Chapter 3

IFN-β affects the angiogenic potential of circulating angiogenic cells by activating calpain 1

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ABSTRACT

Circulating angiogenic cells (CACs) are monocyte-derived cells with endothelial characteristics, which contribute to both angiogenesis and arteriogenesis in a paracrine way. Interferon-beta (IFN-β) is known to inhibit these divergent processes in animals and patients. We hypothesized that IFN-β might act by affecting the differentiation and function of CACs. CACs were cultured from peripheral blood mononuclear cells (MNCs) and phenotypically characterized by surface expression of monocytic and endothelial markers. IFN-β significantly reduced the number of CACs by 18-64%. Apoptosis was not induced by IFN-β, neither in MNCs during differentiation, nor after maturation to CACs. Rather, IFN-β impaired adhesion to- and spreading on fibronectin, which was dependent on α5β1 (VLA-5) integrin. IFN-β affected the function of VLA-5 in mature CACs, leading to rounding and detachment of cells, by induction of calpain 1 activity. Cell rounding and detachment was completely reversed by inhibition of calpain 1 activity in mature CACs. During in vitro capillary formation, CAC addition and calpain 1 inhibition enhanced sprouting of endothelial cells to a comparable extent, but were not sufficient to rescue tube formation in the presence of IFN-β. We show that the IFN-β-induced reduction of the numbers of in vitro differentiated CACs is based on activation of calpain 1, resulting in an attenuated adhesion to extracellular matrix proteins via VLA-5. In vivo this could lead to inhibition of vessel formation due to reduction of the locally recruited CAC numbers and their paracrine angiogenic factors.
INTRODUCTION

The viability and function of the myocardium after a coronary artery obstruction is largely dependent on formation of a sufficient collateral network, requiring a remodelling process termed arteriogenesis\(^1\). Pre-existing collateral arteries grow in response to increased fluid shear stress, whereas circulating immune cells like monocytes/macrophages\(^2,3\) and also CACs\(^4,5\) are important effectors of this remodelling. CACs, previously considered to be a subpopulation of endothelial progenitor cells (EPCs)\(^6\), are now regarded as a monocyte-derived cell population that acquires expression of endothelial markers upon exposure to an angiogenic environment\(^4,7,8\). Further immunophenotypic analysis indicates that CACs are closely similar to alternatively activated M2 macrophages, which are anti-inflammatory and promote angiogenesis, tissue repair and vascular remodeling\(^9,10\). Neovascularization is more efficiently supported by CACs than by normal monocytes and macrophages, as was shown in a murine model of hind limb ischemia\(^4\). CACs do not enhance angiogenesis and collateral artery formation by their incorporation in the endothelium, but rather by secretion of angiogenic factors, including growth factors, chemokines, cytokines and matrix metalloproteinases, that support increased endothelial migration and proliferation\(^5,8,11,12\). The mobilization and homing of CACs to the vessel wall is regulated by numerous chemokines, growth factors, and adhesion molecules\(^11\).

The extent of collateral artery formation greatly varies between individuals. Previously, we have demonstrated that monocytes from patients with insufficient coronary collateral artery development show increased expression of IFN-β and its downstream genes\(^13\). In addition, we have shown that IFN-β acts as a potent inhibitor of collateral artery formation upon femoral artery ligation in a murine hind limb model of arteriogenesis\(^13,14\). Interestingly, a strong negative effect of IFN-β on angiogenesis, that is, the sprouting of new capillaries from existing vessels, has been widely documented\(^15-17\). Arteriogenesis and angiogenesis differ considerably in their mechanisms and driving forces. Whereas angiogenesis is primarily driven by hypoxia with HIF1alpha and VEGF as effectors, arteriogenesis is driven by the increase in shear stress that results from the (partial) occlusion of a proximal artery. In both processes, however, CACs play a similar role through paracrine delivery of pro-angiogenic and pro-arteriogenic factors\(^18,19\). We therefore hypothesize that part of the adverse effects of IFN-β on arteriogenesis and angiogenesis results from its interference with CAC function. In the present study we determined the effect of IFN-β during and after in vitro differentiation of CACs from mononuclear cells. Here, we report that IFN-β affects CAC numbers by reducing VLA-5 mediated CAC adhesion to fibronectin through activation of calpain 1. Furthermore, we report that IFN-β inhibition of angio/arteriogenesis, is partly dependent on activation of the protease calpain 1.
METHODS

Isolation and cultivation of CACs

CACs were cultured according to previously published methods\textsuperscript{7,20,21}. Briefly, mononuclear cells (MNCs) were isolated from human peripheral blood buffy coats of healthy volunteers obtained from the Blood Bank (Sanquin, Amsterdam, The Netherlands). Blood was diluted 1:4 with phosphate buffered saline (PBS) containing 1% citrate (Sigma-Aldrich, Zwijndrecht, The Netherlands), and overlayed on Lymphoprep (1.077 g/ml; Axis-Shield, Oslo, Norway). Cells were centrifuged at 700 x g for 30 minutes at room temperature (RT). The MNCs were collected and washed five times in PBS/citrate at 200 x g for 10 minutes to remove platelets. Isolated MNCs were resuspended to a concentration of 8 x 10\textsuperscript{6}/ml in endothelial basal medium (EBM; Lonza, Breda, The Netherlands) supplemented with 20% Fetal Bovine Serum (FBS; Lonza) and the EGM SingleQuots (Lonza) containing bovine brain extract, human epidermal growth factor, hydrocortisone, gentamicin, and amphotericin B. The MNCs were seeded on tissue culture plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) precoated with 10 μg/ml human fibronectin (Sigma-Aldrich) for at least 30 minutes at RT. After three days of culture, the non-adherent cells were discarded by thorough washing with PBS for two times and fresh medium was added. The remaining adherent cells were maintained in culture for a total of 6 days. For analyzing the effect of IFN-β and calpain 1 on CACs during their differentiation period, MNCs were directly stimulated after seeding with recombinant human IFN-β (Merck, Darmstadt, Germany) with and without the chemical calpain 1 inhibitor (1 μM; CPI-1, Sigma-Aldrich) or vehicle (0.05% DMSO; Sigma-Aldrich). To investigate the effect of IFN-β and calpain 1 on CACs after their maturation, CACs at day three were stimulated with IFN-β with and without the calpain 1 inhibitor (1 μM) or vehicle (0.05% DMSO). In both culture conditions, the inhibitor was added 30 minutes prior to addition of IFN-β, and the cells were left in culture with these stimuli for three days unless otherwise specified. For the different experiments, adherent cells were used unless mentioned otherwise. For each independent experiment, blood from a different volunteer was used.

