant human CyPA (R&D Systems) was injected in ischemic adductor muscles at five different sites (10 μl/injection) along the projection of the femoral artery.

For AMD3100 treatment, Alzet® Osmotic Pumps (Alzet®) were implanted in mice to continuously deliver AMD3100 (360 μg/day) or an equivalent volume of saline over a period of 3 days. Mice that underwent osmotic pump implantation were previously anaesthetized with intraperitoneal injection of medetomidine 0.5 mg/kg and ketamine 100 mg/kg.

Carprofen (5 mg/kg body weight) was given by intramuscular injection to provide post-operative analgesia.

2.3. Limb perfusion in mice after ischemia

At 0, 3, 7, 14, and 21 days post-ischemia, mice were anaesthetized with an intraperitoneal injection of a mixture containing medetomidine 0.5 mg/kg and ketamine 100 mg/kg and blood flow ratios in the ischemic (left) versus control (right) limbs were measured using a laser Doppler PeriScan PIM II Imager (Perimed AB). Images were obtained by two operators blinded to the treatment and analysed with PeriScan System Software — LPDIwin (Perimed AB). Data represent ischemic/contralateral non-ischemic hind-limb ratios.

2.4. Immunofluorescence

Mice were anaesthetized, perfused with normal saline and then fixed with 10% phosphate-buffered formalin for 10 min at 100 mm/Hg, *via* left ventricle. Fixed adductor muscles were paraffin embedded and 5-μm-thick sections were cut from each sample with muscle fibres oriented in the transverse direction. Capillary density was determined by counting the capillary structures in 30–40 random fields using an antibody against CD31 (BD Bioscience). In other sections, arterioles were identified by using an antibody against α-smooth muscle actin (AbCam) and counted. Counts were performed by two readers blinded to the treatment and similar results were obtained. Analyses were performed using a Zeiss LSM 710 confocal microscope. The number of capillaries and arterioles was normalized to the section area calculated with ZEN 2008 software (Carl Zeiss).

2.5. Immunohistochemistry

Formaldehyde-fixed paraffin sections were incubated with the primary antibody overnight (O/N) at 4 °C. Antibodies raised against CyPA (Santa Cruz Biotechnology), CD117 (Cell Signaling Technology), CD45 (BD Bioscience), Mac-3 (BD Bioscience), and CD3 (BD Bioscience) were used. As a negative control, species- and isotype-matched IgGs were incubated in place of the primary antibodies. Slides were viewed with an AxioSkop microscope equipped with an AxioCam camera (Carl Zeiss). Densitometric analyses were performed with AxioVision 4.7 software (Carl Zeiss) by two readers blinded to the treatment.

2.6. CyPA serum levels

Mice were anaesthetized by 2% isoflurane and blood samples were collected by retro-orbital puncture. Serum was obtained from blood samples at 0, 3, 7, 14, and 21 days after ischemia. CyPA serum levels were detected with an ELISA kit (USCN Life Science Inc.) following the manufacturer's instructions.

2.7. Cell isolation and culture

8-Week-old male WT (C57Bl/6j) mice were anaesthetized with 2% isoflurane and euthanized by cervical dislocation. BM-derived CD117$^+$ cells were isolated by flushing the BM of femurs and tibiae. Thereafter, immune-magnetic cell sorting was conducted by using CD117 MicroBeads following the manufacturer's instructions (MACS; Miltenyi Biotech). Only preparations with >90% BM-derived CD117$^+$ cells were used. Cells were grown in StemSpan SFEM Medium supplemented with the StemSpan CC100 Cocktail containing IL-3, IL-6, SCF and FLT3 (STEMCELL Technologies).

2.8. BM-derived CD117$^+$ cell recruitment

BM-derived CD117$^+$ cells were isolated as reported above and incubated O/N in StemSpan SFEM Medium. The day after, cells were labelled with the CellTrace™ Carboxyfluorescein succinimidyl ester (CFSE) reagent (Life Technologies) following the manufacturer's instructions. BM-derived CD117$^+$ labelled cells (6×10^5 cells) were resuspended in 200 μl saline solution and injected retro-orbitally in each ischemic mouse (5 treated with CyPA, 5 treated with saline). Two days after injection, mice were anaesthetized, perfused with normal saline and then fixed with 10% phosphate-buffered formalin for 10 min at

100 mm/Hg, *via* the left ventricle. Adductor muscles were dissected, washed O/N with Phosphate Buffer Saline (PBS; Gibco), treated with different concentrations of sucrose (Sigma-Aldrich) solutions (ranging from 15% to 30%), and embedded in OCT (Bio-Optica). 5-μm-Thick cryo-sections were cut from each sample with muscle fibres oriented in the transverse direction. To evaluate whether recruited BM-derived $CD117^+$ cells co-express an endothelial marker, an immunofluorescence assay was performed with an anti-CD31 antibody (BD Bioscience). Nuclei were stained with Hoechst 33342 (Life Technologies) for 10 min. Analyses were performed by two blinded operators with the AxioObserver Z.1 microscope (Carl Zeiss) equipped with an AxioCam MRm camera (Carl Zeiss). Fluorescence intensity was normalized to section area by using the image analyser AxioVision 4.7 software (Carl Zeiss).

2.9. Cell proliferation

BM-derived $CD117^+$ cells were plated at a density of 2×10^5 cells/ml (96-well plate; Costar) and incubated with exogenously added CyPA at different concentrations (ranging from 10 ng/ml to 1000 ng/ml) or vehicle. Cell counting was performed by an investigator blinded to the treatment protocol. Cell proliferation was measured daily from day 1 to day 5 by cell counting in the presence of Trypan blu (Sigma-Aldrich).

2.10. Cell death and cycle

CyPA effect on BM-derived $CD117^+$ cell death and cell cycle were evaluated following serum deprivation for 48 h in RPMI medium (Gibco) and upon exposure to exogenously added CyPA (ranging from 10 ng/ml to 1000 ng/ml).

For cell death experiments, after treatment cells were resuspended in FACS buffer (0.1% Bovine Serum Albumin, BSA and 5 mM EDTA in PBS) and incubated with AnnexinV-FITC antibody (eBioscience) for 10 min at room temperature. Then, cells were washed, resuspended in FACS buffer and incubated with 20 μg/ml of Propidium Iodide (PI; eBioscience).

FACS analysis was performed with a Gallios flow cytometer (Beckman Coulter). Data from 10,000 events per sample were collected and the percentage of the elements was calculated using Kaluza software (Beckman Coulter).

The cell cycle distribution after cell starvation was evaluated by flow cytometry by bromodeoxyuridine/PI (BrdU/PI) assay. Briefly, cells were treated with BrdU (Sigma) solution at a final concentration of 30 μM for 1 h in a CO_2 incubator at 37 °C. Then, cells were washed in PBS, resuspended in PBS, fixed with 95% ethanol, and stored O/N at 4 °C. The day after, cells were pelleted, treated with 2 N HCl (Sigma), 0.2% Triton X-100 (Sigma) for 30 min at room temperature and then resuspended with 0.1 M $Na_2B_4O_7$ (Sigma), pH 8.5 to neutralize acid. Cells were washed in PBS, 0.1% BSA, 0.5% Tween 20 (Sigma), and incubated with antibody anti-BrdU-FITC (BD Bioscience) for 30 min at room temperature. Cells were centrifuged and resuspended in PBS containing 5 ng/ml of PI (Sigma). FACS analysis was performed with a Gallios flow cytometer (Beckman Coulter). Data from 20,000 events per sample were collected and the relative percentages of the cells in G0/G1, S, and G2/M phases of the cell cycle were calculated using Kaluza software (Beckman Coulter).

2.11. Adhesion assay

BM-derived $CD117^+$ cells were treated with increasing concentrations of CyPA (from 10 ng/ml to 1000 ng/ml) for 20 h in StemSpan SFEM Medium. 96-Well plates were coated with 10 μg/mL of fibronectin (Sigma-Aldrich) O/N at 4 °C. The day after, wells were washed 2 times with PBS, blocked for 2 h with 1% BSA (Sigma-Aldrich) and washed again with PBS. Treated BM-derived $CD117^+$ cells (5×10^5 in 100 μl) were added to each well and allowed to adhere for 3 h. Each well was washed with PBS with Ca^{2+}/Mg^{2+} (Gibco) to remove debris and floating cells, then adherent cells were fixed for 10 min with 10% formalin and again washed 3 times. Cells were stained with 0.05% Crystal Violet (Sigma-Aldrich) for 30 min and washed 3 times with distilled water. To quantify the cell number, the dye was solubilized with methanol and the OD of each well was quantified at 540 nm with an absorbance plate reader Mithras LB 940 (Berthold Technologies).

2.12. Migration assay

BM-derived $CD117^+$ cells (1×10^6) were seeded into the upper chamber of a transwell plate with a pore size of 3 μm (Sigma-Aldrich) in Migration Medium (0.1% FCS, 25 mmol/l HEPES in RPMI). CyPA 100 ng/ml was added to the lower chamber of the transwell plate. Cell migration towards CyPA was determined after 4 h of incubation at 37 °C. The number of migrating cells was determined by the Crystal Violet (Sigma-Aldrich) assay as reported above or by cell counting in the presence of Trypan blue. Migration was calculated by dividing the number of cells which traversed the transwell membrane in the presence of CyPA by the number of cells which migrated in response to medium alone. Cell counting was performed by an investigator blind to the treatment protocol.

