International Journal of Cardiology 212 (2016) 324–335

Contents lists available at ScienceDirect

International Journal of Cardiology

journal homepage: www.elsevier.com/locate/ijcard

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

Gianluca L. Perrucci [a,b], Stefania Straino [c,d], Maria Corlianò [b], Alessandro Scopece [b], Monica Napolitano [c], Bradford C. Berk [e], Federico Lombardi [a,g], Giulio Pompilio [a,b,f], Maurizio C. Capogrossi [c], Patrizia Nigro [b,*]

[a] Department of Clinical Sciences and Community Health, University of Milan, Via Festa del Perdono 7, 20122 Milan, Italy
[b] Unit of Vascular Biology and Regenerative Medicine, Centro Cardiologico Monzino-IRCCS, Via C. Parea 4, 20138 Milan, Italy
[c] Laboratory of Vascular Pathology, Istituto Dermopatico dell'Immacolata-IRCCS, Via Monti di Creta 104, 00167 Rome, Italy
[d] Explora Biotech S.r.l., Via G. Peroni 386, 00131 Rome, Italy
[e] Department of Medicine, University of Rochester School of Medicine and Dentistry, Rochester, NY 14642, USA
[f] Department of Cardiovascular Surgery, Centro Cardiologico Monzino-IRCCS, Via C. Parea 4, 20138 Milan, Italy
[g] Unit of Cardiology, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Via Francesco Sforza 35, 20122 Milano, Italy

ARTICLE INFO

*Article history:*
Received 28 October 2015
Received in revised form 11 March 2016
Accepted 19 March 2016
Available online 24 March 2016

*Keywords:*
Cyclophilin A
CD117$^+$ cells
Neo-angiogenesis
SDF-1/CXCR4 axis
Hind-limb ischemia

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

* Corresponding author at: Unit of Vascular Biology and Regenerative Medicine, Centro Cardiologico Monzino-IRCCS, Via privata Carlo Parea 4, 20128 Milan, Italy.
*E-mail address:* patrizia.nigro@ccfm.it (P. Nigro).

http://dx.doi.org/10.1016/j.ijcard.2016.03.082

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 ($6 \times 10^5$ 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