Characterization of CACs

CACs were immunophenotyped using fluorescence microscopy or flow cytometric analysis. Microscopy. Briefly, adherent cells were incubated with 2.4 μg/ml Dil-labeled ac-LDL (Tebu-Bio BV; Heerhugowaard, The Netherlands) and 10 μg/ml fluorescein isothiocyanate (FITC)-labeled \textit{Ulex europaeus} agglutinin type I lectin (Ulex-Lectin; Sigma-Aldrich) at 37°C with 5% CO\textsubscript{2} for one hour. Thereafter the cells were fixed with 4% paraformaldehyde (PFA) in HBSS (Invitrogen, Bleiswijk, The Netherlands) for 30 minutes at RT. After fixation, cell nuclei were stained with 5 μg/ml of Hoechst (Invitrogen) for 30 minutes at RT. The adherent cells were observed and photographed at 20x magnification using an inverted fluorescent microscope.
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(Nikon Eclipse TE300, Tokio, Japan). The CAC morphology was assessed and cells positive for both FITC-Ulex-lectin and Dil-ac-LDL were judged to be CACs. A total of five randomly selected images were acquired from triplicate wells of each donor, and the number of adherent CACs were determined using WCIF Image J software (National Institutes of Health, Bethesda, Maryland, USA).

**Flow cytometry.** To further characterize these cells, the lineage and surface marker phenotype of the CACs was determined by flow cytometry. Briefly, adherent cells were detached with 1 mM EDTA (Merck) in PBS, and blocked with normal mouse serum (NMS) for 10 minutes at 4°C. Subsequently, the cells were labeled for 30 minutes at 4°C with the fluorescent monoclonal antibodies: mouse FITC-conjugated anti-human CD14 (Immunotools, Friesoythe, Germany), APC-conjugated anti-human CD45 (Immunotools), PE-conjugated anti-human CD31 (Immunotools), Alexa-fluor 647 anti-human VEGFR-2/KDR (Biolegend, San Diego, CA, USA), APC-conjugated anti-human CD105 (Immunotools), PE-conjugated anti-human Tie2/TEK (R&D Systems, Abingdon, UK), and PE-Cy7-conjugated anti-human CD117/c-kit (Biolegend). Fluorescent anti-human isotype-matched antibodies (Immunotools) were used as negative controls. The analysis of surface expression of β1 integrin subunits in adherent CACs was performed after IFN-β stimulation, compared to unstimulated control cells. After detachment, the cells were labeled with an APC-conjugated anti-human β1 monoclonal antibody (Immunotools). After labeling, the cells were washed, taken up in 0.5% BSA (Roche Diagnostics, Almere, The Netherlands) in PBS and analyzed on a Cyan ADP High Performance Research Flow Cytometer (Beckman Coulter, Woerden, The Netherlands). The data were analyzed using the Summit V4.3 (Dako, Fort Collins, CO, USA) program.

**Actin staining**

To visualize the effect of IFN-β treatment on subcellular localization of actin, CACs were cultured on hydrophobic chamber slides (2.1 x 10⁶ cells/well; Ibidi, Planegg/Martinsried, Germany) precoated with 10 μg/ml fibronectin. Cells were fixed in 4% PFA in HBSS (Invitrogen) for 10 minutes at RT, and then stained with 10 μg/ml Ulex-Lectin for one hour at RT. Next, cells were permeabilized with 0.1% Triton X-100 (Merck) in PBS for 5 minutes at RT and stained with 0.4 μg/ml phalloidin-tetramethyl rhodamine iso-thiocyanate (TRITC; Sigma-Aldrich) for one hour at RT. Cells were visualized and photographed at 63 times magnification with the confocal laser scanning microscope (Leica TCS SP2 AOBs, Leica Microsystems B.V., Rijswijk, The Netherlands). A total of five randomly selected fields were acquired with Leica confocal software version 2.61 (Leica Microsystems, Wetzlar, Germany), and analyzed using WCIF Image J software (National Institutes of Health).
Adhesion assay

The effects of IFN-β or neutralizing antibodies against α4, α5 or β1 integrin subunits on CAC adhesion were assessed by analyzing CAC adhesion to fibronectin in vitro. In brief, CACs were washed once with pre-warmed PBS (37°C) to remove the dead cells. The adherent cells were detached with 1 mM EDTA/PBS. CACs were washed once with PBS, counted and resuspended in PBS to a concentration of 7 x 10⁶/ml. To label the CACs, 25 μg calcein-AM (Invitrogen) per ml of cells was added, and incubated for 30 minutes in a 37°C waterbath in the dark. After incubation, cells were washed once with PBS and resuspended to a concentration of 0.8 x 10⁶/ml in EBM/0.5% BSA. For the adhesion assay 40.000 calcein-labeled cells/well were added in a 96-wells tissue culture plate (Greiner Bio-One) precoated with 10 μg/ml fibronectin, and allowed to adhere for 30 minutes at 37°C in 5% CO₂ in a humidified incubator. Thereafter, the non-adherent CACs were removed by careful washing with pre-warmed (37°C) EBM/0.5% BSA for two times, and 100 ul/well lysis buffer containing 50 mM Tris (Merck), 0.1% SDS (Sigma-Aldrich) was added. To measure the percentage of adherent CACs, the same assay was performed with a control plate containing total cells, where the wash step was omitted, and 50 ul/well lysis buffer was added. The fluorescence of the plate was measured in a fluorescence microplate reader at excitation 485 nm/emission 520 nm. Results from triplicate wells were expressed as the percentage adherent cells calculated by dividing the corrected (background subtracted) fluorescence of adherent cells by the total corrected fluorescence of cells and multiplied by 100%. For the inhibition of attachment to fibronectin by neutralizing antibodies, cells were preincubated with 10 μg/ml of the antibody against α4 (R&D Systems), α5 (Abcam, Cambridge, UK) or β1 (R&D Systems) for 15 minutes at RT.