2.13. Differentiation assay

BM-derived $CD117^+$ cells were seeded at a cell density of 2.5×10^5 and treated with CyPA (100 ng/ml) as previously described [34]. Briefly, cells were cultured on 6-multiwell plates coated with 20 μg/ml fibronectin (Sigma) in IMDM medium (Gibco) supplemented with 20% FBS (Lonza) and vascular endothelial growth factor (VEGF, 20 ng/ml), fibroblast growth factor-1 (FGF-1, 20 ng/ml), and insulin growth factor-1 (IGF-1, 20 ng/ml) (Peprotech). Cells were cultured for 7 days and the medium was changed every 2 days. BM-derived $CD117^+$ cell differentiation towards the endothelial lineage was determined by flow cytometry. Cells were collected, washed with the previously described FACS buffer and incubated with CD31-PE (BD Bioscience) and CD45-PerCP (clone 30-F11, BD Bioscience) antibodies. FACS analysis was performed with a Gallios flow cytometry (Beckman Coulter). Data from 10,000 events per sample were collected and percentage analyses were performed by Kaluza software (Beckman Coulter).

2.14. $CD117^+$ cell tube formation assay

BM-derived $CD117^+$ cells were seeded onto a Cultrex (Cultrex Reduced Growth Factor Basement Membrane Extract, Trevigen Inc.) artificial cell basal membrane as previously reported [35]. Briefly, Cultrex (250 μl) was allowed to polymerize onto 12-well plates at 37 °C, 5% CO_2 for 30 min. BM-derived $CD117^+$ cells were seeded at a concentration of 5×10^4 cells/well in IMDM medium (Gibco) supplemented with 20% FBS (Lonza) and VEGF (20 ng/ml), FGF-1 (20 ng/ml), and IGF-1 (20 ng/ml) (Peprotech) for 7 days.

2.15. mRNA extraction and qRT-PCR

BM-derived $CD117^+$ cells were treated for 16 h with recombinant CyPA (ranging from 10 ng/ml to 1000 ng/ml). RNA was isolated with a Total RNA Purification kit (Norgen Biotek Corp.). For *in vivo* analysis, adductor muscles were harvested and RNA was isolated with an RNeasy® Microarray Tissue Kit (Qiagen). RNA quantification was determined with a spectrophotometer (ND-1000, NanoDrop®, EuroClone®). Reverse transcription was conducted with the SuperScript III (Invitrogen™) following the manufacturer's instructions. qRT-PCR was performed with the use of the iQ™ SYBR Green Super Mix (BIO-RAD) and RT^2 qPCR Primers (Qiagen). All reactions were performed in a 96-well format with the iQ5™ (BIO-RAD). The relative quantities of specific mRNAs were obtained with the use of the comparative Ct method and

were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.16. Western blot analysis

Adductor muscle tissues and BM-derived CD117$^+$ cells were lysed in cell lysis buffer (Cell Signaling Technology) supplemented with a protease inhibitor cocktail (Sigma Aldrich). Total cell proteins were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane. The membranes were blocked for 1 h at room temperature in 5% non-fat dry milk in Wash Buffer (0.1% Tween 20 in Tris Buffer Sulfate) and then incubated O/N at 4 °C with the appropriate primary antibody. The primary antibodies used were specific for CyPA (Sigma Aldrich), CXCR4 (Abcam), phospho-CXCR4 (Abcam), protein kinase B (AKT, Cell Signaling Technology), phospho-AKT (Cell Signaling Technology), ERK1/2 (Santa Cruz Biotechnology), phospho-ERK1/2 (Cell Signaling Technology), and tubulin (Sigma Aldrich). The membranes were incubated with peroxidase-conjugated secondary antibodies for 1 h. Signals were visualized using enhanced chemiluminescence Western blotting detection system (GE Healthcare). Images were acquired with the Alliance Mini 2M (UVITec Cambridge), and densitometric analysis of membranes was performed using the Alliance Mini 4 16.07 software (UVITec Cambridge). Proteins were normalized according to α-tubulin and/or GAPDH.

2.17. Statistical analyses

Quantitative results are expressed as mean ± SD. Statistical significance was evaluated with GraphPad Prism 5.

Variables were analysed by Student's t test. A value of $p \leq 0.05$ was deemed statistically significant.

3. Results

3.1. CyPA levels increase during HLI

To evaluate the potential involvement of CyPA during the *in vivo* response to ischemia, we used an established mouse model of HLI consisting of the unilateral removal of the femoral artery. Interestingly, the endogenous expression of CyPA in the adductor muscle of WT mice was remarkably increased 3 days after ischemia and peaked at 7 days post-surgery. This effect was evident both at RNA (Fig. 1A) and protein (Fig. 1B, C and D) levels.

Since CyPA is a protein secreted in response to inflammatory stimuli [36] and limb ischemia is a pathologic condition characterized by elevated inflammation in the injured muscle, we evaluated whether CyPA is also secreted during HLI. Serum levels of CyPA rose after ischemia (Fig. 1E) suggesting that it might be secreted in order to modulate the in vivo response to HLI.

3.2. Local administration of CyPA increases limb perfusion and neoangiogenesis

The CyPA expression and secretion data prompted us to evaluate the effect of exogenously administered recombinant CyPA in the neoangiogenic response to ischemia. Specifically, CyPA (10 ng) was locally injected in the ischemic muscle immediately after HLI. To evaluate whether CyPA affects neo-angiogenesis, hind-limb perfusion, capillary density and arteriole number were analysed.

As depicted in Fig. 2A and B, exogenous CyPA significantly improved blood flow *vs.* saline 0, 3, 7, 14, and 21 days after HLI. Furthermore, in adductor muscles treated with CyPA, a significant increase both in capillary density (Fig. 2C and D) and arteriole number (Fig. 2E and F) was observed 7 days after ischemia.

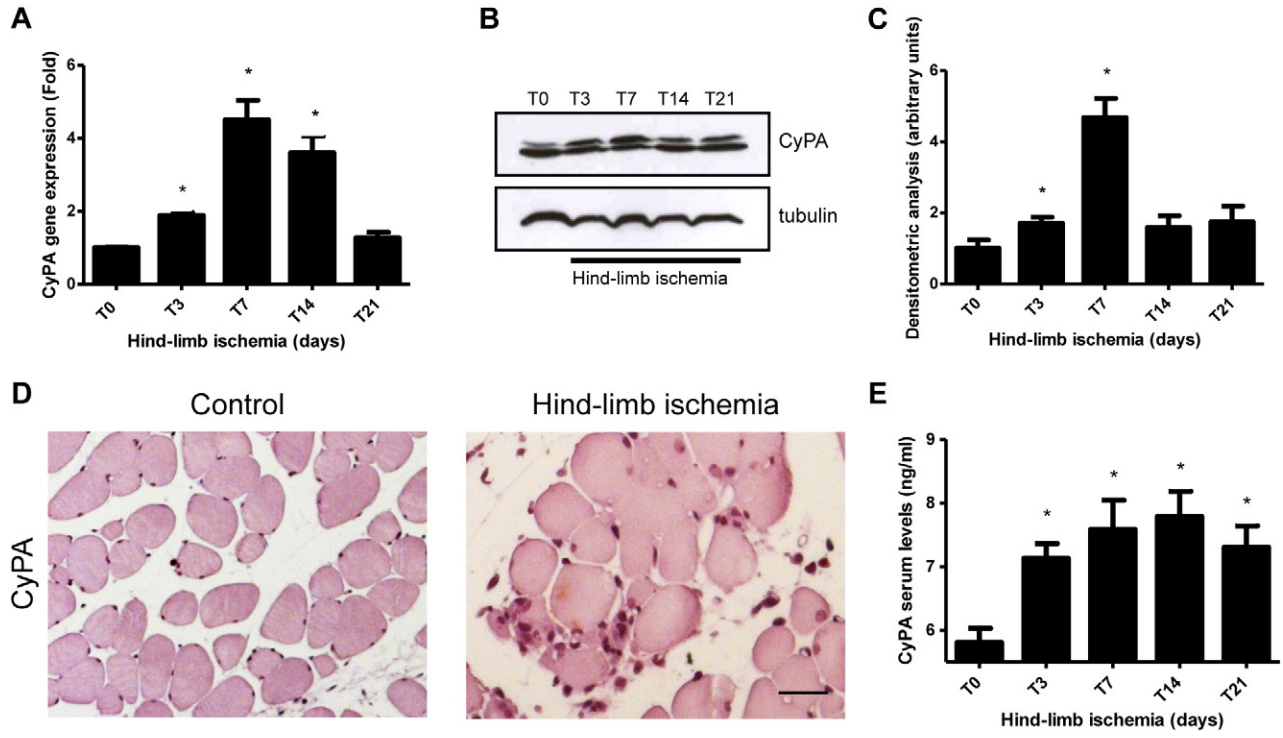

Fig. 1. Intracellular and extracellular levels of CyPA increase during HLI. CyPA gene (A) and protein (B, C) expression at different days after HLI (qRT-PCR data are fold change ± SD, Western blot quantification data are mean ± SD, $n = 5$/group/time point). (D) Representative images of immunohistochemistry for CyPA in ischemic (right panel) and contralateral non-ischemic (left panel) adductor muscle sections 3 days after HLI. (E) Serum levels of CyPA at different time points after HLI (data are mean ± SD, $n = 5$/time point). Student's t-test: $^*p < 0.05$. Magnification = 20×. Scale bar = 100 μm.

