



## Original Articles

## Acetyl-L-carnitine is an anti-angiogenic agent targeting the VEGFR2 and CXCR4 pathways



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## ABSTRACT

Carnitines play an important role in the energy exchange in cells, and are involved in the transport of fatty acids across the inner mitochondrial membrane. L-Acetylcarnitine (ALCAR) is an acetic acid ester of carnitine that has higher bioavailability and is considered a fat-burning energizer supplement. We previously found that in serum samples from prostate cancer (PCa) patients, 3 carnitine family members were significantly decreased, suggesting a potential protective role of carnitine against PCa. Several studies support beneficial effects of carnitines on cancer, no study has investigated the activities of carnitine on tumor angiogenesis.

We examined whether ALCAR acts as an "angiopreventive" compound and studied the molecular mechanisms involved. We found that ALCAR was able to limit inflammatory angiogenesis by reducing stimulated endothelial cell and macrophage infiltration *in vitro* and *in vivo*. Molecularly, we show that ALCAR downregulates VEGF, VEGFR2, CXCL12, CXCR4 and FAK pathways. ALCAR blocked the activation of NF-κB and ICAM-1 and reduced the adhesion of a monocyte cell line to endothelial cells. This is the first study showing that ALCAR has anti-angiogenic and anti-inflammatory properties and might be an attractive candidate for cancer angioprevention.

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## 1. Introduction

Angiogenesis is a process characterized by the formation of new blood vessels from pre-existing ones, acting as a crucial vascular orchestrator both in physiological and pathological conditions [22,24,41,82]. A strict physiological balance between endogenous pro-angiogenic and anti-angiogenic factors regulate the endothelial cell growth and angiogenesis [29]. Induction of aberrant angiogenesis represents a shared hallmark in several chronic inflammatory diseases, such as diabetes, cardiovascular diseases, metabolic syndrome and cancer [22]. Tumor angiogenesis is essential for oxygen and nutrient delivery to growing tumors, and provides a roadmap to disseminate to distant organs [22,29]. Increased attention has been addressed to approaches aimed at preventing cancer by suppressing angiogenesis, leading to the

concept of angioprevention [6]. Current clinically employed anti-angiogenic agents target the vascular endothelial growth factor (VEGF) pathway [47]. However, most of clinically employed anti-angiogenic drugs are only effective in a subset of patients, usually relapse occurs, and they are not without toxicities [80]. Therefore, the identification of new anti-angiogenic compounds which could overcome these drawbacks are urgently needed. During the last decades, great efforts have been addressed to diverse diet derived compounds (nutraceuticals), that have been explored for their ability to prevent or slow down cancer, given their anti-proliferative, anti-inflammatory, anti-oxidant and pro apoptotic activities [1,6,10,13,46,54,62,75–77,81]. Many of these agents also have been observed to block tumor progression by inhibiting angiogenesis [6,25,26,74,78]. Major features of these agents are represented by their low toxicity, and high tolerability over long term administration.

Carnitine ( $\beta$ -hydroxy- $\gamma$ -N-trimethylaminobutyric acid) is a naturally occurring quaternary ammonium compound and its derivatives acetyl-L-carnitine and propionyl-L-carnitine, are essential

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## List of abbreviations

ALCAR	Acetyl – l – Carnitine	PCa	Prostate Cancer
ANGPT1	Angiopoietin-1	PECAM-1	Platelet and Endothelial Cell Adhesion Molecule-1
BPH	Benign Prostatic Hyperplasia	ERK 1/2	Extracellular signal – Regulated protein Kinases 1 and 2
CPT1A	Carnitine Palmitoyl Transferase 1	PLC $\gamma$ 1	Phospholipase C, gamma 1
CPT1C	Carnitine O – Palmitoyl Transferase 1	FAK	Focal Adhesion Kinase
CXCR4	C-X-C chemokine receptor type 4	Src	Proto-Oncogene Tyrosine – Protein Kinase
FAO	Fatty Acid Oxidation	SACI	Surface – Activated Chemical Ionization
FGF2	Fibroblast Growth Factor 2	SANIST	Rapid mass spectrometric SACI/ESI data acquisition and elaboration platform
Hif-1 $\alpha$	Hypoxia-inducible Factor 1-alpha	CXCL12	SDF-1; Stromal Cell-Derived Factor-1
HUVECs	Human Umbilical Vein Endothelial Cells	THP-1	Leukemic Monocyte
ICAM-1	Intracellular Cell Adhesion Molecule – 1	TNF- $\alpha$	Tumor Necrosis Factor $\alpha$
CCL2	MCP-1; Monocyte Chemoattractant Protein-1	VCAM-1	Vascular Cell Adhesion Molecule – 1
NF- $\kappa$ B	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells	VEGF	Vascular Endothelial Growth Factor
p-38 MAPK	Cytokinin Specific Binding Protein	VEGFR2	Vascular Endothelial Growth Factor Receptor 2

for lipid energy metabolism within the mitochondria, contributing to the transport of long-chain acyl-CoA into the mitochondrial matrix where the enzymes for  $\beta$ -oxidation are located. Other roles of carnitine include buffering of branched-chain amino acids metabolism, removal of excess acyl groups, and peroxisomal fatty acid oxidation [18,32]. Carnitine deficiency has been observed in diverse disorders, such as diabetes, sepsis, cardiomyopathy, malnutrition, cirrhosis, endocrine disorders and those related with aging [32]. Low plasma carnitine levels have been found in cancer patients, ascribed to malnutrition [64]. Clinically, L-carnitine (LC) and its derivatives (acetyl-LC; propionyl-LC) are under study to combat wasting and chemotherapy-induced peripheral neuropathy [21,68].

Through metabolomics approaches we had previously found that in serum samples from PCa patients, 3 molecules from the carnitine family (decanoyl-L-Carnitine, octanoyl-L-carnitine and 5-cis-tetradecenoyl carnitine) were significantly decreased, compared to those from individuals with BPH, suggesting a potential protective role of carnitine against PCa [3]. Carnitine supplementation in several experimental models has been shown to slow down tumor growth by inhibiting histone deacetylases (HDAC) [39]. Further inhibition of carnitine palmitoyltransferases (CPT1A and CPT1C) also results in inhibition of tumor growth [55,67,73,79]. Obese mice consuming curcumin, a known angiogenesis inhibitor, showed enhanced carnitine CPT1 activity [28]. In addition carnitine showed a synergism with curcumin in a colon cancer model [56].

Considering the key role of fatty acid oxidation (FAO) as an important regulator of angiogenesis [69,79], we investigated whether carnitines may exert anti-angiogenic and angiopreventive properties *in vitro* and *in vivo*, and the potential molecular pathways involved. We focused our experiments on the acetylated form of L-carnitine, acetyl-L-carnitine (ALCAR), given that it has higher bioavailability than L-carnitine [66]. ALCAR is a component of several supplement formulations and is largely available on the nutraceutical market.

Angiogenesis and inflammation are two host-derived hallmarks of cancer that are linked together [36]. Here, we investigated for the first time whether ALCAR targets inflammatory angiogenesis by limiting key functional activities on cytokine-activated human umbilical vein endothelial cells (HUVEC) in normoxic, hypoxic and inflammatory environments. ALCAR significantly inhibited angiogenesis, it downregulated VEGF and VEGFR2, together with key down-stream protein kinases, such as pTyr397-FAK, pTyr416-Src,

p-38 MAPK, and p-Ser1248-PLC $\gamma$ 1. Within the tumor microenvironment, the interaction of CXCL12 with its receptors represents a potential target in tumor angiogenesis [42]. We found that ALCAR affected migration and invasion of endothelial cells, and inhibited the CXCL12/CXCR4 axis. We also found that ALCAR reduced the TNF- $\alpha$ -induced adhesion of macrophages (THP-1) to an endothelial cell monolayer and inhibited inflammatory angiogenesis by hindering NF- $\kappa$ B activation and reduced the expression of ICAM-1. These results were consistent with *in vivo* data in the matrigel sponge assay, where we showed an inhibition of angiogenesis and inflammation by a substantial and significant lowering of endothelial cells and macrophage recruitment into the matrigel plugs. Our results identified cellular and molecular mechanisms related with ALCAR anti-angiogenic and angiopreventive properties and provide the rational for the employment of ALCAR as supplement for approaches of interception and prevention of cancer.

## 2. Materials and methods

### 2.1. Chemicals and reagents

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), acetyl-L-Carnitine (ALCAR) were from Sigma Aldrich.

### 2.2. Cell culture

HUVE cells (human umbilical vein endothelial cells) were from Promocell and cultured in endothelial cell basal medium (EBM<sup>TM</sup>, Lonza) supplemented with endothelial cell growth medium (EGM<sup>TM</sup>SingleQuots<sup>TM</sup>, Lonza), 10% Fetal Bovine Serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin and were used between the 3–5 passage. MRC-5 (PD 30) cell line from Sigma were cultured on EMEM (EBSS) supplemented with 10% FBS, 2 mM L-glutamine, 1% non-essential amino acids, 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin. The human monocytic cell line (THP-1) from ATCC were cultured in cell suspension in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 0.5 mM  $\beta$ -mercaptoethanol, 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin. Peripheral blood mononuclear cells (PBMCs) were obtained by whole blood samples subjected to a density gradient stratification and maintained at 10<sup>6</sup> cells/mL density in RPMI 1640 supplemented with 10% FBS 2 mM L-glutamine, 100 UI/mL recombinant human IL2 (R&D), 100 U/ml penicillin and 100  $\mu$ g/mL streptomycin.