RNA isolation, reverse transcriptase-polymerase chain reaction, real-time PCR

Effect of IFN-β on the expression levels of α5 and β1 integrin, calpain 1, and calpain 2 gene was analyzed in adherent CACs. Briefly, total RNA was isolated from adherent cells using a RNeasy mini kit (Qiagen, Venlo, The Netherlands) according to the manufactures instructions, including the DNase I (Qiagen) digestion step to remove genomic DNA. RNA samples were speedvacced for 30 minutes to concentrate the RNA. RNA purity and concentration was measured using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Breda, The Netherlands). For cDNA synthesis, 200 ng of total RNA per sample was reverse transcribed using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions. Quantitative real-time polymerase chain reaction (PCR) was carried out in an ABI PRISM 7900HT system (Applied Biosystems, Foster City, CA, USA) with the following primers (Invitrogen) designed by Primer Express version 2.0 (Applied Biosystems):
**Western blot**

Adherent CACs (initiated with $20 \times 10^6$ cells) were lysed during 15 minutes at 4°C in 120 μl RIPA lysis buffer containing 50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Nonidet P40 (octyl phenoxypolyethoxylethanol), 1 tablet protease inhibitor per 10 ml (Roche Diagnostics), 0.5 mM sodium orthovanadate and 5 mM NaF. After lysis, the samples were snap-frozen in liquid nitrogen and stored at -80 °C until usage. The samples were mixed with 5x SDS sample buffer containing 57% glycerol, 173 mM SDS, 50 mM Tris pH 6.8, 0.01% bromophenol blue, and 0.2 M DTT, and denatured at 95 °C for 3 minutes. The samples were loaded onto 7.5% SDS polyacrylamide gels and blotted onto nitrocellulose membranes. Membranes were blocked with Odyssey blocking buffer (Li-Cor, Lincoln, NE, USA) diluted 1:1 in PBS-0.1% Tween-20 (PBS-T) for one hour at RT and probed overnight with primary antibodies directed against human α5 integrin subunit (mouse; 1:2500; BD Biosciences, San Jose, CA, USA), human β1 integrin subunit (mouse 1:2500; BD Biosciences), human calpain 1 (mouse; 1:1000; Abcam), and human GAPDH loading control (mouse; 1:1000; Abcam) or a human α-tubulin loading control (mouse; 1:2000; Cedarlane, Hornby, Ontario, Canada) at 8°C. Following extensive washings with PBS-T, the blots were incubated for one hour at RT with IRDye 800CW or IRDye 680RD conjugated goat anti-mouse secondary antibodies (1:7500; Li-Cor). After washing with PBS-T, the blots were scanned with an Odyssey Imager (Li-Cor) according to the instructions of the manufacturer, and signals were quantified using Odyssey application software v. 2.1 (Li-Cor).
Calpain 1 activity assay

Calpain 1 activity was determined in IFN-β-treated mature CACs using the fluorogenic Calpain Activity Assay kit (EMD Millipore, San Diego, CA, USA). In brief, calpain activity was analyzed by measuring the cleavage of a synthetic calpain substrate, Suc-Leu-Leu-Val-Tyr-7-Amino-4-methylcoumarin (Suc-LLVY-AMC) in mature CAC lysates, according to the manufacturer’s instructions. Cell lysates were incubated for 15 min at RT in the presence of calpain 1 inhibitor (1 µM) or vehicle (0.05% DMSO), and the cleavage product AMC was measured fluorometrically at an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

Quantification of dead cells

The percentage of dead cells in the non-adherent and adherent CACs was determined after IFN-β treatment. The non-adherent cells were collected and adherent cells were detached with 1 mM EDTA/PBS. Next, the cells were washed twice with ice-cold PBS, counted and resuspended to a concentration of 1 x 10⁶/ml in 1 x binding buffer containing 0.01 M Hepes (pH 7.4; Invitrogen), 0.14 M NaCl (Sigma-Aldrich), 2.5 mM CaCl₂ (Sigma-Aldrich). To 100 ul of the solution 5 ul of FITC-Annexin V (BD Biosciences) was added, and incubated for 15 minutes at RT. Before flow cytometric analysis, 0.69 μM of Sytox Blue nucleic acid stain (Invitrogen) was added. The data were analyzed using the Summit V4.3 program. Dead cells were defined as positive for Annexin V binding and Sytox Blue staining.

Time lapse video

MNCs were cultured on hydrophobic chamber slides precoated with 10 µg/ml fibronectin at a density of 0.825 x 10⁶ cells/well. Three days later, non-adherent cells were removed and adherent cells were stimulated with and without 100 IU/ml IFN-β and 1 µM calpain 1 inhibitor. The movement of CACs was followed over time using an inverted time-lapse video microscope (Olympus IX81 microscope, Zoeterwoude, The Netherlands) housed in a 60% humidified, 5% CO₂ gassed, temperature controlled (37°C) chamber. The cells were recorded after one hour adhesion for 48 hours every 12 minutes at 20 times magnification. The movies were converted to avi format with Cell^R software, and subsequently splitted with Quick avi splitter (v. 2.0 goldzsoft).