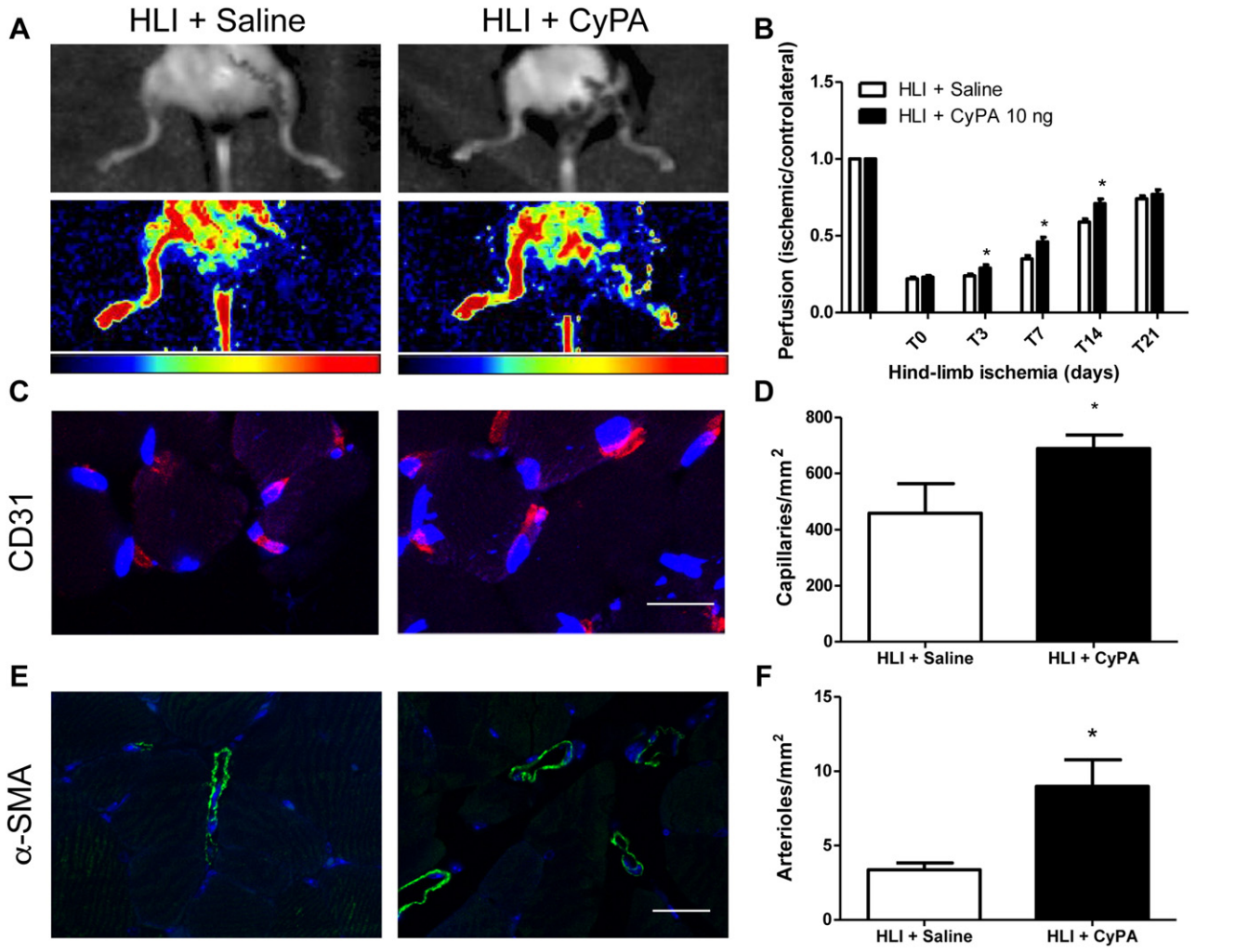

Fig. 2. CyPA increases limb perfusion and angiogenesis. (A) Representative contrast images (upper panels) and correspondent laser Doppler images (lower panels) of hind-limbs 3 days after surgery and treatments. Colour scale in Doppler images represents a gradient from less perfused (blue) to highly perfused (red) areas. (B) Bar chart of perfusion rates on different days post-HLI. (C) CD31 immunofluorescence for capillary detection and relative quantification (D) 7 days post-surgery. Magnification = 40×. Scale bar = 15 μm. (E) α-smooth muscle actin (α-SMA) immunofluorescence and relative arteriole quantification (F) 7 days post-surgery. Magnification = 20×. Scale bar = 40 μm. All data are mean ± SD, n = 5/group/time point. Student's t-test: *$p < 0.05$.

Collectively, these data suggest that an acute local administration of CyPA improves post-ischemic reparative neovascularization and blood flow recovery in WT mice.

3.3. CyPA augments the number of inflammatory cells following HLI

Key cellular processes observed during an ischemic stimulus are the activation, recruitment, and invasion of a number of inflammatory cells that are involved in inducing and promoting angiogenesis by releasing multiple pro-angiogenic cytokines, chemokines and growth factors [37]. Thus, we evaluated the involvement of CyPA in the recruitment of inflammatory cells into ischemic tissue.

CyPA treatment increased the number of leukocytes ($CD45^+$ cells; Fig. 3A and B), macrophages ($Mac\text{-}3^+$ cells; Fig. 3C and D) and T cells ($CD3^+$ cells; Fig. 3E and F) into the ischemic tissue at 3 days post-surgery. It is worth noting that inflammation decreased in CyPA-treated mice at longer time points post-ischemia (14 days, data not shown), suggesting that CyPA did not exacerbate a chronic and deleterious inflammatory process.

Collectively, these experimental findings imply that exogenous CyPA accelerates an inflammation-driven neoangiogenic process favouring tissue repair.

3.4. CyPA regulates the recruitment of $CD117^+$ cells following HLI

Emerging data suggest the clinical potential of $CD117^+$ cells to restore blood flow to the ischemic limb [6,7,18]. Thus, we measured the number of these cells after HLI in the presence or absence of CyPA. Interestingly, CyPA significantly increased the number of $CD117^+$ cells in ischemic muscles compared to saline-treated group (Fig. 4A and B). Consistently, an increased expression of the gene encoding CD117 (Fig. 4C) and its protein (Fig. 4D and E) was observed after 3 days from the initial treatment with CyPA, administered at the time of the surgery. This result is likely due to their increased recruitment in the ischemic tissue. To evaluate whether the increased number of cells was linked to the capacity of CyPA to act as chemoattractant, $CD117^+$ cells were isolated from mouse BM and labelled with the green fluorescence marker CFSE CellTrace™ reagent. Subsequently, these cells were delivered by retro-orbital injection in the venous sinus of WT mice (6×10^5 cells/mouse) at day 1 post-ischemia (Fig. 5A). Intravenously administered $CD117^+$ cells were detected in adductor muscles harvested 3 days post-ischemia along with the endothelial marker CD31 (Supplementary Fig. 1S). CFSE-positive cells were typically found in small clusters in the border zone of the ischemic tissue and their recruitment into the ischemic tissue was significantly increased in mice treated with