### 2.3. Functional *in vitro* angiogenesis assays

#### 2.3.1. Tube formation

The effects of ALCAR on endothelial cell ability to form capillary-like structures on basement membrane matrix was assessed *in vitro*, using the morphogenesis assay. HUVE cells were grown in EBM2 complete medium and when 80% confluent were serum starved overnight. A 24-well plate, pre-chilled at  $-20^{\circ}\text{C}$ , was carefully filled with 300  $\mu\text{L}$  of liquid matrigel (BD Biosciences) per well at  $4^{\circ}\text{C}$  and then polymerized for 1 h at  $37^{\circ}\text{C}$ .  $5 \times 10^4$  of HUVE cells/well were suspended in 1 mL of EBM2 medium containing 100 ng/mL VEGF and 100 ng/mL FGF2 alone or with 1–10 mM ALCAR, and layered on the top of the polymerized matrigel. Positive and negative controls received 10% FBS or serum free EBM2 medium, respectively. The effects on HUVEC tube formation were captured after 6 h incubation using a Zeiss Microscope associated with a Nikon camera (Axio Observer A1, Zeiss, Germany) and quantified using ImageJ software and the “Angiogenesis Analyzer” tool. Experiments were performed on HUVE cells either at basal level or activated with TNF- $\alpha$  (10 ng/ml), or incubated in a hypoxic chamber (Eppendorf, Germany) at an atmosphere of 1% O<sub>2</sub>, 94% N<sub>2</sub>, 5% CO<sub>2</sub> at  $37^{\circ}\text{C}$  for 72 h, while the control cells were incubated in an atmosphere of 21% O<sub>2</sub> and 5% CO<sub>2</sub> at  $37^{\circ}\text{C}$ .

#### 2.3.2. Adhesion

HUVE cells were pre-treated with 1 or 10 mM ALCAR for 24 h in complete medium. Control cells received complete medium alone. Following treatment,  $3 \times 10^3$  cells were seeded on 4-well chamber slides, pre-coated with 2  $\mu\text{g}/\text{ml}$  fibronectin (Sigma Aldrich), for 45 min at  $37^{\circ}\text{C}$ , 5% CO<sub>2</sub>. Following 90 min of incubation, medium was removed, cells were washed with PBS and fixed with 4% paraformaldehyde (PFA) and stained with DAPI (Sigma Aldrich). Assays were performed in triplicates. Cells within five blinded fields for each condition were counted using a Zeiss microscope. Experiments were performed on HUVE cells either at basal level or activated with TNF- $\alpha$  (10 ng/ml), as indicated.

#### 2.3.3. Migration and invasion

A modified Boyden Chamber, as described in Refs. [2,5] was used to perform migration and invasion assays. HUVE cells were pre-treated with 1 or 10 mM ALCAR for 24 h. 10  $\mu\text{m}$  pore-size polycarbonate filters, pre-coated with matrigel (1 mg/ml, BD) for chemoinvasion assay and with collagen IV (50  $\mu\text{g}/\text{ml}$ , Sigma Aldrich) for chemotaxis assay [2,5] were used as the interface between the two chamber compartments. Following 6 h (chemotaxis) or 24 h (chemoinvasion) of incubation at  $37^{\circ}\text{C}$  in 5% CO<sub>2</sub>, the filters were collected, cells on the upper surface mechanically removed using a cotton swab and migrated or invaded cells on the lower filter surface were fixed with absolute ethanol and stained with DAPI. Migrated/invaded cells were counted in a double-blind manner in 5 consecutive fields using a Zeiss Microscope associated with a Nikon camera (Axio Observer A1, Zeiss, Germany).

### 2.4. Real time PCR

Total RNA was extracted using small RNA miRNeasy Mini Kit (Qiagen) and quantified in a Nanodrop Spectrophotometer. Reverse transcription was performed using SuperScript VILO cDNA synthesis kit (Thermo Fisher). Real-time PCR was performed using SYBR Green Master Mix (Applied Biosystems) on QuantStudio 6 Flex Real-Time PCR System Software (Applied Biosystems). All reactions were performed in triplicate. The relative gene expression was expressed relative to non-treated cells normalized to the housekeeping gene. Gene expression analysis was performed using

the primers shown in Supplementary Table 1. Experiments were performed on HUVE cells either at basal level or activated with TNF- $\alpha$  (10 ng/ml), as indicated.

### 2.5. Western blotting

Cells were lysed in RIPA buffer containing protease and phosphatase inhibitor cocktails (Roche Diagnostics GmbH, Mannheim, Germany). Protein concentration was determined by Bradford assay using bovine serum albumin (BSA; Sigma-Aldrich) as standard. Proteins (25  $\mu\text{g}$ ) were separated on the NuPageNovex 10% Bis-Tris gel or on 4–12% Bis-Tris Gel (Life Technologies) and then transferred to a PVDF membrane (Amersham Hybond). The membrane was blocked with 5% (v/v) non-fat dry milk in Tween 20 (Sigma-Aldrich) in PBS for 1 h and then incubated overnight at  $4^{\circ}\text{C}$  with the following primary antibodies: anti-VEGFR2, anti-HIF-1 $\alpha$ , anti-NF- $\kappa$ B p65, anti-p-38 MAPK, anti-p-Src, anti-FAK, anti-p-PLC $\gamma$ 1 (all from Cell Signaling Technology), anti-VEGF (Santa Cruz), anti-ICAM-1 and anti-VCAM-1 (Abcam).

After a triple wash with 0.1% (v/v) PBS/Tween, membranes were incubated with the secondary antibody horseradish-peroxidase-linked anti-rabbit IgG or anti-mouse IgG (GE Healthcare Life science) diluted at 1:3000 for 1 h at room temperature. Specific protein bands were detected with Pierce ECL Western Blotting Substrate (ThermoFisher Scientific). Protein expressions were normalized to beta-Actin at 1:5000 (Abcam). Western-blot data were analyzed using ImageJ software to determine optical density (OD) of the bands. Experiments were performed on HUVEC cells either at basal level or activated with TNF- $\alpha$  (10 ng/ml) as specifically indicated.

### 2.6. Flow cytometry for cytokine detection

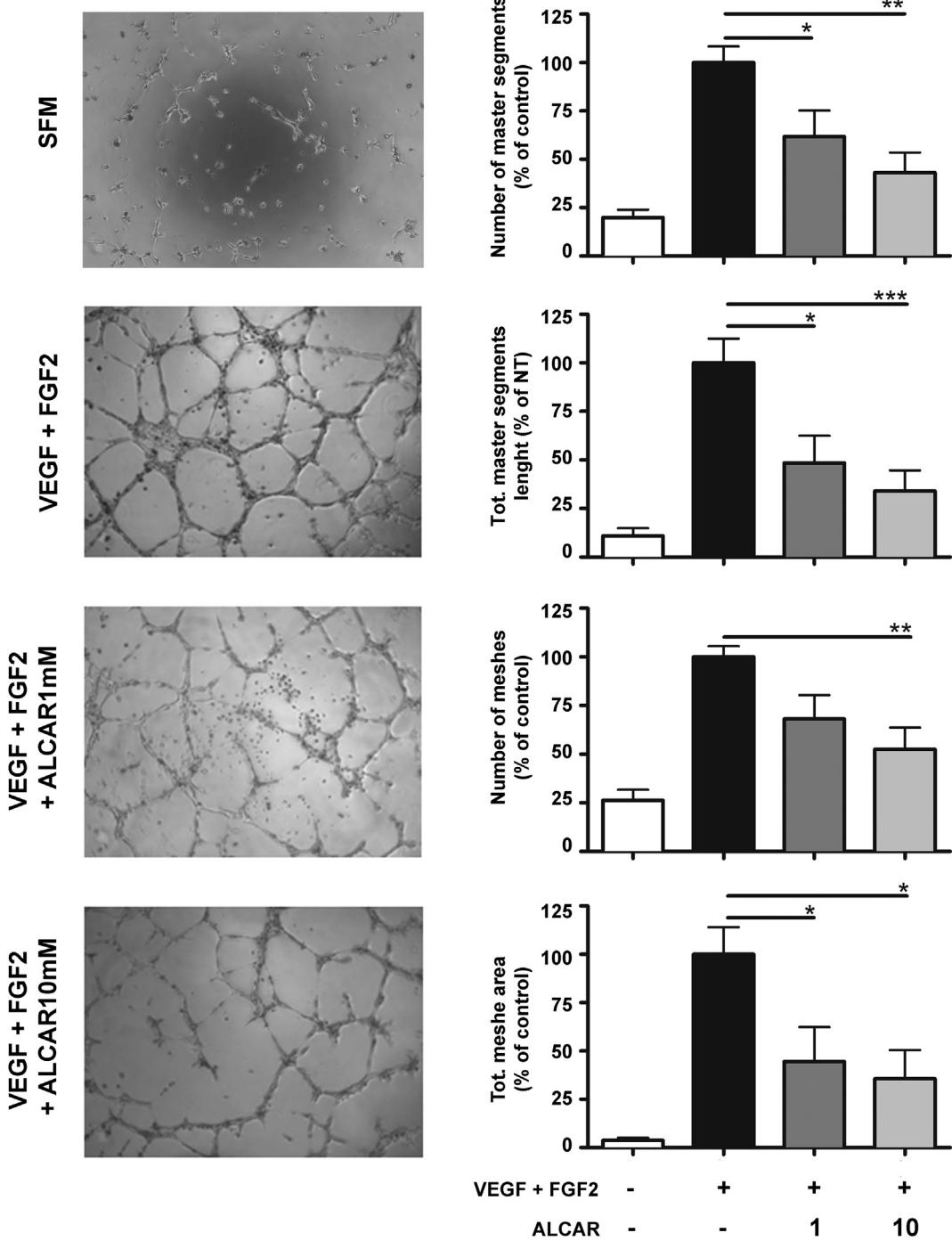
The effects of ALCAR on the modulation of selected surface antigens (VEGFR2, CXCR4, PECAM-1) and cytokine release (VEGF, CCL2, CXCL12, Angiopoietin-1) was investigated by flow cytometry. HUVE cells where treated with ALCAR (1 or 10 mM) for 24 h. Following treatment, cells ( $3 \times 10^5$  per FACS tube) were detached and stained for surface antigens with the following PE conjugated mabs: anti-human VEGFR2/KDR (Clone #89106, R&D Systems) or CD184/CXCR4 (Clone #12G5, BD Pharmingen) for 30 min at  $4^{\circ}\text{C}$ . For cytokine detection, pre-surface antigen stained cells were fixed and permeabilized using the CytoFix/Cytoperm kit (BD), accordingly to manufacturer, and stained with the following PE-conjugated Mabs: anti-human VEGF (Clone #23410, R&D Systems), CXCL12 (Clone #79018, R&D Systems); CCL2 (Clone #RE4248, Miltenyi Biotec). For Angiopoietin-1 detection, following primary antibodies incubation and washing, the anti-rabbit PE-conjugated secondary antibody (R&D Systems) was added. Fluorescence intensity for surface antigens and intracellular cytokines was detected by flow cytometry, on viable (SSC Vs FSC) gated cells, using a FACS Canto II analyzer. Experiments were performed on HUVEC cells either at basal level or activated with TNF- $\alpha$ , as indicated.