In vitro tube formation assay

In vitro tube formation assays were performed in three-dimensional human fibrin matrices as described previously²². In brief, fibrinogen (2 mg/ml; Kordia, Leiden, The Netherlands) was dissolved in M199 (Lonza) with 1% penicillin/streptomycin (P/S; Lonza) in a 37°C waterbath for one hour. After dissolving fibrinogen was sterilized by filtering through a 0.2
μm filter, thrombin Ila (Organon Technica, Boxtel, The Netherlands) was added at 0.05 U/ml and a 96-wells plate was filled with 100 μl/well. Gelation then occurred by incubation for one hour at RT, and subsequently one hour at 37°C in 5% CO₂. After polymerization, M199 supplemented with 10% heat-inactivated human serum, 10% heat-inactivated new born calf serum, 1% P/S, and 1% L-glutamin (HMEC medium) was added to inactivate thrombin, with an incubation time of two hours at 37°C in 5% CO₂. Next, human foreskin microvascular endothelial cells (HMVECs) at passage 10 were resuspended in HMEC medium, and seeded at confluent density on fibrin matrices resulting in ~27,500 cells HMVECs/cm². After 16 hours of growth starvation in HMEC medium, the endothelial cells were stimulated with 10 ng/ml TNF-α (Sigma-Aldrich) and 10 ng/ml bFGF (Peprotech, London, UK) to initiate tube formation. After 24 hours, the medium was replaced with medium containing TNF-α and bFGF, now in the absence or presence of IFN-β and/or CPI-1, and untreated mature CACs. Four days after stimulation, the experiment was terminated by fixing the plates with pre-warmed 2% PFA (pH 7.4) in PBS for two hours at RT. Tubular structures of HMVECs in the 3D fibrin matrix were photographed at 2.5x magnification by phase-contrast microscopy (Olympus-CK2 microscope), and the total length of tube-like structures was quantified using Optimas image analysis v. 6.5 software. Five microscopic fields per well were used to calculate the total length of the tube-like structures, expressed as mm/cm², and percentage of control conditions.

Statistical analysis
All data are presented as mean ± standard error of the mean (SEM). Statistical significances for comparisons between two conditions were performed using Student’s t-test (two-sided) or Wilcoxon paired rank test for nonparametric data. Multiple comparisons were performed using one-way ANOVA with Tukey’s multiple comparison test. Differences were considered statistically significant if probability values (P) were less than (*) 0.05, (**) 0.01, (***) 0.001, or (****) 0.0001. All experiments were performed at least in triplicate, and all statistics were performed with Graphpad Prism version 6.00 (Graphpad Software, San Diego, CA, USA).

RESULTS
Characterization of CACs
Human mononuclear cells were isolated and cultured under endothelial cell growth conditions on fibronectin-coated tissue-culture plates. After 4 days of cultivation, elongated spindle shaped cells were observed, that were double-positive for the uptake of ac-LDL and binding of Ulex-Lectin (Fig. 1A), a standard method to establish successful differentiation to CACs. Flow cytometric analysis of endothelial cell surface antigens revealed that all of the adherent cells were positive for vascular endothelial growth factor receptor 2 (VEGFR-2/
KDR), endoglin (CD105), Tie2 (TEK), and CD31 (PECAM-1) (Fig. 1B). In addition, all adherent cells expressed the pan-leukocyte marker CD45, the monocyte/macrophage marker CD14 and CD16, and stem/progenitor marker CD117 (c-kit). These data indicate that the adherent cells have both endothelial and myeloid characteristics, fully in line with previously published data.  

Figure 1. Phenotypic characteristics of CACs at day 4. CACs show double-positive staining for both ac-LDL (red) and Ulex-Lectin (green). Scale bar represents 100 μm (A). Representative histograms of the flow cytometric analysis for the expression of endothelial-, myeloid-, and stem/progenitor cell markers are shown (B). Open histograms indicate isotype control, filled histograms show specific staining by respective antibody.

**IFN-β decreases CAC numbers independent from apoptosis**

Homing of CACs to the vessel wall involves adhesion of these cells to specific extracellular matrix (ECM) proteins. One of the ECM proteins that is expressed during collateral artery growth is fibronectin. To assess whether IFN-β alters the adhesive properties of CACs to fibronectin, CAC numbers were determined during differentiation from MNCs or after full maturation, either in the presence or absence of IFN-β. The experimental setup is outlined in Fig. 2A. The number of CACs was determined by counting the ac-LDL/Ulex-lectin double-positive cells. Consistent with literature, approximately 6% of the initially seeded MNCs developed into CACs under these conditions (Supporting information A). Upon differentiation in the presence of IFN-β CAC numbers were significantly reduced, i.e. by 18±5% at 100 IU/ml, and by 64±6% at 1000 IU/ml (Fig. 2B).
Figure 2. IFN-β dose-dependently reduces CAC numbers. For studying the effect of IFN-β during differentiation, MNCs were treated with IFN-β for 72 hours, and thereafter were analyzed. For the effect of IFN-β after CAC maturation, day 3 CACs were treated with IFN-β for a time-period of 24, 48, or 72 hours (A). CAC numbers are represented as DP, indicating the number of double-positive cells for the uptake of acetylated LDL and binding of Ulex-Lectin. Shown are effects of different concentrations of IFN-β on cell numbers either during differentiation from MNCs or after maturation, at the indicated time points (B). Cell numbers are presented as percentage of untreated controls. *P < 0.05, **P < 0.01, ***P < 0.001 untreated vs. IFN-β treatment. Representative images of CACs stained for uptake of acetylated LDL (red), binding of Ulex-Lectin (green), and Hoechst (blue; cell nucleus) either untreated or IFN-β treated are depicted in C. Scale bar represents 100 μm.
When IFN-β was added after maturation of MNCs to CACs, the number of CACs was significantly reduced after 72 hours treatment with IFN-β. Here, IFN-β reduced the CAC number by 45±19% at 100 IU/ml, and by 62±19% at 1000 IU/ml (Fig. 2B). Thus, both during CAC differentiation and after maturation, a dose-dependent effect of IFN-β on the number of CACs was observed. Although IFN-β reduced the number of CACs, all remaining CACs were positive for Ulex-lectin staining and uptake of ac-LDL uptake (Fig. 2C).