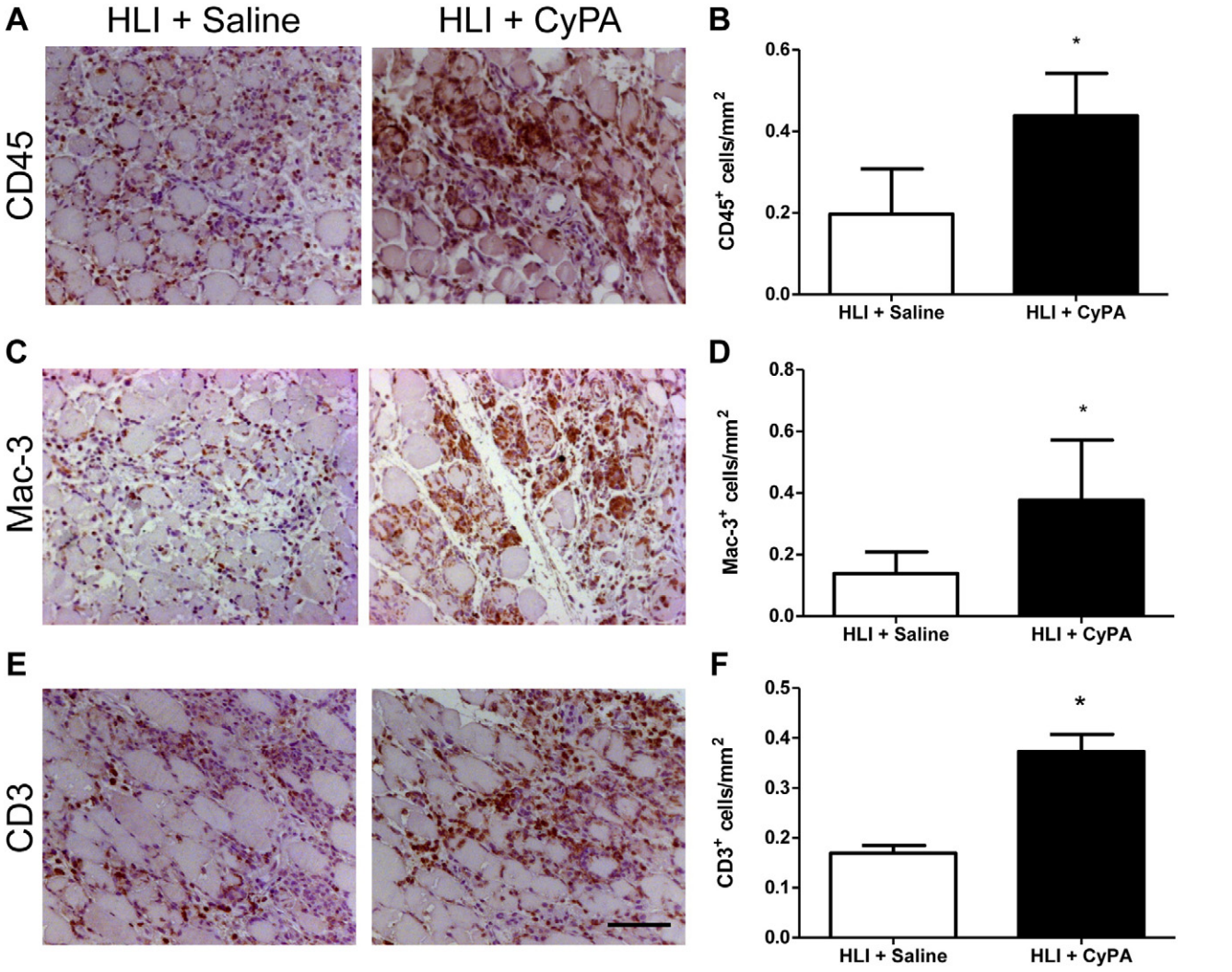

Fig. 3. CyPA augments inflammatory cell number following HLI. Immunohistochemistry for CD45 (A), Mac-3 (C), and CD3 (E) on adductor muscle slices of ischemic mice treated with saline or with 10 ng CyPA for 3 days. Immunohistochemistry quantification (B, D, F) data are shown as mean ± SD, $n = 5$/group. Student's t-test: $^*p < 0.05$. Magnification = 20×. Scale bar = 40 μm.

CyPA compared to saline (Fig. 5B and C). Taken together these results indicate that CyPA modulates the recruitment of $CD117^+$ cells.

3.5. CyPA is involved in $CD117^+$ cell survival, adhesion and migration in vitro

Based on experimental evidence underlying the importance of CyPA in the recruitment of $CD117^+$ cells into ischemic tissue, we next evaluated the effect of CyPA on isolated $CD117^+$ cells *in vitro*.

Hence, $CD117^+$ cells were isolated from the BM of WT mice and the ability of exogenous CyPA to directly stimulate these cells was tested at different concentrations (10, 100 and 1000 ng/ml) and time points (0, 24, 48, and 72 h). A low concentration (100 ng/ml) of CyPA induced the proliferation of $CD117^+$ cells, while the higher concentration (1000 ng/ml) was effective in reducing cell number (Fig. 6A). This effect was significant ($p < 0.05$) after 48 h and 72 h of treatment.

We further analysed whether the reduced number of $CD117^+$ cells due to a high concentration of CyPA was related to a cell cycle progression block (anti-proliferative effect) and/or to the occurrence of cell death (cytotoxicity). Cell cycle distribution analysis of $CD117^+$ cells exposed to increasing doses of CyPA revealed no significant differences in cell cycle distribution between control and treated cells (Supplemental Fig. 2S). However, $CD117^+$ cell death analysis following serum deprivation for 48 h and upon exposure to exogenous CyPA revealed that while 100 ng/ml CyPA decreased the number of $AnnexinV^-/PI^+$ cells, 1000 ng/ml CyPA exacerbated necrosis (Fig. 6D). In accordance with results regarding cell proliferation, these data further establish that the behaviour of CyPA on $CD117^+$ cells is biphasic, exerting both positive and negative effects depending on its concentration.

Since both migration and the subsequent adhesion of BM-derived cells occur into the newly vascularized tissue during the formation of new blood vessels after ischemia [38], we next tested the effects of CyPA on the migratory and adhesive activity of $CD117^+$ cells. Intriguingly, exogenous CyPA directly stimulated the migration of $CD117^+$ cells (Fig. 6B) and enhanced cell adhesion to fibronectin-coated dishes in a dose-dependent manner (Fig. 6C). Considering that $CD117^+$ cells are able to differentiate into endothelial cells [38], we appraised whether CyPA might affect their differentiation potential. As shown in Fig. 6E, CyPA did not increase the expression of CD31 and did not improve the ability of $CD117^+$ cells to form tubules *in vitro* (Fig. 6F).

Since the leukocyte fate of $CD117^+$ cells is also important for the neoangiogenesis process, we evaluated whether CyPA could affect

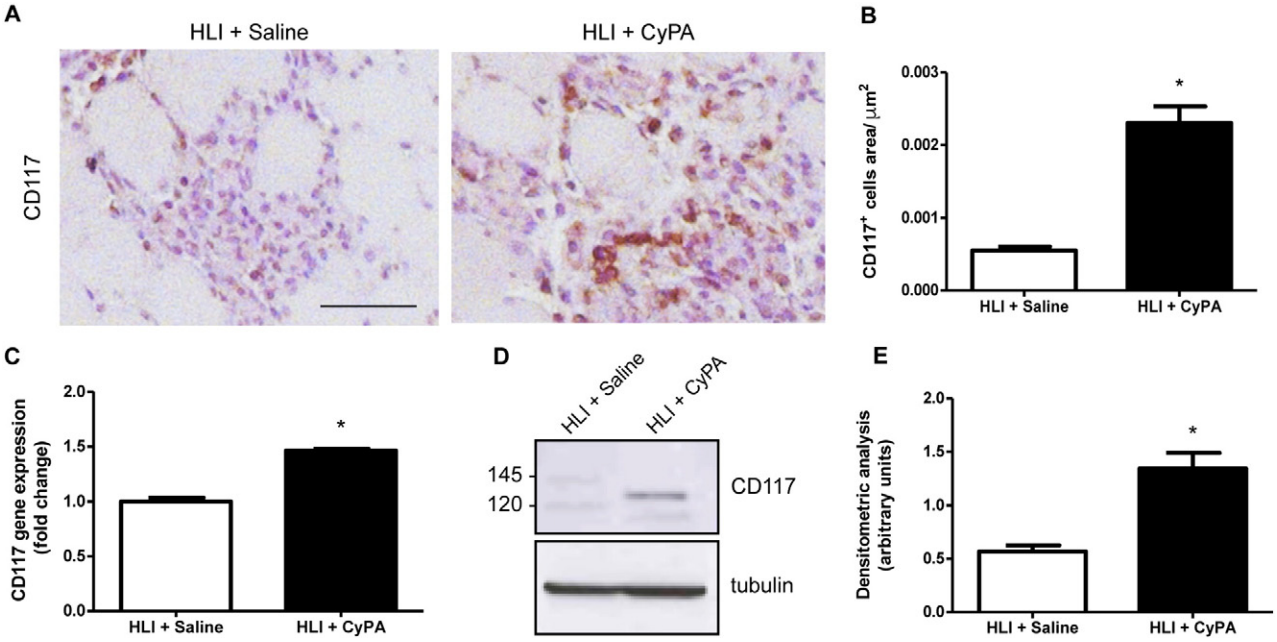

Fig. 4. CyPA increases the number of BM-derived CD117$^+$ cells. Immunohistochemistry for CD117 on adductor muscle sections of ischemic mice treated with saline or with 10 ng CyPA (A) and relative quantification (B). Ischemic samples were collected 7 days after HLI. Data are shown as mean ± SD, $n = 5$/group. CD117 gene (C) and protein (D, E) expression in adductor muscle tissues (qRT-PCR data are fold change ± SD, Western blot quantification data are mean ± SD, $n = 5$/group). Student's t-test: $^*p < 0.05$. Magnification = 40×. Scale bar = 50 μm.

CD45 expression in isolated cells. As reported for CD31, CyPA did not alter the expression of this pan leukocyte marker (Fig. 3S).

These results clearly suggest that the main mechanism of action for CyPA is to increase the survival, migration, and adhesion of CD117$^+$ cells instead of promoting differentiation towards the endothelial lineage.

3.6. CyPA regulates CXCR4 expression and activity in vitro and in vivo

In light of the major importance of SDF-1/CXCR4 signalling in the regulation of BM-derived CD117$^+$ cell recruitment and neo-angiogenesis during ischemia [16,18], we examined whether CyPA modulates CXCR4 expression. CD117$^+$ cells exposed to increasing concentrations of CyPA for 16 h exhibited a progressive increase in CXCR4 mRNA levels (Fig. 7A). However, the highest dose of CyPA (1000 ng/ml) did not induce CXCR4 gene up-regulation, probably due to cytotoxic effects shown above.