### 2.7. *In vivo* matrigel sponge assay

The ability of ALCAR to inhibit angiogenesis *in vivo* was investigated using the matrigel sponge assay. Unpolymerized liquid matrigel (10 mg/mL, Corning) was mixed with a cocktail of inflammatory pro-angiogenic factors that includes 100 ng/ml VEGF-A (PeproTech), 2 ng/ml TNF- $\alpha$  (PeproTech) and 25 U/ml heparin (Sigma Aldrich), either alone or in combination with 1 or 10 mM ALCAR. The mixture was brought to a final volume of 0.6 ml and injected subcutaneously into the flanks of 6- to 8-week-old C57/BL6

male mice [Charles River Laboratories, Calco (Lecco), Italy]. Two days before matrigel injection, mice received 1 or 10 mM ALCAR that was intraperitoneally (ip) injected in PBS to a total volume of 200 microL. The ALCAR administration was repeated the day of matrigel injection and after two days. All animals were housed in a conventional animal facility with 12 h light/dark cycles and fed ad libitum. *In vivo* experiments were performed in accordance with the Italian and European Community guidelines (D.L. 2711/92

No.116; 86/609/EEC Directive), the 3 R's declaration and within an approved protocol by the institutional ethics committee. Groups of 6 mice were used for each treatment. Four days after injection, the gels were recovered, minced digested with 1 mg/mL Collagenase II (Sigma Aldrich) for 30 min at 37 °C, 5% CO<sub>2</sub>. The cell suspension obtained was analyzed by multicolor flow cytometry for the detection of endothelial cells and macrophages.

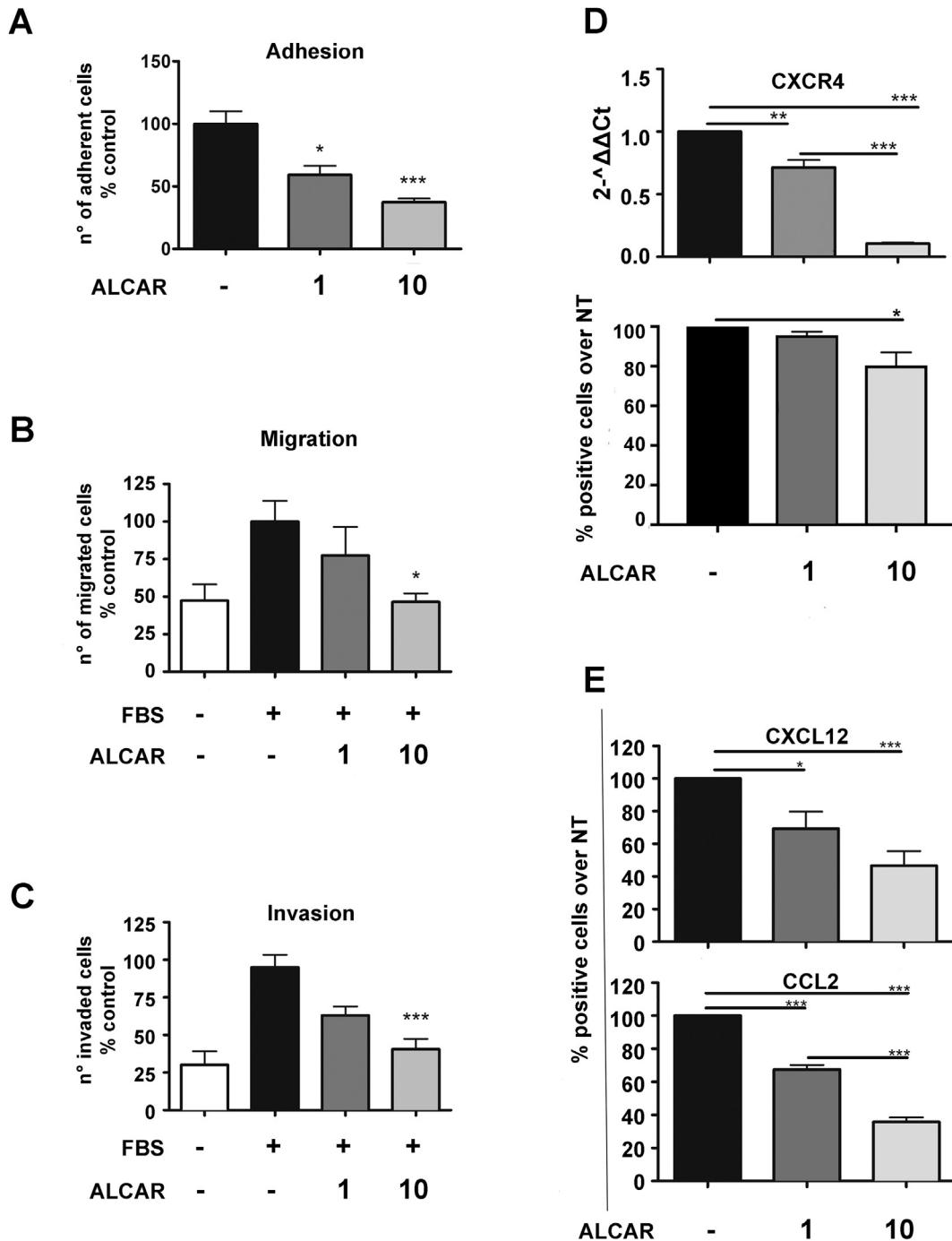


**Fig. 1. Effects of ALCAR on capillary-like structure formation of HUVE cells.** HUVE cells were pretreated with ALCAR at 1 and 10 mM for 24 h or incubated with vehicle alone, were then placed in 96-well plates coated with a layer of Matrigel ( $15 \times 10^3$  per well). SFM: cells cultured in serum-free EGM-2 medium as a negative control; NT: cells treated with vehicle alone in EGM-2 medium supplemented with VEGF + FGF2. Six hours after plating on matrigel, tubular structures were photographed at 5× magnification and quantified by the Angiogenesis analyzer ImageJ tool kit. Pre-treatment with ALCAR at 1 and 10 mM for 24 h inhibited HUVEC ability to form capillary-like structures on matrigel compared to vehicle treated cells (NT). Data are showed as Mean ± SEM. \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.0001 versus VEGF + FGF2 alone or as indicated by the bars.

### 2.8. Flow cytometry for detection of endothelial cells and macrophage infiltration in vivo

The cell suspension obtained from the excised matrigel plugs was used to determine the infiltration of endothelial cells and

macrophages.  $3 \times 10^5$  cells per tube were stained for 30 min at  $4^\circ\text{C}$  with the following anti-mouse monoclonal antibodies: PerCP-conjugated CD45, V500-conjugated CD3, FITC-conjugated CD31, APC-conjugated F4/80, all purchased from Immunotools (Friesoythe Germany). For FACS analysis, viable cells were gated



**Fig. 2. Effects of ALCAR on chemotactic motility of HUVE cells.** ALCAR at 1 and 10 mM concentrations can interfere with crucial steps of angiogenesis by decreasing HUVEC (A) adhesion, (B) migration and (C) invasion, as compared to vehicle treated cells (NT); FBS- cells in serum-free EGM-2 medium as a negative control; FBS + cells in medium containing 10% FBS as a positive control. All experiments were performed three times in duplicate. (D) qPCR and FACS analysis showed an inhibition of CXCR4 transcript and protein levels in HUVECs treated with ALCAR at 1 and 10 mM. The gene expression of CXCR4 is quantified relatively to NT cells, normalized to the housekeeping gene expressed as  $[2^{-\Delta\Delta Ct}]$ , GAPDH ( $n = 3$  independent experiments). (E) FACS analysis for CXCL12 and CCL2 confirmed the downregulation of markers involved in cell motility metastasis and invasion ( $n = 4-5$  independent experiments). Results are expressed as percentage of positive cells over NT and showed as Mean  $\pm$  SEM \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.0001$  as indicated by the bars (One-Way ANOVA).

according to physical parameters (FSC/SSC). Endothelial cells were identified as CD31<sup>+</sup>CD45<sup>-</sup>CD3<sup>+</sup>F4/80<sup>-</sup> cells and macrophages as CD45<sup>+</sup>CD3<sup>-</sup>CD31<sup>+</sup>F4/80<sup>+</sup> cells.