As IFN-β has been shown to induce apoptosis in a number of cell types, we next investigated its pro-apoptotic potential in CACs, again both during differentiation from MNCs and after maturation. We performed FACS analysis using annexin-V and sytox blue staining to establish the percentage of dead cells in adherent and non-adherent cell fractions. Percentages of

Figure 3. Apoptosis is not responsible for the IFN-β-induced reduction of CAC numbers. FACS analysis of late apoptotic/dead cells during differentiation from MNCs and after maturation in absence and presence of IFN-β. The number of late apoptotic/dead cells are expressed as percentage of total gated cells. Results are presented as mean ± SEM from at least 3 independent experiments.
dead cells did not significantly differ between untreated and IFN-β treated CACs either during differentiation or after maturation in the adherent cell fraction. Likewise, in the non-adherent fraction, we could not detect a significant increase in the percentage of dead cells (Fig. 3). We conclude from these observations that IFN-β-induced apoptosis cannot explain the reduction in cell numbers observed upon addition of IFN-β.

**IFN-β increases integrin mRNA and protein expression levels in mature CACs**

The fact that IFN-β reduced the numbers of adherent CACs during differentiation and after maturation, prompted us to investigate the underlying mechanisms. First, we analyzed IFN-β-induced morphological and cytoskeletal changes and adhesive properties of CACs. Morphologically, CACs treated with IFN-β became round-shaped and showed a decrease in size compared to untreated CACs. This was accompanied by changes in the actin cytoskeleton (Supporting information B).

Organization of the actin cytoskeleton as well as adhesion and homing of CACs to the vessel wall is highly dependent on the expression of integrins. Specifically, integrins α4β1 and α5β1 have been reported to mediate binding of CACs to newly deposited fibronectin during vascular remodeling\(^6,25-27\). In addition, α5β1 integrin was found to be highly expressed in the vascular wall during arteriogenesis\(^28\). Therefore, we studied the effect of neutralizing antibodies against α4, α5 and β1 integrin subunits on CAC adhesion to fibronectin. α5 and β1 integrin inhibitory antibodies almost completely blocked adhesion, indicating that interaction of CACs with fibronectin is mostly dependent on the α5β1 (VLA-5) integrin (Fig. 4A).

To determine whether IFN-β affects VLA-5 integrin expression, mRNA levels of the individual α5 and β1 subunits were measured in CACs treated with IFN-β during differentiation from MNCs or after maturation. Surprisingly, gene expression of both the α5 and β1 subunit was significantly and dose-dependently increased in IFN-β treated CACs (Fig. 4B). However, this did not result in significant changes in expression levels of α5 protein and surface β1 protein during CAC differentiation, as observed by western blotting and flow cytometry (Fig. 4C), which could indicate an increased turnover of the α5β1 integrin protein by IFN-β during differentiation or a block in translation. However, in mature CACs, the increase of α5β1 gene expression by IFN-β was paralleled by an increase of α5β1 protein expression levels (Fig. 4C; Fig. 5D). These results indicate that IFN-β inhibits adhesion of mature CACs by affecting integrin activity rather than integrin protein expression. A candidate protein that is involved in the inactivation of integrins is the calcium-dependent cysteine protease calpain.
Figure 4. CAC adherence to fibronectin is dependent on VLA-5, and VLA5 levels are increased by IFN-β in mature CACs. CAC adherence to fibronectin is shown, in the presence of blocking antibodies to integrin α5 and -β1, expressed as percentage adherent CACs without additions (A). Effects of IFN-β on integrin expression are expressed at gene expression level as percentage of untreated sample by real-time RT-PCR analysis (B) and at the protein level as fold change relative to untreated sample, by Western blot and FACS analysis, shown in panel C. Dotted line, open/grey/black histograms in the FACS analysis indicate isotype control, untreated, 100 IU/ml and 1000 IU/ml IFN-β respectively. Results are presented as mean ± SEM from at least 3 independent experiments. *P < 0.05, **P < 0.01 isotype control vs. neutralizing antibody, untreated vs. IFN-β treatment.

**IFN-β induces detachment of mature CACs through activation of calpain 1**

The calpain family of proteases has been described in cellular processes as cell adhesion, migration, and apoptosis\textsuperscript{29,30}. Active calpains may induce integrin inactivation through destabilization of integrin-ligand and integrin-cytoskeletal interactions involving cleavage of β integrins, cytoskeletal and signaling proteins, leading to cell detachment\textsuperscript{31}. We tested the hypothesis that the inhibitory effect of IFN-β on CAC adhesion to fibronectin is mediated by calpain 1. IFN-β stimulated the expression of calpain 1 transcript levels significantly and dose-dependently in mature CACs, but not during differentiation of MNCs to CACs. Calpain 2 was not significantly induced, neither during differentiation nor after maturation (Fig. 5A). Conversely, the increase of calpain 1 gene expression by IFN-β was not paralleled by a
concomitant increase of calpain 1 protein in mature CACs by western blotting (Fig. 5B), which prompted us to study calpain 1 activity in mature CACs. Interestingly, mature CACs treated with IFN-β exhibited a significant and dose-dependent increase in calpain activity, relative to untreated control, as measured with a fluorescent calpain substrate assay. Furthermore, calpain 1 inhibitor (CPI-1) at 1 µM significantly decreased calpain activity by ≥60%, relative to vehicle controls (Fig. 5B). These findings suggest that calpain 1 activity is involved in reduced adhesion of mature CACs by IFN-β. To confirm this, the influence of IFN-β on CAC numbers was determined in the presence or absence of CPI-1 at 1 µM. Indeed, inhibition of calpain 1 activity by calpain 1 inhibitor in IFN-β-treated mature CACs led to a significant increase in CAC numbers, whereas inhibition during differentiation in the absence or presence of IFN-β did not significantly alter CAC numbers (Fig. 5C). The activity of calpain 1 was not affecting α5β1 integrin protein expression in mature CACs (Fig. 5D), strongly suggesting that IFN-β-induced calpain 1 activity influences α5β1 integrin activity but not protein turnover. Morphologically, cells appeared more elongated under conditions of calpain 1 inhibition in mature CACs as observed by time-lapse video microscopy (Supporting information C and supplementary video files 1 and 3), suggesting a more efficient spreading and adhesion. When mature CACs were treated with IFN-β, this led to rounding and detachment of the CACs. This deleterious effect of IFN-β was completely absent in the presence of the calpain 1 inhibitor (Supporting information C and supplementary video files 2 and 4).