In addition, CyPA increased CXCR4 protein activation (phosphorylation at serine 346, Fig. 7B and C) and consequently its downstream signalling pathway (ERK1/2 and AKT, Fig. 7B). Inhibition of the SDF-1/CXCR4 axis by the CXCR4 antagonist AMD3100 decreased CyPA-mediated activation of CXCR4 cell signalling (Fig. 7B and C).

The critical role for SDF-1/CXCR4 pathway in CyPA-mediated effects was corroborated in the murine model of HLI. In particular, ischemic muscles of mice treated with CyPA showed increased CXCR4 gene (Fig. 7D) and protein expression (Fig. 7E–F and Supplementary Fig. 4S). Interestingly, CyPA also induced the up-regulation of SDF-1 gene expression (Fig. 7G) and its serum levels (Fig. 7H).

These data demonstrate that CyPA modulates the SDF-1/CXCR4 pathway in CD117$^+$ cells during HLI.

3.7. Inhibition of SDF-1/CXCR4 signalling reverses CyPA-mediated neo-angiogenesis in vivo

To substantiate that the action of CyPA during HLI is mainly mediated by the SDF-1/CXCR4 axis, neo-angiogenesis was determined after CXCR4 blockade with the antagonist AMD3100. AMD3100 (360 μg/day) was infused continuously over 3 days *via* an implanted osmotic mini-pump. At day 1, mice underwent HLI and treatment with CyPA or saline. After 4 days, ischemic adductor muscles were harvested to measure tissue neovascularization. Results revealed that AMD3100 abrogated the CyPA-mediated increase in blood perfusion (Fig. 8A) as well as capillary density (Fig. 8B) and arteriole number (Fig. 8C).

Additionally, the influx of BM-derived CD117$^+$ cells into injured adductor muscles was not significantly increased in the AMD3100 + CyPA treatment group compared to mice receiving AMD3100 alone (Fig. 8D and E).

Altogether, these results suggest that CyPA drives the angiogenic response to ischemia, at least in part, through the SDF-1/CXCR4 signalling pathway.

4. Discussion

The present study addressed the role of CyPA as a novel agent regulating ischemia-induced neo-angiogenesis. Specifically, we demonstrated that the treatment of ischemic hind-limbs with exogenous CyPA improves tissue revascularization and repair. Notably, blood flow perfusion and new blood vessel formation were significantly accelerated in mice treated with CyPA compared with saline.

Our results were surprising and counter-intuitive because we always considered CyPA as a pro-inflammatory cytokine mediating tissue damage. Nonetheless, recent insights have shed light on the cellular and molecular processes through which conventional inflammatory cytokines (e.g. IL-6 and TNFα) promote tissue repair and regeneration [39]. In this regard, we believe that CyPA levels increase during HLI to promote reparative inflammation. Indeed, we found that CyPA increases the number of inflammatory cells into ischemic limbs. These results are consistent with a recent evidence which highlights the tight interconnection between the processes of inflammation and angiogenesis [37]. It has been established that newly formed blood vessels enable the continuous recruitment of inflammatory cells, which release a

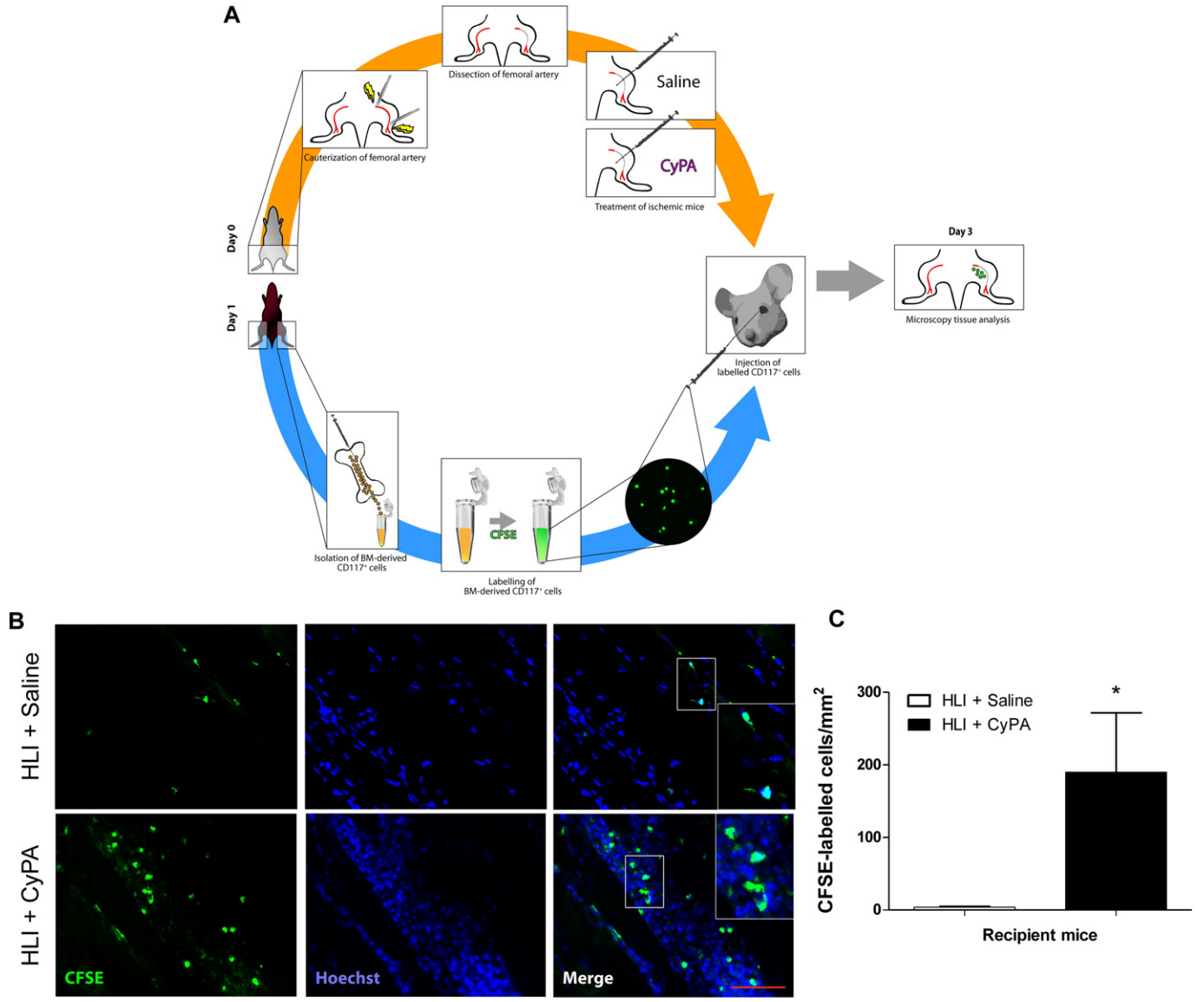

Fig. 5. CyPA enhances BM-derived CD117$^+$ cell recruitment in ischemic mice. (A) Methodological approach for HLI surgery and BM-derived CD117$^+$ cell isolation, labelling, and retro-orbital injection. WT C57Bl/6j mice underwent surgical femoral artery removal by cauterization and dissection (recipient mice). Ischemic mice were treated by intramuscular injection of saline solution ($n = 5$) or 10 ng CyPA ($n = 5$). The day after surgery, WT C57Bl/6j mice ($n = 5$; donor mice) were sacrificed to explant BM-derived selected CD117$^+$ cells from hind-limb bones. Once isolated, cells underwent CFSE labelling and injection into recipient HLI mice. (B) Representative images of CFSE-labelled BM-derived CD117$^+$ cells (green) in adductor muscle cryo-sections and relative signal quantification (C). Highlighted areas correspond to 40× magnification. Data are shown as mean ± SD, $n = 5$/group. Student's t-test: $^*p < 0.05$. Magnification = 20×. Scale bar = 200 μm.

variety of pro-angiogenic cytokines, chemokines, and growth factors and further promote angiogenesis. However, self-limiting tissue inflammation is essential for proper restorative responses. In this regard, it is important to point out that in our experimental setting CyPA did not trigger a positive feedback loop which ultimately leads to chronic inflammation. In fact, we observed that the inflammatory process accelerated by CyPA was decreased 14 days after HLI; thus inflammation was transient and not chronic in nature.