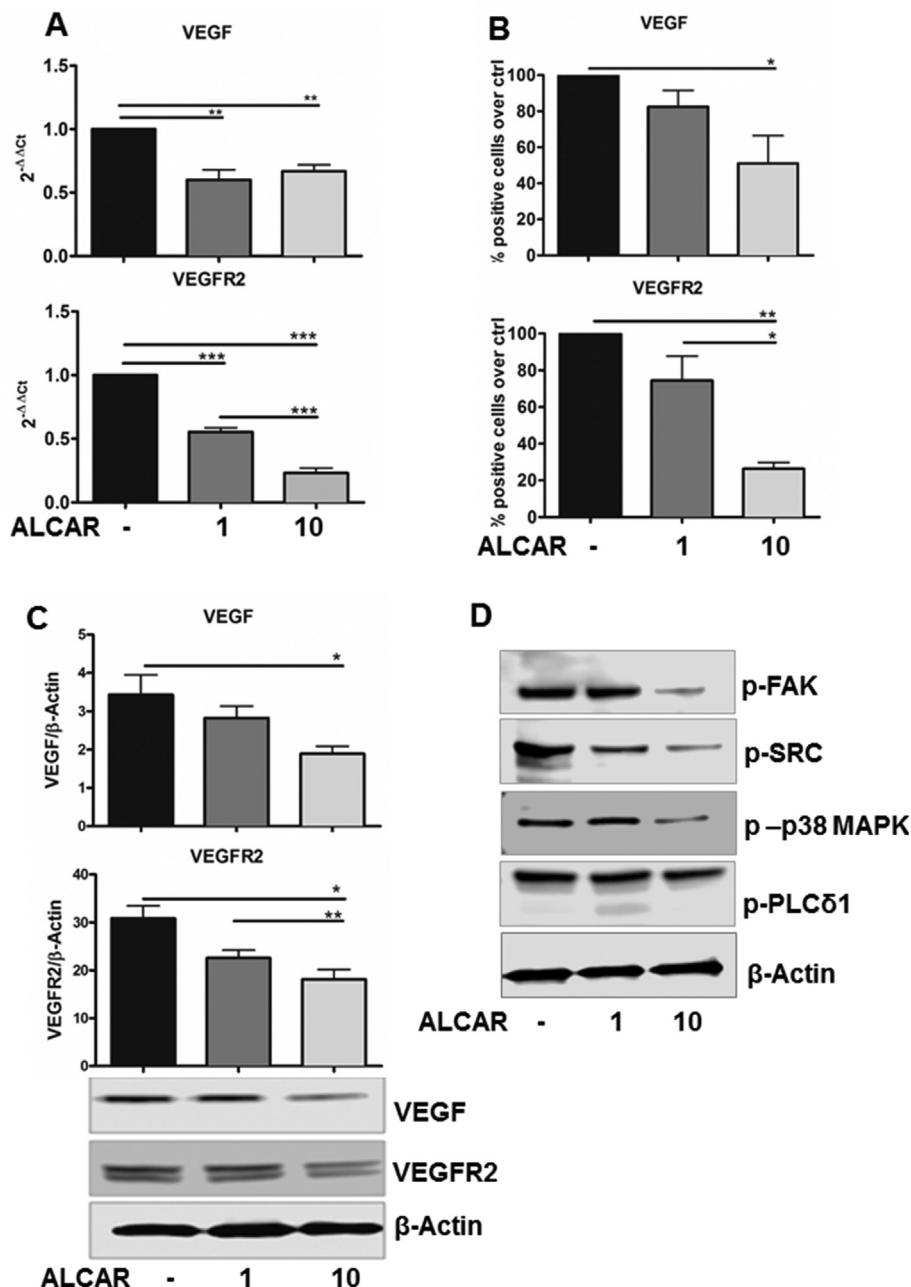
### 2.9. Statistical analysis

The statistical significance between multiple data sets was determined by one-way ANOVA, differences in cell growth curves were determined by two-way ANOVA using Graph-Pad PRISM. FACS data were analyzed by FACSDiva Software 6.1.2. Data are expressed as means  $\pm$  SEM.

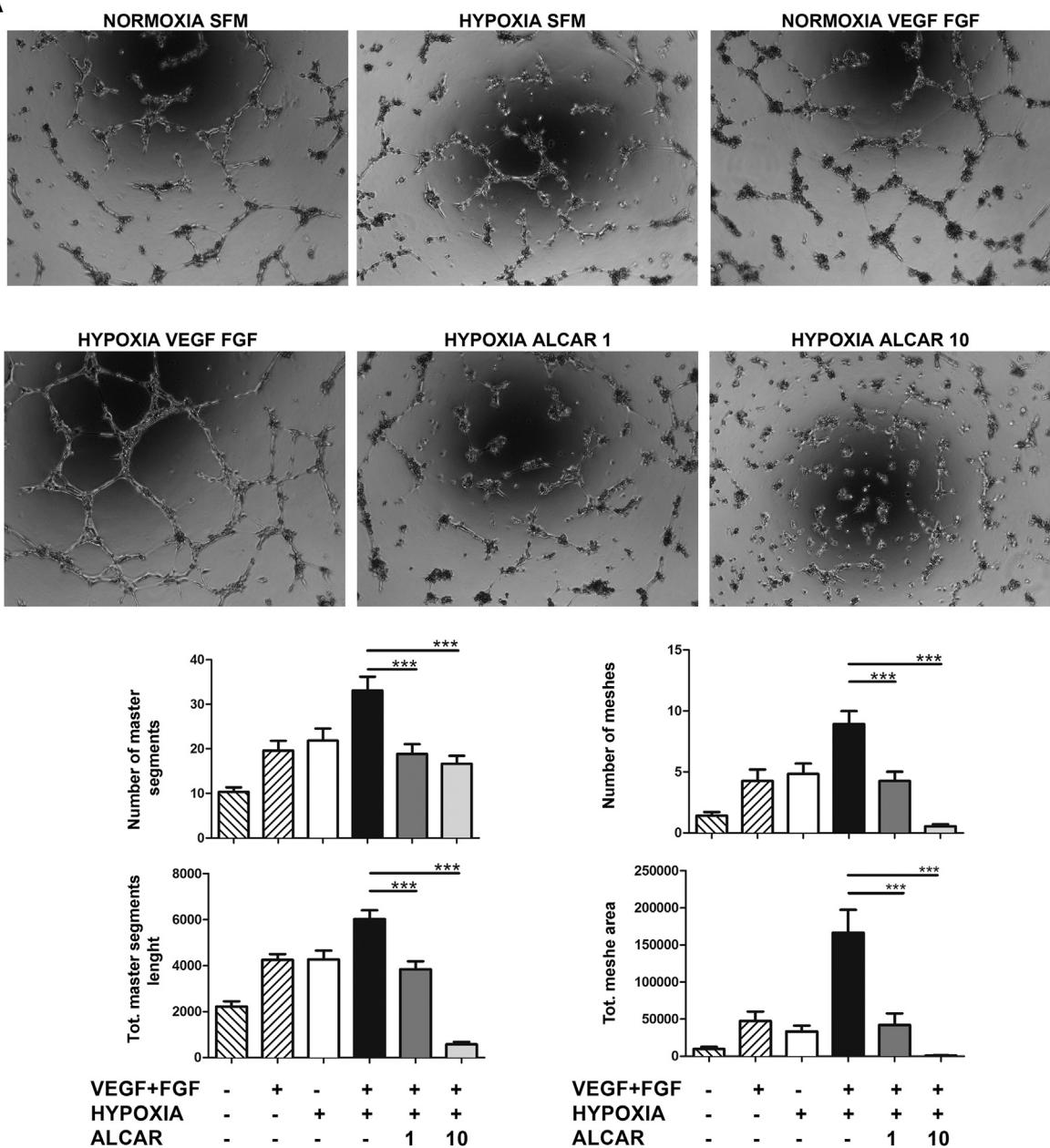
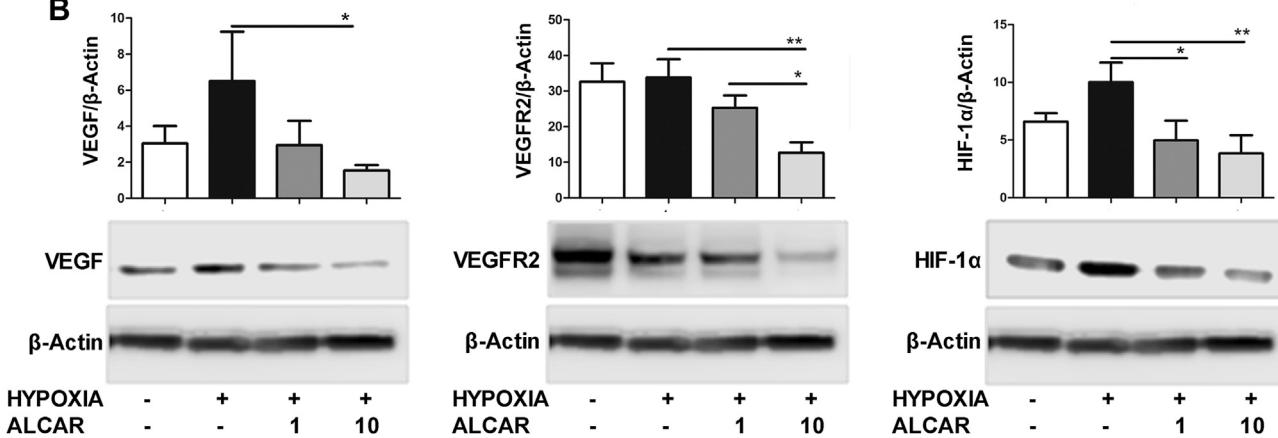
## 3. Results