Taken together, these results indicate that the inhibitory effect of IFN-β on adhesion of mature CACs is mediated by calpain 1.

**IFN-β attenuates the stimulatory effects of mature CACs on endothelial tube formation in vitro, which cannot be reversed by calpain 1 inhibition**

Next, we assessed the effect of IFN-β on function of mature CACs in an in vitro endothelial capillary tube formation assay. First, the capacity of various numbers of CACs to promote capillary formation by HMVECs in the presence of bFGF and TNF-α was investigated in our established model system using HMVECs and fibrin matrix. CACs indeed dose-dependently increased the capillary tube formation with optimal effects observed at 5% CACs relative to the number of HMVEC (Fig. 6A).

Next, the effects of 5% CACs on the capillary tube formation by HMVECs under pro-angiogenic stimulation with bFGF and TNF-α and in the absence or presence of IFN-β was studied. To that end, we added equal numbers of control and IFN-β treated CACs to the tube forming assay, in the absence of IFN-β, which showed equally stimulatory effects (data not shown). When control CACs were added in the presence of IFN-β in the assay, however, the promoting effect of CACs on capillary tube formation in vitro was significantly diminished (Fig. 6B and C). Thus, we observed in this model that CACs significantly enhance endothelial cell tube formation and that continuous presence of IFN-β abrogates this promoting effect of CACs.
Chapter 3

A

During differentiation

CAPN1

CAPN2

relative mRNA expression (% of control)

U/ml IFN-β

0  50  100  150

-  100  1000

relative mRNA expression (% of control)

U/ml IFN-β

0  25  50  75  100

-  100  1000

After maturation

CAPN1

CAPN2

relative mRNA expression (% of control)

U/ml IFN-β

0  50  100  150

-  100  1000

relative mRNA expression (% of control)

U/ml IFN-β

0  25  50  75  100

-  100  1000

B

After mat.

Vehicle

CPI-1

U/ml IFN-β

-  100  1000

calpain 1

α-tubulin

fold change calpain 1 protein expression

U/ml IFN-β

-  100  1000

Relative Calpain Activity (%)

U/ml IFN-β

-  100  1000

After maturation

Vehicle

CPI-1

C

During differentiation

Number of DP-cells/mm² (% of control)

U/ml IFN-β

-  100  1000

After maturation

Number of DP-cells/mm² (% of control)

U/ml IFN-β

-  100  1000

none

Vehicle

CPI-1
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Figure 5. IFN-β reduces adhesion of mature CACs to fibronectin by inducing calpain 1 activity. Effects of IFN-β on the gene expression levels of CAPN1 and CAPN2 both during differentiation and after maturation are expressed as percentage of untreated CACs (A). Results are presented as mean ± SEM from at least 3 independent experiments. *P < 0.05 untreated vs. IFN-β treatment. Effect of IFN-β on the calpain 1 protein expression level after maturation measured by a western blot are expressed as fold change relative to untreated sample (B; left). Results are presented as mean ± SEM from at least 3 independent experiments. Calpain activity assay in mature CACs (B; right). IFN-β-induced calpain activity is inhibited by calpain 1 inhibitor at 1 μM. Results are presented as mean ± SEM from at least 3 independent experiments. **P < 0.01, ***P < 0.001 untreated vs. IFN-β treatment; ****P < 0.0001 vehicle (0.05% DMSO) vs. CPI-1. Effect of inhibition of IFN-β-induced calpain 1 activity by 1 μM CPI-1 on CAC number is indicated by DP as number of double-positive cells for the uptake of acetylated LDL and binding of Ulex-Lectin and expressed as percentage of controls (C). Results are presented as mean ± SEM from at least 3 independent experiments. *P < 0.05, **P < 0.01 vehicle (0.05% DMSO) vs. CPI-1. Effects of IFN-β (100 and 1000 IU/ml) and calpain 1 inhibitor (1 μM) on the α5β1 integrin protein expression levels after maturation in a western blot are expressed as fold change relative to vehicle untreated sample. Results are presented as mean ± SEM from at least 3 independent experiments. *P < 0.05, **P < 0.01 untreated vs. IFN-β treatment (D).

The role of calpain in angiogenesis has been shown in several studies. To explore the importance of calpain 1 inactivation in vascular repair, we assessed the consequences of calpain 1 inactivation on the formation of capillary-like structures in vitro, through direct effects on either CACs or endothelial cells. Therefore, we first exposed HMVECs to the indicated concentrations of calpain 1 inhibitor in the absence or presence of IFN-β. Calpain 1 inactivation significantly enhanced endothelial cell tube formation in the absence of CACs, whereas calpain 1 inactivation did not show this effect in the presence of IFN-β. The maximal effective concentration of CPI-1 to stimulate endothelial cell tube formation was 1 μM. (Fig. 6D and E). To evaluate the effect of calpain 1 inactivation on CAC-induced endothelial cell tube formation, we added calpain 1 inhibitor to HMVECs and CACs.
Chapter 3

**A**

![Graph showing tube formation](image)

**B**

![Graph showing tube formation](image)

**C**

![Images comparing - CACs and + CACs](image)

**D**

![Images showing different treatments](image)

**E**

![Graph showing tube formation with different treatments](image)
IFN-β affects the angiogenic potential of CACs by calpain 1 activation

Figure 6. Effect of CACs, IFN-β and calpain 1 inhibition on capillary tube formation in vitro. CACs dose-dependently increase capillary-like structures in vitro with the highest increase at 5% (A). The concentration of CACs is expressed as a percentage of confluent seeded HMVECs. Results are expressed as percentage of control, and as mean ± SEM from at least 3 independent experiments. *P < 0.05, ***P < 0.001 without CACs vs. 0.1%, 1%, 5%, or 10% CACs. CACs at 5% in the presence of IFN-β during the tube formation assay loose their stimulatory activity on the capillary formation (B). Note a minor effect of IFN-β on endothelial cells in the absence of CACs. Results are expressed as percentage of control, as mean ± SEM from at least 3 independent experiments. **P <0.01, untreated vs. IFN-β treatment, without CACs vs. with CACs. Representative images of capillary tube formation are shown in C. Calpain 1 inhibition increases endothelial cell tube formation in the absence of CACs (D and E). Furthermore, calpain 1 inhibition does not restore IFN-β-affected endothelial cell tube formation neither in the absence nor in the presence of CACs. Representative images of capillary tube formation are shown (D). Results are expressed as percentage of control, and as mean ± SEM from at least 3 independent experiments (E). *P < 0.05 vehicle (0.05% DMSO) vs. CPI-1, without CACs vs. with CACs. #P < 0.05, indicates the effect of IFN-β treatment vs. the same experimental condition without IFN-β. Scale bars represent 200 μm.