In addition to inflammatory cells, the process of neo-angiogenesis in the ischemic limb critically involves BM-derived cells that incorporate into the damaged blood vessel endothelium and promotes new blood vessel formation [40]. Cells expressing the CD117 marker have been extensively studied as haematopoietic and vascular progenitors in experimental models of HLI. In particular, it was reported that these cells are recruited into ischemic tissues and participate in the process of revascularization [8,9,18]. The transplantation of BM-derived CD117$^+$ cells into ischemic hind-limbs was associated with improved microvessel density and recovery of blood perfusion [8]. Interestingly, CyPA significantly improved CD117$^+$ cell recruitment into ischemic tissues. These findings led us to explore the direct effect of CyPA in cultured CD117$^+$ cells *in vitro*. We found that CyPA functionally modulates CD117$^+$ cells by enhancing proliferation, migration and adhesion, critical steps of the neo-angiogenic process. Specifically, low doses of CyPA augmented CD117$^+$ cell proliferation in addition to enhanced cell migration and adhesion. Conversely, high doses of CyPA induced cell death. This dose-dependent response to CyPA might be related to the capacity of CyPA to induce the accumulation of reactive oxygen species [21,22]. In fact, low doses of CyPA might increase low levels of oxidative stress that can act as *bona fide* messengers to control multiple cellular functions, including proliferation. On the other hand,

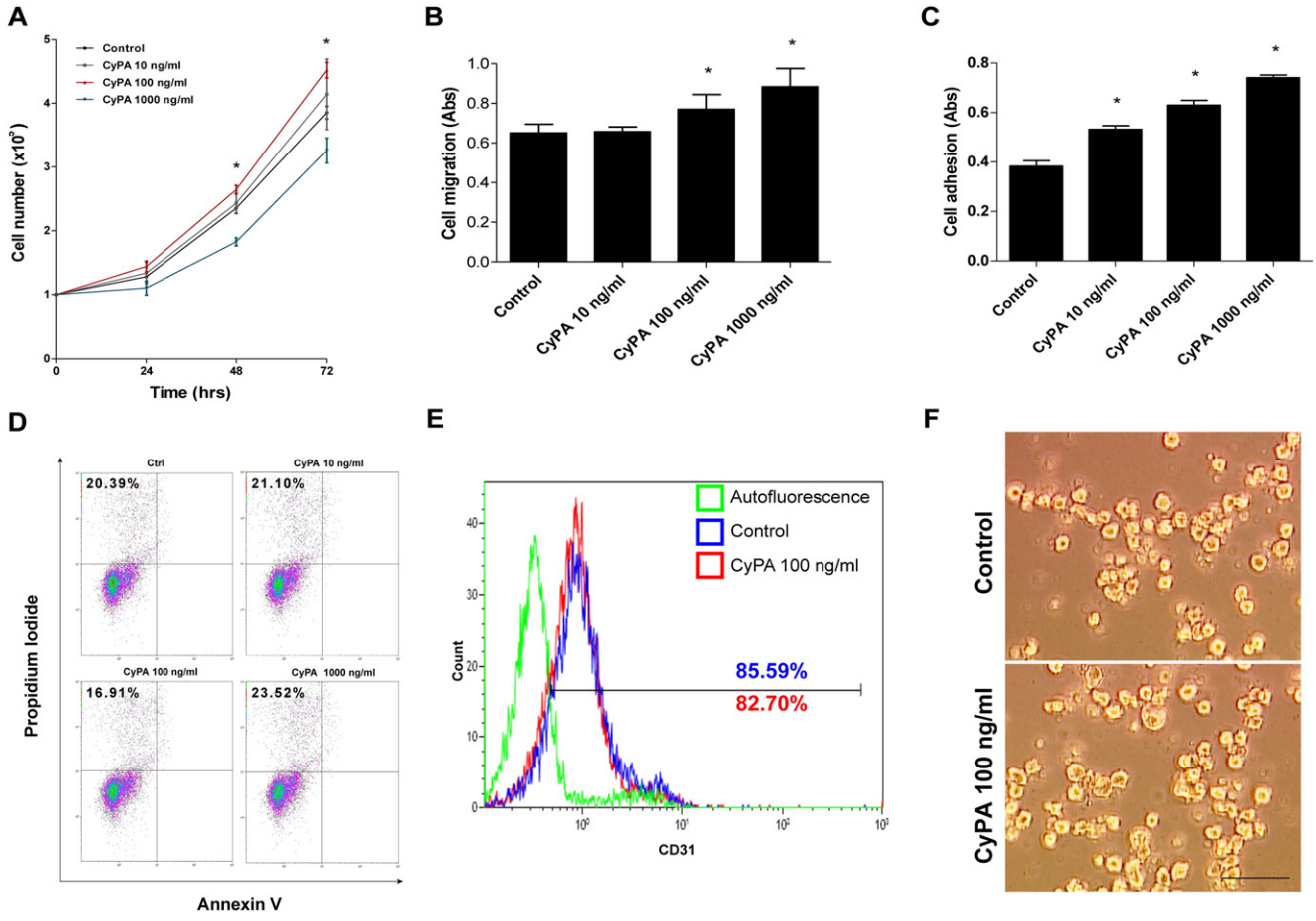

Fig. 6. CyPA is involved in BM-derived CD117$^+$ cell survival, migration and adhesion. Assay graphs for cell number up to 72 h (A), migration up to 4 h (B), and adhesion after 24 h (C) of treatment with increasing doses of CyPA. (D) FACS analysis reporting the percentage of AnnexinV negative and PI positive BM-derived CD117$^+$ cells after serum deprivation and treatment with 100 ng/ml CyPA for 48 h. (E) FACS analysis and percentage of CD31 in BM-derived CD117$^+$ cells after 7 days of treatment with 100 ng/ml CyPA. (F) Representative images of tube formation assay in BM-derived CD117$^+$ cells ± CyPA (100 ng/ml). Results are means ± SD of three experiments performed in triplicate. Student's t-test: *$p < 0.05$. Magnification = 20×. Scale bar = 50 μm.

high doses of CyPA might induce excessive formation of reactive oxygen species leading to cell death.

In our attempts to decipher the molecular mechanisms underlying the process of CyPA-induced neo-angiogenesis, we found that CyPA up-regulates the SDF-1/CXCR4 axis, a critical pathway involved in the recruitment of BM-derived cells during peripheral limb ischemia [16]. The importance of the SDF-1/CXCR4 axis in BM-derived stem/progenitor cell recruitment has been established with the observation that selective expression of SDF-1 in injured tissue correlates with adult stem cell recruitment and tissue regeneration [41]. Additionally, SDF-1 and CXCR4 were recently reported to be expressed by or in close proximity to angiogenic vessels in ischemic muscles of patients with peripheral artery disease, suggesting that SDF-1/CXCR4 signalling might be relevant in post-natal neovascularization. The involvement of this pathway in the process of angiogenesis was further confirmed by Tachibana et al. showing that CXCR4$^{-/-}$ mice die *in utero* and are defective in vascular development, haematopoiesis and cardiomyogenesis [42].

Recently, it was reported that intracellular CyPA is essential for SDF-1-mediated chemotactic actions [20] and cell survival [43]. However, the effect of exogenous administered CyPA on CD117$^+$ cells was never investigated. Here, we found that CD117$^+$ cells treated with exogenous CyPA exhibited up-regulated expression of CXCR4, which paralleled their migratory capacity in response to SDF-1. Moreover, we provided evidence that CyPA directly regulates CXCR4 downstream events. Explicitly, we studied the role of CyPA on the modulation of ERK1/2 and AKT activity, serine-threonine kinases that are involved in the CXCR4 downstream intracellular pathway. We showed that exogenous CyPA activates ERK1/2 and AKT and this effect was abrogated using the CXCR4 antagonist AMD3100. These results demonstrated that CyPA, by activating CXCR4, modulates ERK1/2 as well as AKT activity in CD117$^+$ cells.

In line with these *in vitro* findings, we found that exogenous CyPA strongly up-regulates SDF-1 and CXCR4 gene expression *in vivo*. Notably, CyPA-dependent CXCR4 up-regulation may have interesting therapeutic implications. In fact, a recent study has demonstrated that up-regulation of CXCR4 by gene transfer improves *in vitro* functional properties of human endothelial progenitor cells and enhances arterial re-endothelialization in mice subjected to carotid artery injury [44]. Remarkably, the stimulatory effect of CyPA on neovascularization and CD117$^+$ cell recruitment was not observed when the SDF-1/CXCR4 axis was blocked by the infusion of AMD3100.

It is important to point out that in this study we focused on CD117$^+$ cells since transplantation of these cells in the ischemic limb has been reported to lead to muscle and vascular regeneration [8,9,18]. However, we cannot exclude that CyPA might induce neo-angiogenesis by acting on other cell types by parallel mechanisms. We and others have shown that CyPA increases endothelial cell and vascular smooth muscle cell proliferation [29,45,46], as well as inflammatory cell recruitment [21,26], both crucial and necessary mechanisms in the process of adult neo-angiogenesis [47].

Therefore, in future investigations, we plan to address the effects of CyPA on multiple cellular and biochemical targets during HLI.