### 3.1. ALCAR inhibits capillary-like tube formation in vitro

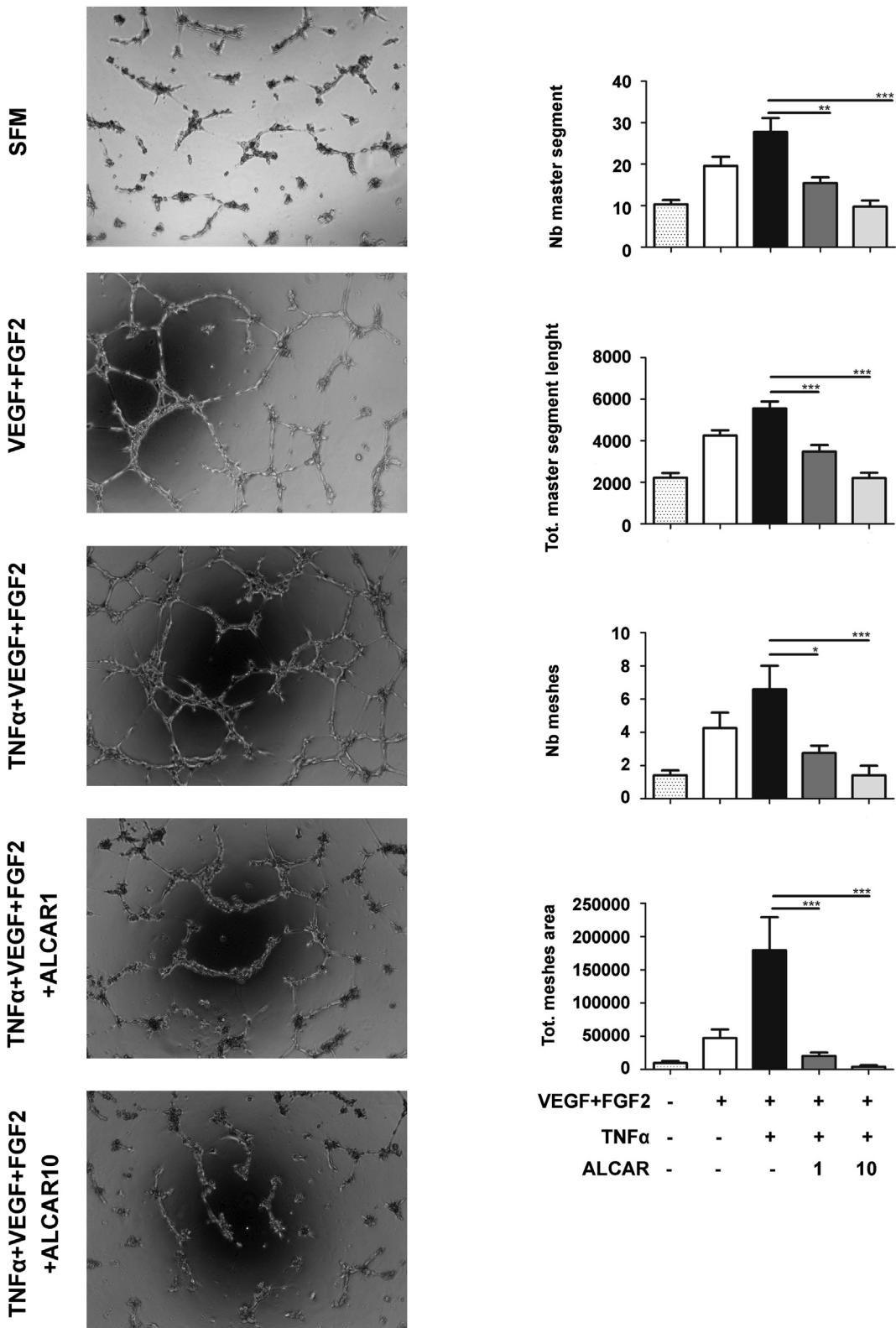
First, we investigated the effects of ALCAR on HUVEC proliferation by crystal violet (Supplemental Fig. 1A) and MTT assay (Supplemental Fig. 2A) showing that ALCAR acts on HUVEC proliferation in a dose-dependent manner. We also examined the impact of ALCAR on cell survival and growth inhibition of other human cell lines such as MRC-5 and peripheral blood mononuclear cells (PBMCs) from healthy volunteers (Supplemental Figs. 1A–C). We found that ALCAR exerted little impact on the proliferation of normal



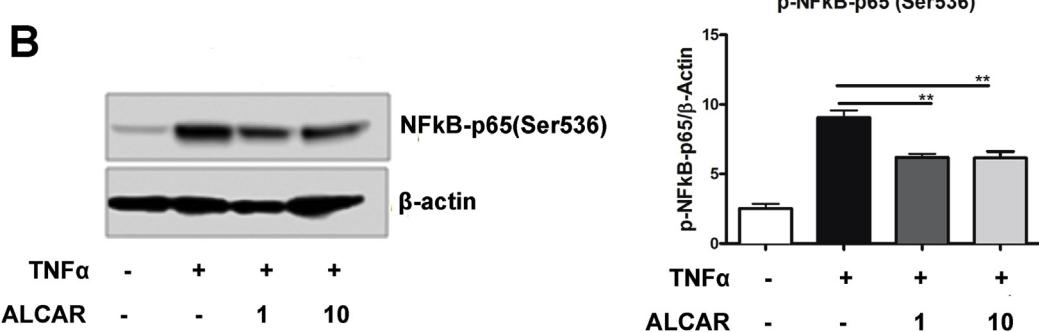
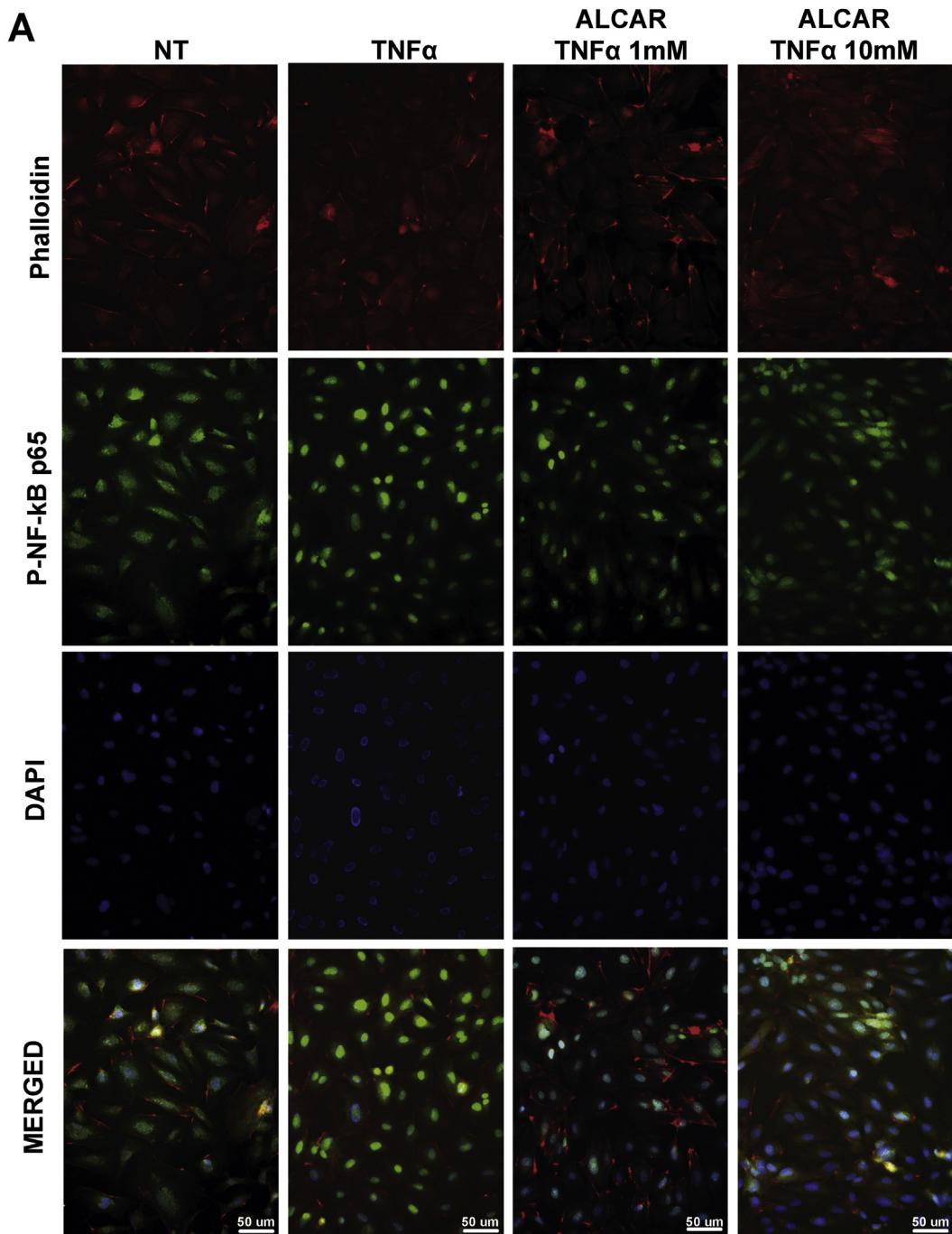
**Fig. 3. Effects of ALCAR on VEGF and VEGFR2 synthesis of HUVE cells.** (A) qPCR analysis demonstrates an inhibition of VEGF/VEGFR2 transcripts in HUVECs treated with ALCAR 1 and 10 mM after 24 h. mRNA levels [ $2^{-\Delta\Delta Ct}$ ] were calculated relatively to NT cells, normalized to the housekeeping gene, GAPDH ( $n = 4$  independent experiments). (B–C) FACS analysis for VEGF/VEGFR2 confirmed the inhibition at protein level ( $n = 4$ –5 independent experiments). Further validation was obtained by western blot analysis, the graphs show quantification of VEGF and VEGFR2 ( $n = 4$  independent experiments). (D) ALCAR inhibited the activation of VEGFR2 downstream cascade: pTyr397-FAK, pTyr416-Src, p-38 MAPK, p-Ser1248-PLC $\gamma$ .