In the presence of the calpain 1 inhibitor, CACs only slightly increased endothelial cell tube formation (not significant). We next addressed whether calpain 1 inactivation could rescue the IFN-β-induced CAC dysfunction in endothelial cell tube formation. To this aim, we added calpain 1 inhibitor to HMVECs and CACs in the presence of IFN-β. Remarkably, calpain 1 inactivation did not reduce the IFN-β-induced CAC dysfunction in endothelial cell tube formation (Fig. 6E). These results suggest that the major effect of calpain 1 thus seems to be the reduction of the numbers of mature CACs, whereas after addition of normalized numbers of CACs to the angiogenesis assay, their functionality is not affected by CPI-1.

DISCUSSION

Transplantation of CACs after in vitro conditioning has been shown to improve neovascularization in a murine model of hind limb ischemia4,5. Given the in vivo inhibitory effect of IFN-β on both angiogenesis and arteriogenesis, we now investigated the effect of IFN-β on CACs, and the possible involvement of calpain 1 in vitro. We show that IFN-β reduces CAC numbers both during their differentiation from MNCs and after their maturation. This reduction of CACs is not caused by apoptosis but by interference with adhesion of the CACs to fibronectin. This results in rearrangement of the actin cytoskeleton, leading to rounding and detachment of the cells. The reduction in numbers of mature CACs can be completely abolished by the calpain 1 inhibitor CPI-1. As calpain 1 activity is induced by IFN-β in mature CACs, our data indicates a direct involvement of calpain 1 in the adverse effects of IFN-β on CAC-matrix adhesion.

During angiogenesis and arteriogenesis the expression of fibronectin is highly increased24,38, and blocking of fibronectin strongly impairs angiogenesis39. The integrin receptor responsible for adhesion of endothelial progenitor cells to fibronectin is α5β1 (VLA-5) integrin27. In accordance with these previous findings, we show that adhesion of CACs to
fibronectin is mediated by VLA-5. Since IFN-β reduced CAC adhesion, we examined whether this might be ascribed to reduced expression of VLA-5. Gene expression of both α5 and β1 subunits was significantly increased by IFN-β in a dose-dependent manner both during CAC differentiation and after maturation. However, at the protein level no significant changes in α5 and β1 integrin expression were detected during CAC differentiation, indicating an increased turnover of the protein, potentially by increased degradation, which might affect CAC adhesion. Conversely, mature CACs showed increased α5 and β1 integrin protein expression, which suggest that reduction of mature CAC adhesion by IFN-β was not attributable to the expression levels of VLA-5 per se, but rather to a reduction of VLA-5 functional activity.