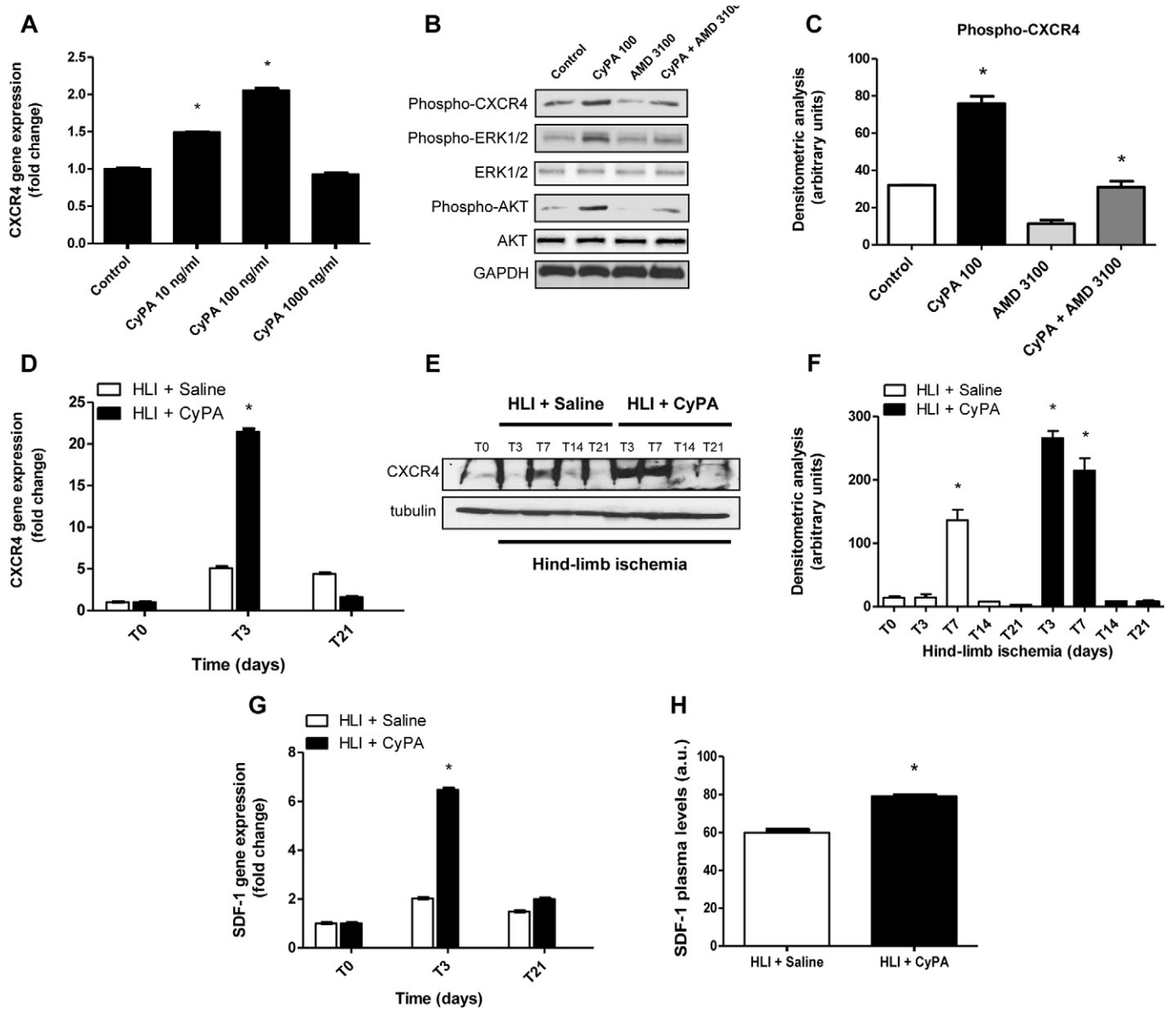

Fig. 7. CyPA regulates CXCR4 expression and activity *in vitro* and *in vivo*. CXCR4 gene expression (A) in BM-derived CD117$^+$ cells treated with CyPA. qRT-PCR was performed in triplicate and data are shown as fold change ± SD. Western blot analysis (B) for active CXCR4 (phospho-CXCR4) and downstream mediators in BM-derived CD117$^+$ cells treated for 5 min with 100 ng/ml CyPA and/or 25 μg/ml AMD3100. Cells were pre-treated with AMD3100 for 30 min before CyPA addition. Gene expression of CXCR4 (D) and SDF-1 (G) were evaluated in adductor muscles of ischemic mice treated with saline or 10 ng CyPA (qRT-PCR data are fold change ± SD $^*p < 0.05$; $n = 5$/group/time point). CXCR4 protein expression (E) was analysed on adductor muscles by Western blot. Protein quantification (C, F) was performed by densitometric analysis ($^*p < 0.05$). SDF-1 levels (H) were measured by ELISA in the serum of mice, 3 days after surgery and treatment with 10 ng CyPA. Student's t-test: $^*p < 0.05$.

5. Conclusion

The present study suggest that CyPA enhances neo-angiogenesis and restore revascularization in ischemic tissues by regulating SDF-1/CXCR4 pathway and concomitantly modulating the recruitment of BM-derived CD117$^+$ cells (Supplementary Fig. 5S). Thus, CyPA may serve as a useful therapeutic strategy for accelerating angiogenesis in ischemic cardiovascular diseases, such as CLI.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijcard.2016.03.082.

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Conflict of interest

The authors report no relationships that could be construed as a conflict of interest.

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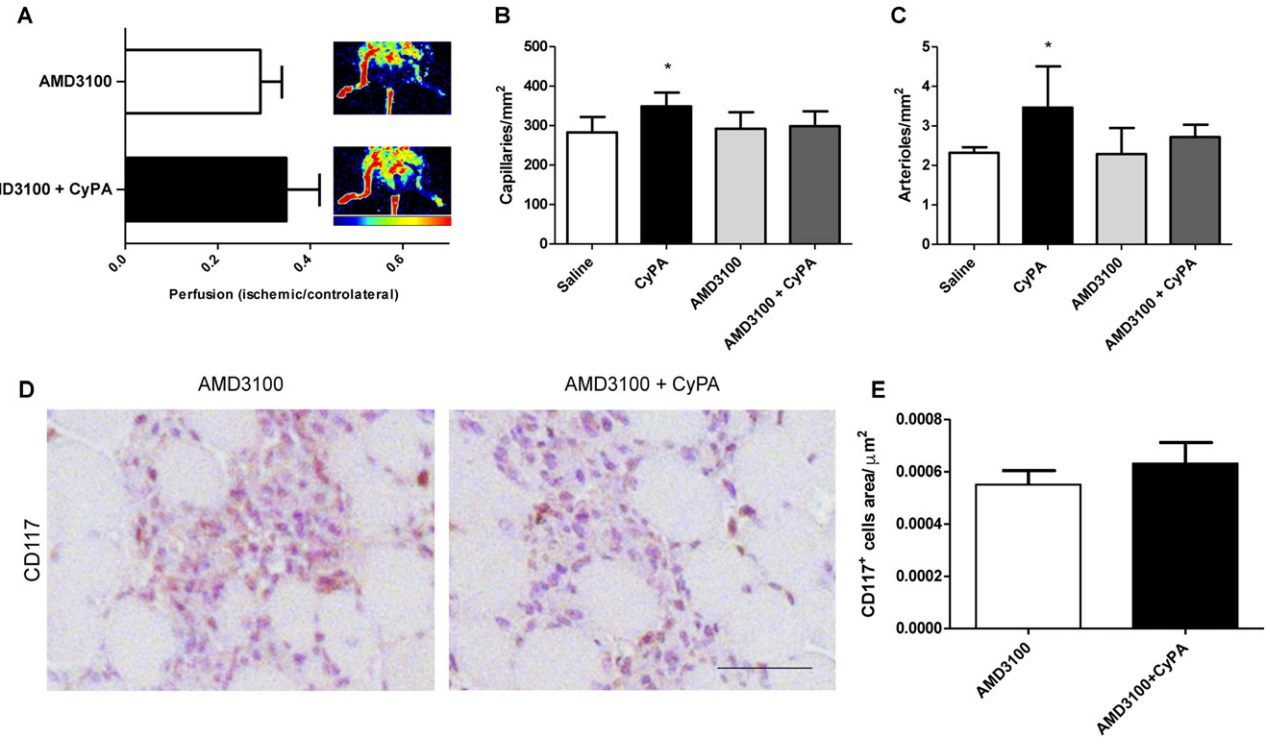

Fig. 8. Inhibition of CXCR4 signalling reverses CyPA-mediated neo-angiogenesis *in vivo*. Bar charts depict perfusion levels (A), number of capillaries (B), and arterioles (C) in mice treated with saline, 10 ng CyPA, AMD3100 (360 μg/die) + saline or AMD3100 (360 μg/die) + 10 ng CyPA after 3 days post-surgery. Immunohistochemistry (D) and its densitometric analysis (E) for CD117 in adductor muscle tissue sections from ischemic mice treated with AMD3100 or co-treated with 10 ng CyPA 3 days post-surgery. Data are means ± SD, $n = 5$. Student's t-test: $^*p < 0.05$. Magnification = 20×. Scale bar = 50 μm.