**A****B**

**Fig. 4. Effect of ALCAR on the hypoxia-induced angiogenesis.** (A) Activation of HUVE cells in hypoxic conditions for 72 h, followed by treatment with ALCAR 1 and 10 mM for 24 h resulted in significantly reduced ability to form capillary-like structures on matrigel compared to vehicle treated HUVE cells. Microphotographs were taken at 5 $\times$  magnification, representative images are shown. Images were quantified by Angiogenesis analyzer ImageJ tool kit. Data are showed as Mean  $\pm$  SEM. \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.0001 vs cells in normoxic conditions or as indicated by the bars. (B) WB showing that ALCAR reduces hypoxia-induced angiogenesis by downregulating VEGF and VEGFR2 following treatment with ALCAR (n = 3 independent experiments).



**Fig. 5. Effect of ALCAR on TNF- $\alpha$ -induced capillary-like structure formation.** Pre-treatment with ALCAR at 1 and 10 mM concentrations, of HUVE cells stimulated with 10 ng/ml of TNF- $\alpha$  (10 ng/ml) for 6 h resulted in significantly reduced ability to form capillary-like structures on matrigel. Microphotographs were taken at 5 $\times$  magnification, representative images are shown. Images were quantified by Angiogenesis analyzer ImageJ tool kit. Data are showed as Mean  $\pm$  SEM. \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.0001 vs VEGF + FGF2+TNF- $\alpha$  as indicated by the bars.



cells at the highest concentration (10 mM), while showed a significantly higher effect on the proliferation of endothelial cells (*Supplemental Figs. 1A–C*). ALCAR C capillary-like might modulate activities on angiogenesis by selectively targeting endothelial cells. Based on data obtained from cell proliferation assay, detection of apoptosis and cell cycle arrest (*Supplemental Figs. 2A–C*), we selected two ALCAR concentrations, 1 and 10 mM. The selected concentrations are consistent with other preclinical studies [52,60] and in line with several clinical trials. We assessed the effects of ALCAR on endothelial cell morphogenesis induced by FGF2 and VEGF by determining the ability of HUVECs to organize into capillary-like networks. We observed that ALCAR significantly inhibited the network like formation induced by VEGF/FGF2 in HUVE cells cultured on a matrigel layer (*Fig. 1*), as determined by the quantification of number and total length of master segments and number and total meshes area ( $P = 0.0002$ ). These results indicated that ALCAR inhibits HUVE C capillary-like morphogenesis *in vitro*.

### 3.2. ALCAR reduces oxidative stress in hypoxic conditions

ALCAR and other carnitine acyl esters effectively protect from oxidative damage [84] by acetylation membrane proteins [58], removing long-chain acyl-CoAs from cell membranes [16] and by scavenging free radicals [34]. We tested the potential role of ALCAR on attenuating mitochondria-derived ROS generation in hypoxic conditions after 72 h. We found that ALCAR reduced significantly the production of superoxides in the mitochondria of HUVE cells (*Supplemental Fig. 3*) under a partial reduction in oxygen (1%), that could be involved in its protective role in preventing inflammation and endothelial dysfunction.

### 3.3. ALCAR blocks HUVEC migration and invasion by inhibiting CXCR4/CXCL12 axis

Adhesion to the extracellular matrix, migration and invasion are key steps in tumor-induced neovascularization. We therefore assessed the effects of ALCAR on these processes on HUVE cells *in vitro*. ALCAR significantly decreased ( $P < 0.0001$ ) HUVE cell adhesion on a fibronectin layer (*Fig. 2A*) in a dose dependent manner. Accordingly, ALCAR at 10 mM significantly interfered with HUVEC migration ( $P = 0.0201$ ) on collagen IV (*Fig. 2B*) and invasion ( $P < 0.0001$ ) through a matrix layer, using FBS as chemoattractant, according to published methods [2,5] (*Fig. 2C*), in a dose dependent manner. Since we observed that ALCAR functionally inhibits HUVE cell adhesion, migration and invasion, we then investigated which molecular pathways involved in cell motility metastasis and invasion were targeted by ALCAR. We observed significant down regulation of PECAM-1, FAK transcript levels and trends in down regulation of P-selectin (*Supplemental Fig. S3*). CXCR4 and its ligand, CXCL12, known as key regulators in pro-angiogenic migratory phenotype [42,50], were significantly downregulated by ALCAR at mRNA ( $P < 0.0001$ ; *Fig. 2D*) and protein levels ( $P = 0.031$  and  $P = 0.0010$ ; *Fig. 2D–E*). CCL2, that acts on mononuclear cells and indirectly acts on endothelial cells to sustain inflammatory angiogenesis [38], was also inhibited by ALCAR ( $P < 0.0001$ ) (*Fig. 2E*).

**Fig. 6. ALCAR inhibits the TNF- $\alpha$ -induced p65 phosphorylation and nuclear translocation.** (A) HUVE cells were incubated with ALCAR 1 and 10 mM for 6 h and followed by TNF- $\alpha$  (10 ng/ml) stimulation for 30 min. After fixation, cells were stained with specific anti-NF $\kappa$ B-p65 (Ser536) antibody followed by secondary antibody Alexa Flour 488 (green). Actin filaments were stained with rhodamine phalloidin, while nucleus was counterstained with DAPI (blue) and captured with a 20 $\times$  magnification objective (Axio Observer A1, Zeiss, Germany). Scale bars: 50  $\mu$ m. Images were acquired for each fluorescence channel and were merged using AxioVision Software. (B) Representative western blot images showed a significantly reduced phosphorylation of anti-NF $\kappa$ B-p65 (Ser536) in HUVE cells incubated with ALCAR 1 and 10 mM for 6 h, followed by TNF- $\alpha$  (10 ng/ml) stimulation for 30 min. The graphs show quantification of anti-NF $\kappa$ B-p65 (Ser536) indicating significantly differences ( $P < 0.0001$ ) of ALCAR treated vs non-treated cells. Data are expressed as Mean  $\pm$  SEM ( $n = 5$  independent experiments).

### 3.4. ALCAR suppresses VEGF and VEGFR2 synthesis in endothelial cells

VEGF/VEGFR2 interaction acts as a major regulator of angiogenesis [14,29]. We investigated whether anti-angiogenic activities of ALCAR may target this axis both at gene expression and protein levels. Quantitative RT-PCR indicated that ALCAR treatment reduced, in a dose-dependent manner, VEGFR2 and VEGF mRNA in endothelial cells (*Fig. 3A*). This was confirmed by flow cytometry analysis (*Fig. 3B*) and by western blot (*Fig. 3C*).

The endothelial VEGF/VEGFR2 signaling network represents the key regulator of angiogenesis leading to endothelial cell proliferation, migration, survival and new vessel formation. We then moved to the specific protein and downstream signaling pathways involved. Focal Adhesion Molecule (FAK), which plays a crucial role in cell proliferation, survival and mobilization was found to be inhibited by ALCAR both at transcriptional (*Supplemental Fig. S3*) and protein phosphorylation level (pTyr397-FAK) (*Fig. 3D*). The downstream pathways and VEGFR2 signaling intermediates pTyr416-Src, p-38 MAPK and p-Ser1248-PLC $\gamma$ 1 were also inhibited by ALCAR at 1 and 10 mM (*Fig. 3D*). Taken together, our results indicated that ALCAR targets multiple angiogenesis-related pathways and exerts a direct effect on VEGF and VEGR2 signaling.