Integrins require a conformational change to increase the affinity for their ligand. This conformational change is regulated by binding of integrin activators, such as talin, to the cytoplasmic tail of integrins. Subsequently, a focal adhesion complex is formed with integrin clustering and binding of talins to the F-actin fibers of the cytoskeleton. The calpains, a family of Ca2+-dependent cysteine proteases, play a crucial role in cell adhesion, migration and apoptosis in certain experimental models. Calpains may induce integrin inactivation by destabilization of integrin-ligand and integrin-cytoskeletal bonds mediated by cleavage of β integrins, cytoskeletal- and signaling proteins, leading to cell detachment. Many knockout and enzyme inhibition studies support these findings. Pharmacological inhibition of calpain reduces adhesive complex disassembly and reduces cell detachment during migration, integrin release, and the migration rate. Calpain inhibition in CHO cells suppresses both α5β1 and αIIbβ3 integrin-mediated cell migration, and increases adhesion on fibronectin and fibrinogen. The calpain-mediated cleavage of β3 integrin inhibits spreading of CHO cells on fibrinogen, and tyrosine phosphorylation of β3 integrin at its cytoplasmic tail induces resistance to calpain cleavage, leading to enhanced cell spreading. Calpain 1 cleaves RhoA in bovine aortic endothelial cells (BAEC) and CHO cells plated on fibronectin, generating a dominant-negative fragment that inhibits integrin-induced stress fiber formation and cell spreading. For integrin-mediated adhesion of monocytes, phosphorylation of the actin-bundling protein L-plastin is required. The integrin β1-L-plastin-actin complex is dissociated by calpain 1, which may be relevant for monocyte-derived CAC adhesion as well. In our model system, calpain 1 inhibition (≥60%) at a concentration of 1 µM CPI-1 indeed enhanced cell spreading, and significantly increased adhesion of mature CACs in the presence of IFN-β, whereas inhibition during differentiation did not result in increased adhesion. Furthermore, calpain 1 inhibition did not increase α5β1 protein expression levels (Fig. 5D), which suggests no role of calpain 1 in α5β1 protein turnover. The specific mechanisms by which IFN-β-induced calpain 1 activity interferes with adhesive capacity of mature CACs still needs to be elucidated. However, our data suggest that these mechanism result in reduced functional activity of VLA-5 integrin.
On the other hand, we observed that high concentrations of calpain 1 inhibitor were toxic for CACs (5 and 10 μM; data not shown), which was also consistent with a previous report that concentrations between 5-10 μM lead to apoptosis and may be linked to impairment of the activity of the proteasome\textsuperscript{47}. High concentrations of calpain 1 inhibitor induces accumulation of proteasome substrates including p53, E6AP, c-Jun, and cyclin D1\textsuperscript{48}. We have now shown the relevance of calpain 1 inhibition in an endothelial cell tube formation assay \textit{in vitro}. The capability of differentiated CACs to promote capillary-like tube formation was evaluated. We show an increased endothelial cell tube formation when CACs are added at numbers of 5% of confluently seeded endothelial cells. IFN-β had a slight inhibitory effect on endothelial cell tube formation in the absence of CACs, and completely abrogated the positive effect of CACs on endothelial cell tube formation. The adhesion of endothelial cells to fibrin(ogen) matrices is mediated by α5β1 and αvβ3 integrins, and both integrins have been shown to stimulate angiogenesis \textit{in vitro}\textsuperscript{49,50}. Calpain cleaves the cytoplasmic domain of β1 and β3 integrins\textsuperscript{51}, leading to inactivation. Calpain also cleaves the cytoplasmic domain of αvβ3 integrins in vascular endothelial cells, leading to reduced adhesiveness\textsuperscript{52}. Given the role of calpain in integrin cleavage, we suggest that calpain 1 inactivation in endothelial cells probably leads to enhanced VLA-5 and αvβ3 integrin activation, enhancing endothelial cell-matrix adhesion and thus tube formation. Our results show that calpain 1 inhibition significantly increased tube formation by endothelial cells in the absence of CACs, but could not reverse the negative effects of IFN-β on tube formation. This suggests that inhibitory effects of IFN-β on endothelial cell tube formation are independent of calpain 1 activity and are likely dependent on other factors. A role of calpain in angiogenesis has been confirmed in several studies, but with contradictory results. Some studies show that calpain activity is required for angiogenesis and endothelial cell motility in response to VEGF, which has been shown to induce calpain activity in endothelial cells\textsuperscript{37}. On the other hand, calpain inhibition has been reported to suppress angiogenesis \textit{in vivo} and to block capillary morphogenesis \textit{in vitro}\textsuperscript{32,37}. However, the used concentrations of calpain inhibitors in these studies were very high, pointing to toxicity as observed by us. Others have reported that moderate calpain inhibition prevents architectural and functional abnormalities, and reduces retinal hypoxia in an established model of ischemic retinopathy. Furthermore, restored organization of the actin cytoskeleton in retinal endothelial cells during capillary morphogenesis \textit{in vitro}, and neovascularization \textit{in vivo} was observed\textsuperscript{34}. Moderate inhibition of calpain 1 by retroviral transduction of endothelial cells with dominant negative (DN) calpain 1 also stimulates neovascular integration, lumen formation and perfusion during VEGF-induced pathological angiogenesis. Similarly, improved VEGF-driven angiogenesis was also observed with moderate calpain 1 inhibition using calpain 1 inhibitor\textsuperscript{33}. Calpain 1 knockdown has been shown to increase adhesion, migration, and cord formation by lymphatic endothelial cells on matrigel, whereas calpain 2 knockdown decreases these processes. The different
functions of both calpains were ascribed to opposite roles in eNOS regulation\textsuperscript{36}. The lack of effect we observed for calpain 1 inhibition on CAC-induced endothelial cell tube formation is consistent with reports that showed that CACs adhere to endothelial cells mainly by β2 integrin, which is important for homing and neovascularization capacity of CACs \textit{in vivo}\textsuperscript{25}. This suggests no role for calpain 1 in the β2 integrin mediated adhesion of CACs to endothelial cells and tube formation. Our results indicate that the IFN-β-mediated reduction of endothelial cell tube formation stimulated by CACs is independent of calpain 1 activation and may involve downregulation of paracrine angiogenic factors by CACs, or a direct effect on endothelial cells. The paracrine function of CACs in enhancing endothelial cell tube formation has been reported elsewhere\textsuperscript{11,12}. Which paracrine angiogenic factors may be affected by IFN-β remains to be elucidated.

In conclusion, our results show that IFN-β plays a crucial role in determining the number of pro-angiogenic CACs through induction of calpain 1 activity, thereby inhibiting their VLA-5-dependent adhesion, resulting in less mature CACs. Furthermore, IFN-β plays a vital role as modulator of angiogenesis \textit{in vitro}. Yet, although calpain 1 inhibition improves adhesion of CACs to the extracellular matrix, it does not restore the full inhibitory effect of IFN-β on endothelial sprout formation \textit{in vitro}. During angiogenesis and arteriogenesis \textit{in vivo}, calpain 1 inhibition might therefore improve the adhesion and thereby numbers of CACs in the neovascularization area, but it will not restore the influence of IFN-β on endothelial cells.

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REFERENCES


**SUPPORTING INFORMATION**

**Supporting information A.** The percentage of differentiated CACs from totally seeded MNCs, and the inhibitory effect of IFN-β on CAC numbers. DP indicates the number of double-positive cells for the uptake of acetylated LDL and binding of Ulex-Lectin. Results are presented as mean ± SEM from at least 3 independent experiments. **P < 0.01, ***P < 0.001 untreated vs. IFN-β treatment.

**Supporting information B.** IFN-β affects CAC adhesion by inducing changes in the CAC morphology. The reduced CAC number both during and after maturation might be attributed to the fact that CACs have lost their spindle-shape by IFN-β, suggestive for reduced adhesion (A). Filamentous F-actin structures are shown, either during differentiation from MNCs or after maturation. Representative images from three independent experiments are shown. Scale bar represent 25 μm.
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Supporting information C. Video time-lapse recording shows effects of IFN-β and calpain 1 inhibition (CPI-1; 1 μM) in CACs after maturation. Representative video images are shown. Scale bar represents 100 μm.

Supplementary Video Files 1-4.
Time laps Video files of mature CACs treated with: 1 vehicle; 2 IFN-β (100 IU/ml); 3 CPI-1 (1 μM); 4 IFN-β (100 IU/ml) and CPI-1 (1 μM). Cells were recorded for 48 hours, at a 20 times magnification (not shown).