References

[1] P. Gresele, C. Busti, T. Fierro, Critical limb ischemia, Intern. Emerg. Med. 6 (Suppl. 1) (2011) 129–134.
[2] S. Attanasio, J. Snell, Therapeutic angiogenesis in the management of critical limb ischemia: current concepts and review, Cardiol. Rev. 17 (2009) 115–120.
[3] W.S. Jones, B.H. Annex, Growth factors for therapeutic angiogenesis in peripheral arterial disease, Curr. Opin. Cardiol. 22 (2007) 458–463.
[4] F. Sedighiani, S. Nikol, Gene therapy in vascular disease, Surgeon 9 (2011) 326–335.
[5] H. Lawall, P. Bramlage, B. Amann, Treatment of peripheral arterial disease using stem and progenitor cell therapy, J. Vasc. Surg. 53 (2011) 445–453.
[6] T. Takahashi, C. Kalka, H. Masuda, et al., Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization, Nat. Med. 5 (1999) 434–438.
[7] Z. Raval, D.W. Losordo, Cell therapy of peripheral arterial disease: from experimental findings to clinical trials, Circ. Res. 112 (2013) 1288–1302.
[8] T.S. Li, K. Hamano, M. Nishida, et al., CD117$^+$ stem cells play a key role in therapeutic angiogenesis induced by bone marrow cell implantation, Am. J. Physiol. Heart Circ. Physiol. 285 (2003) H931–H937.
[9] T.S. Li, M. Hayashi, Z.L. Liu, et al., Low angiogenic potency induced by the implantation of ex vivo expanded CD117(+) stem cells, Am. J. Physiol. Heart Circ. Physiol. 286 (2004) H1236–H1241.
[10] R. Bolli, X.L. Tang, S.K. Sanganalmath, et al., Intracoronary delivery of autologous cardiac stem cells improves cardiac function in a porcine model of chronic ischemic cardiomyopathy, Circulation 128 (2013) 122–131.
[11] T. Ziegelhoeffer, B. Fernandez, S. Kostin, et al., Bone marrow-derived cells do not incorporate into the adult growing vasculature, Circ. Res. 94 (2004) 230–238.
[12] B.J. Capoccia, R.M. Shepherd, D.C. Link, G-CSF and AMD3100 mobilize monocytes into the blood that stimulate angiogenesis in vivo through a paracrine mechanism, Blood 108 (2006) 2438–2445.
[13] K. Tateno, T. Minamino, H. Toko, et al., Critical roles of muscle-secreted angiogenic factors in therapeutic neovascularization, Circ. Res. 98 (2006) 1194–1202.
[14] Y. Shiba, M. Takahashi, T. Hata, et al., Bone marrow CXCR4 induction by cultivation enhances therapeutic angiogenesis, Cardiovasc. Res. 81 (2009) 169–177.
[15] T. Asahara, H. Masuda, T. Takahashi, et al., Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization, Circ. Res. 85 (1999) 221–228.
[16] E. De Falco, D. Porcelli, A.R. Torella, et al., SDF-1 involvement in endothelial phenotype and ischemia-induced recruitment of bone marrow progenitor cells, Blood 104 (2004) 3472–3482.
[17] E. De Falco, D. Avitabile, P. Totta, et al., Altered SDF-1-mediated differentiation of bone marrow-derived endothelial progenitor cells in diabetes mellitus, J. Cell. Mol. Med. 13 (2009) 3405–3414.
[18] C. Cencioni, R. Melchionna, S. Straino, et al., Ex vivo acidic preconditioning enhances bone marrow ckit+ cell therapeutic potential via increased CXCR4 expression, Eur. Heart J. 34 (2013) 2007–2016.
[19] P. Nigro, G. Pompilio, M.C. Capogrossi, Cyclophilin A: a key player for human disease, Cell Death Dis. 4 (2013), e888.
[20] H. Pan, C. Luo, R. Li, et al., Cyclophilin A is required for CXCR4-mediated nuclear export of heterogeneous nuclear ribonucleoprotein A2, activation and nuclear translocation of ERK1/2, and chemotactic cell migration, J. Biol. Chem. 283 (2008) 623–637.
[21] P. Nigro, K. Satoh, M.R. O'Dell, et al., Cyclophilin A is an inflammatory mediator that promotes atherosclerosis in apolipoprotein E-deficient mice, J. Exp. Med. 208 (2011) 53–66.
[22] K. Satoh, P. Nigro, A. Zeidan, et al., Cyclophilin A promotes cardiac hypertrophy in apolipoprotein E-deficient mice, Arterioscler. Thromb. Vasc. Biol. 31 (2011) 1116–1123.
[23] D. Tirziu, K.L. Moodie, Z.W. Zhuang, et al., Delayed arteriogenesis in hypercholesterolemic mice, Circulation 112 (2005) 2501–2509.
[24] Z.G. Jin, M.G. Melaragno, D.F. Liao, et al., Cyclophilin A is a secreted growth factor induced by oxidative stress, Circ. Res. 87 (2000) 789–796.
[25] J. Suzuki, Z.G. Jin, D.F. Meoli, et al., Cyclophilin A is secreted by a vesicular pathway in vascular smooth muscle cells, Circ. Res. 98 (2006) 811–817.
[26] K. Satoh, P. Nigro, T. Matoba, et al., Cyclophilin A enhances vascular oxidative stress and the development of angiotensin II-induced aortic aneurysms, Nat. Med. 15 (2009) 649–656.
[27] B. Sherry, N. Yarlett, A. Strupp, et al., Identification of cyclophilin as a proinflammatory secretory product of lipopolysaccharide-activated macrophages, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 3511–3515.
[28] Q. Xu, M.C. Leiva, S.A. Fischkoff, et al., Leukocyte chemotactic activity of cyclophilin, J. Biol. Chem. 267 (1992) 11968–11971.
[29] K. Satoh, T. Matoba, J. Suzuki, et al., Cyclophilin A mediates vascular remodeling by promoting inflammation and vascular smooth muscle cell proliferation, Circulation 117 (2008) 3088–3098.
[30] S.H. Kim, S.M. Lessner, Y. Sakurai, et al., Cyclophilin A as a novel biphasic mediator of endothelial activation and dysfunction, Am. J. Pathol. 164 (2004) 1567–1574.
[31] I. Tegeder, A. Schumacher, S. John, et al., Elevated serum cyclophilin levels in patients with severe sepsis, J. Clin. Immunol. 17 (1997) 380–386.
[32] H. Kim, W.J. Kim, S.T. Jeon, et al., Cyclophilin A may contribute to the inflammatory processes in rheumatoid arthritis through induction of matrix degrading enzymes and inflammatory cytokines from macrophages, Clin. Immunol. 116 (2005) 217–224.
[33] M.C. Liu, Y.W. Lee, P.T. Lee, et al., Cyclophilin A is associated with peripheral artery disease and chronic kidney disease in geriatrics: the Tianliao Old People (TOP) study, Sci. Rep. 5 (2015) 9937.

[34] E. Pierpaoli, R. Moresi, F. Orlando, et al., Effect of Hyperglycemia on the Number of CD117 Progenitor Cells and Their Differentiation Toward Endothelial Progenitor Cells in Young and Old Ages, Mech. Ageing Dev. (2016).
[35] G. Spaltro, S. Straino, E. Gambini, et al., Characterization of the Pall Celeris system as a point-of-care device for therapeutic angiogenesis, Cytotherapy 17 (2015) 1302–1313.
[36] Z.G. Jin, A.O. Lungu, L. Xie, et al., Cyclophilin A is a proinflammatory cytokine that activates endothelial cells, Arterioscler. Thromb. Vasc. Biol. 24 (2004) 1186–1191.
[37] Y.W. Kim, X.Z. West, T.V. Byzova, Inflammation and oxidative stress in angiogenesis and vascular disease, J. Mol. Med. (Berl) 91 (2013) 323–328.
[38] M. Pesce, A. Orlandi, M.G. Iachininoto, et al., Myoendothelial differentiation of human umbilical cord blood-derived stem cells in ischemic limb tissues, Circ. Res. 93 (2003) e51–e62.
[39] M. Karin, H. Clevers, Reparative inflammation takes charge of tissue regeneration, Nature 529 (2016) 307–315.
[40] C. Urbich, S. Dimmeler, Endothelial progenitor cells: characterization and role in vascular biology, Circ. Res. 95 (2004) 343–353.
[41] V. van Weel, L. Seghers, M.R. de Vries, et al., Expression of vascular endothelial growth factor, stromal cell-derived factor-1, and CXCR4 in human limb muscle with acute and chronic ischemia, Arterioscler. Thromb. Vasc. Biol. 27 (2007) 1426–1432.
[42] K. Tachibana, S. Hirota, H. Iizasa, et al., The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract, Nature 393 (1998) 591–594.
[43] M. Chatterjee, P. Seizer, O. Borst, et al., SDF-1alpha induces differential trafficking of CXCR4–CXCR7 involving cyclophilin A, CXCR7 ubiquitination and promotes platelet survival, FASEB J. 28 (2014) 2864–2878.
[44] L. Chen, F. Wu, W.H. Xia, et al., CXCR4 gene transfer contributes to in vivo reendothelialization capacity of endothelial progenitor cells, Cardiovasc. Res. 88 (2010) 462–470.
[45] K. Satoh, P. Nigro, B.C. Berk, Oxidative stress and vascular smooth muscle cell growth: a mechanistic linkage by cyclophilin A, Antioxid. Redox Signal. 12 (2010) 675–682.
[46] H. Yang, M. Li, H. Chai, et al., Effects of cyclophilin A on cell proliferation and gene expressions in human vascular smooth muscle cells and endothelial cells, J. Surg. Res. 123 (2005) 312–319.
[47] N.I. Moldovan, Role of monocytes and macrophages in adult angiogenesis: a light at the tunnel's end, J. Hematother. Stem Cell Res. 11 (2002) 179–194.