### 3.5. ALCAR inhibits hypoxia induced endothelial cell morphogenesis and VEGF

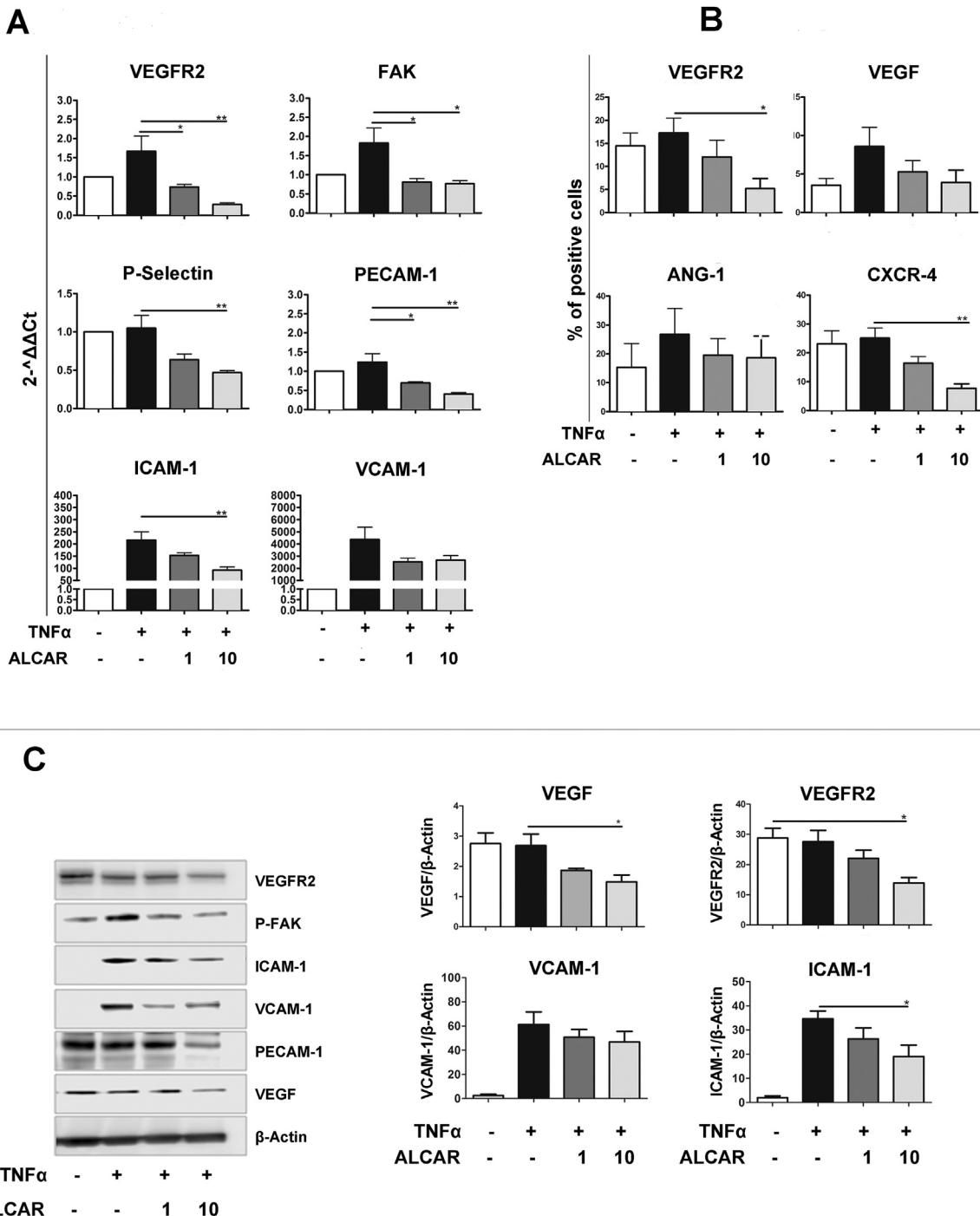
Oxygen availability causes different molecular switches which regulate synthesis and secretion of growth factors and inflammatory mediators within the tissue microenvironment. Hypoxia inducible factor (HIF-1 $\alpha$ ), the central mediator of hypoxic response, regulates several angiogenesis-related genes and VEGF is one of the primary target genes [65,78]. Since hypoxia is present in tumors and is a major controller of the VEGF/VEGFR pathway, we evaluated the effect of ALCAR on the formation of capillary-like structures, and VEGF expression in the hypoxic environment. Hypoxia induced significantly enhanced network formation in both SFM and under the stimulus of VEGF/FGF2 ( $P < 0.01$ ). ALCAR substantially inhibited hypoxia-induced ability of HUVE cells to resemble capillary-like structures on a matrigel matrix as determined by the quantification of number and total length of master segments, number and total area of meshes ( $P < 0.0001$ ) (*Fig. 4A*). This was accompanied by a downregulation of VEGF protein level, reflecting the HIF-1 $\alpha$  trend, while VEGFR2 regulation seems not to be dependent on hypoxia (*Fig. 4*), but was downregulated by ALCAR.

### 3.6. ALCAR inhibits the activation of the NF- $\kappa$ B signaling pathway by TNF- $\alpha$

Inflammation and angiogenesis are closely related events contributing to tumor insurgence and progression while NF- $\kappa$ B activation is considered a master inflammatory-regulator of angiogenesis [36]. Activation of NF- $\kappa$ B requires the degradation of inhibitor kappa B (IkB- $\alpha$ ) that in turn forms a cytoplasmic and inactive complex with the p65-p50 heterodimer and is able to block the nuclear localization of the NF- $\kappa$ B subunits. We explored the effect of ALCAR pre-treatment on NF- $\kappa$ B signaling on endothelial

cells activated with TNF- $\alpha$ , using functional angiogenesis assays, immunofluorescence and western blotting. Endothelial cells exposed to a cocktail of TNF- $\alpha$ , VEGF and FGF2 (100 ng/ml) induced the formation of capillary-like structures on matrigel which was significantly elevated over that of VEGF/FGF2 alone ( $P < 0.05$ ) and was abrogated by ALCAR treatment in a dose-dependent manner

(Fig. 5). Treatment with ALCAR also blocked the translocation of NF- $\kappa$ B p65 into nucleus after the addition of TNF- $\alpha$  (Fig. 6A) and inhibited the phosphorylation of NF- $\kappa$ B (Fig. 6B). This was associated with decreased VEGFR2, FAK, PECAM-1, P-selectin and ICAM-1 at the transcription level, in a dose dependent manner (Fig. 7A). Data from the modulated transcripts were confirmed at the protein



**Fig. 7. ALCAR inhibits TNF- $\alpha$  induced proangiogenic and NF $\kappa$ B-regulated pathways.** (A) qPCR analysis shows an inhibition of VEGFR2, FAK, P-Selectin, PECAM-1, ICAM-1, VCAM-1 transcript in HUVE cells incubated with ALCAR (1 and 10 mM) for 24 h and followed by TNF- $\alpha$  (10 ng/ml) stimulation for 30 min as compared to non-treated cells. mRNA levels [ $2^{-\Delta\Delta Ct}$ ] were calculated relatively to TNF- $\alpha$ -stimulated HUVE cells, normalized to the housekeeping gene, GAPDH ( $n = 4$  independent experiments). (B) FACS analysis shows a downregulation of angiogenic markers (VEGF, VEGFR2 and ANG1OP-1) and invasion markers (CXCR4), expressed as percentage of positive cells over TNF- $\alpha$ -stimulated cells ( $n = 4$ –7 independent experiments). (C) Further validation obtained by WB analysis confirmed the inhibition at protein level of VEGF, VEGFR2 and ICAM-1, the graphs show quantification of VEGF, VEGFR2, ICAM-1, VCAM-1 ( $n = 4$  independent experiments). Results are showed as Mean  $\pm$  SEM \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.0001$  vs TNF- $\alpha$ -treated cells or as indicated by the bars (One-Way ANOVA).

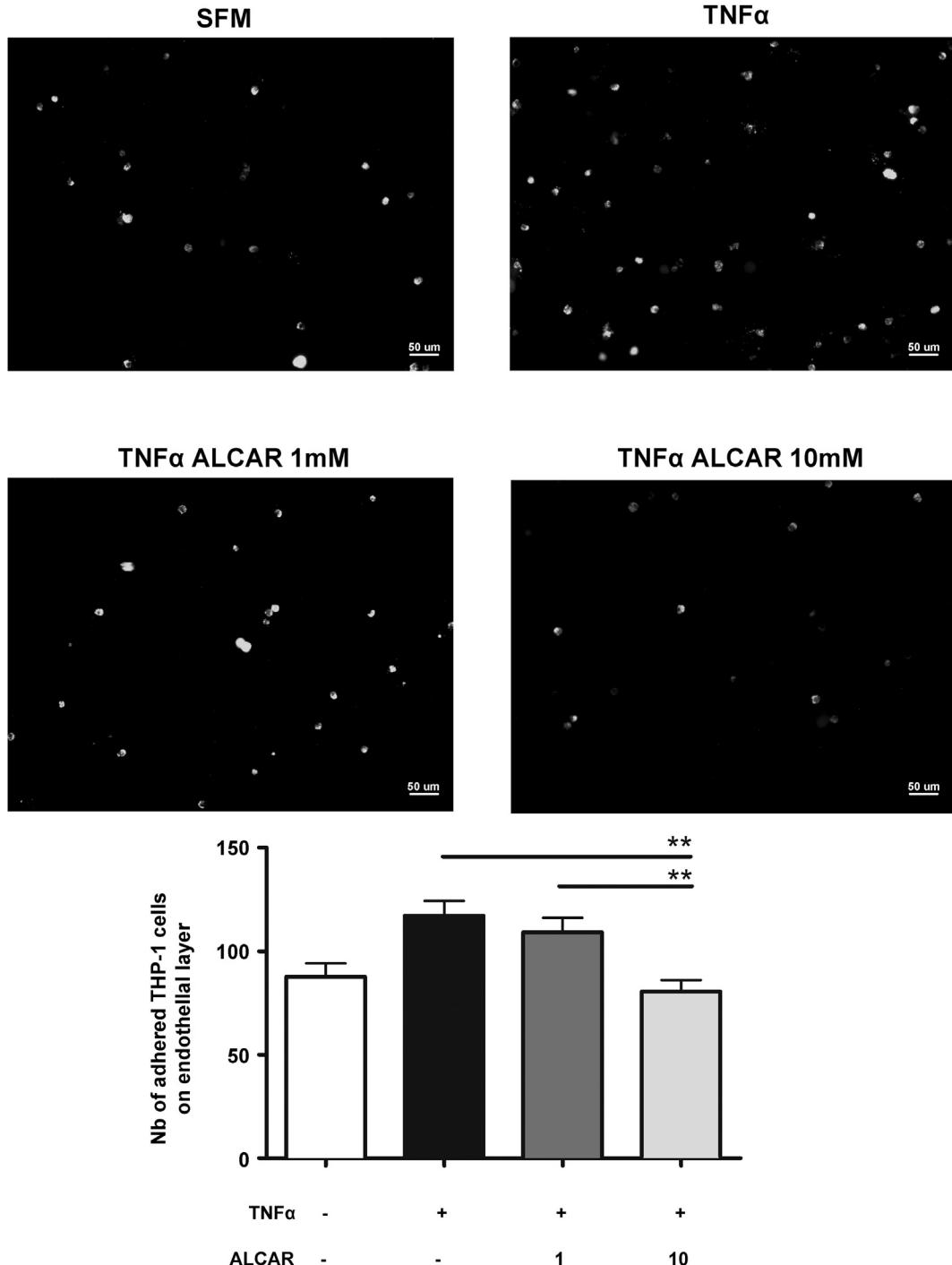
level by flow cytometry (Fig. 7B) and western blot (Fig. 7C). We found that pre-treatment with ALCAR followed by TNF- $\alpha$  exposure downregulates the expression of VEGF, VEGFR2 and CXCR4, which are critical for endothelial cell survival, migration and invasion.

We also investigated whether ALCAR may impact on angiogenesis acting on the inflammatory stimuli by modulating leucocyte recruitment. We mimicked macrophage adhesion (human monocytic THP-1 cells) on an inflammatory (TNF- $\alpha$ ) activated

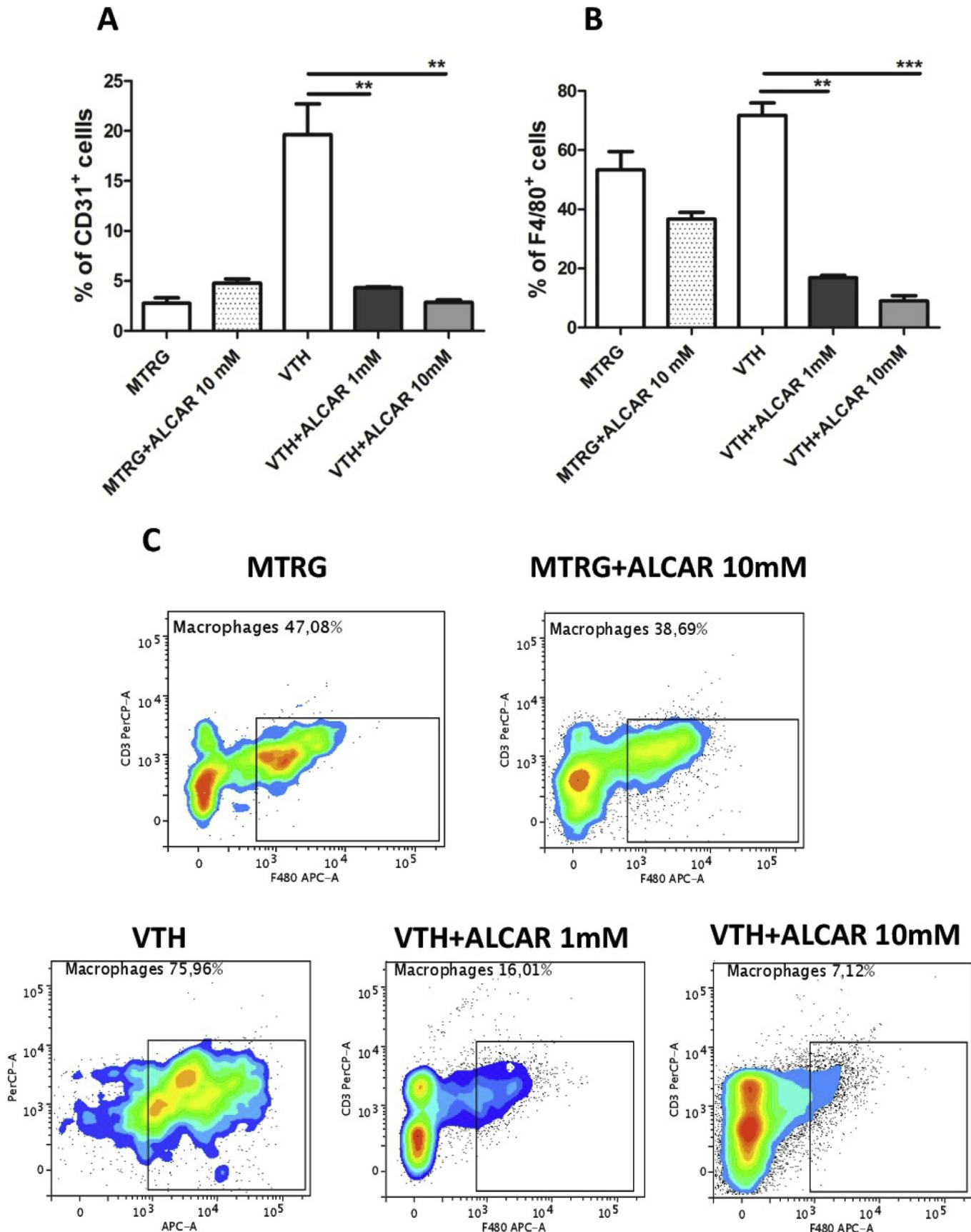
endothelial layer. We found that pre-treatment of HUVE cells with 10 mM ALCAR resulted in significantly ( $P=0.0013$ ) lower number of adhered THP-1 cells on the endothelial cell layer (Fig. 8).

### 3.7. ALCAR inhibits inflammatory angiogenesis *in vivo*

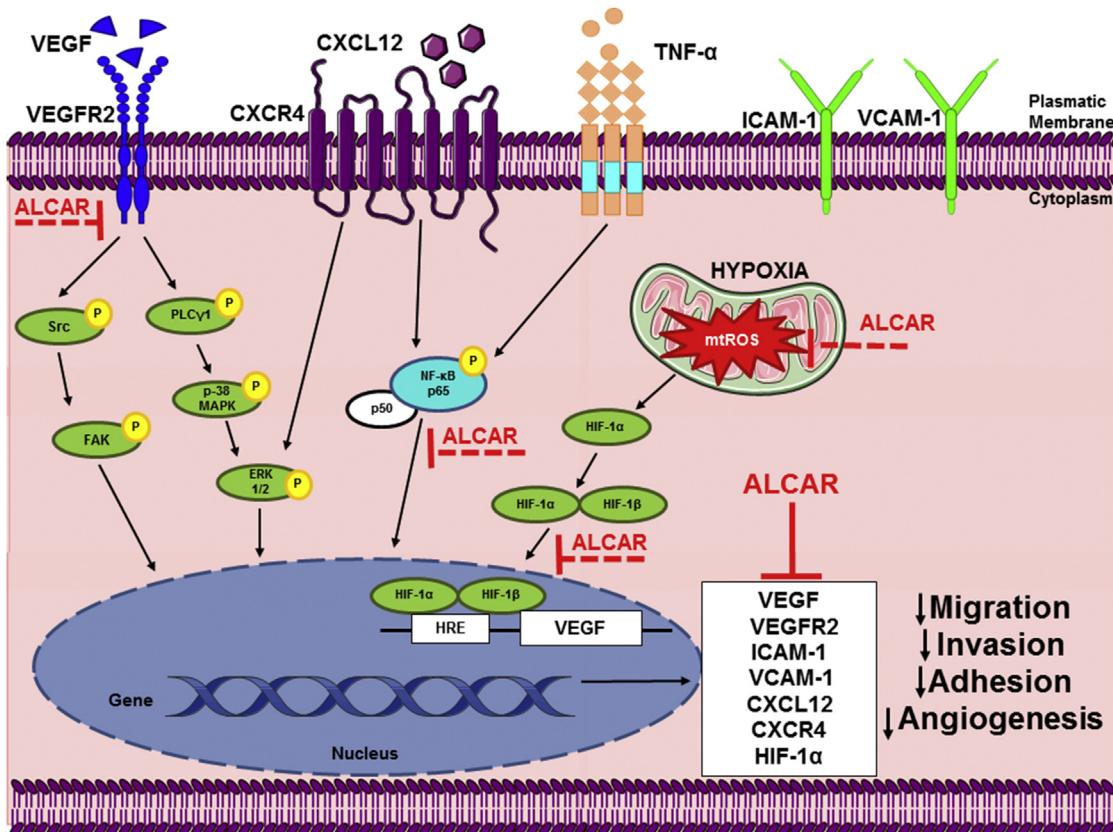
We investigated whether ALCAR was effective in inhibiting angiogenesis and inflammatory angiogenesis *in vivo*. Using the



**Fig. 8. Effects of ALCAR on the adhesion of monocytes to HUVE cells.** THP-1 monocytic cell adhesion to HUVEC is reduced by ALCAR (1 and 10 mM). After treatment of a monolayer of HUVE cells with ALCAR and activation with TNF- $\alpha$  (10 ng/ml), calcein AM-labelled THP-1 cells were added on a top of HUVEC monolayer and incubated for 3 h. After washing to remove non-adherent monocytes, calcein-AM-labelled THP-1 cells (green) adhering to HUVECs were counted. Results are showed as Mean  $\pm$  SEM \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.0001$  vs TNF- $\alpha$ -activated cells as indicated by the bars (One-Way ANOVA).



**Fig. 9.** ALCAR inhibits angiogenesis *in vivo*. Flow cytometry analysis for endothelial cells and macrophage infiltration in the excised Matrigel plugs revealed ALCAR ability to inhibit VTH-induced A) CD31<sup>+</sup> endothelial cell infiltration and B) CD45<sup>+</sup>F4/80<sup>+</sup> macrophage infiltration into the matrigel plugs. C) Representative dot plots for macrophages infiltration in matrigel plugs, as determined by flow cytometry. Results are showed as Mean  $\pm$  SEM \* $p$  < 0.05; \*\* $p$  < 0.01 vs controls, One-Way ANOVA.



**Fig. 10.** Schematic presentation of the anti-angiogenic signaling pathways regulated by ALCAR in HUVE cells. Proposed mechanism for the inhibition of angiogenesis by ALCAR: i) via inhibition of VEGF/VEGFR2-mediated signaling pathways; ii) via downregulation of HIF-1 $\alpha$  and VEGF and iii) by blocking the activation of NF- $\kappa$ B, and downregulation of ICAM; iv) by downregulation of CXCR4. Arrows indicate regulations by ALCAR treatment observed in our experiments.

matrigel sponge assay, that allows to mimic a local inflammatory pro-angiogenic microenvironment in a matrix plug, we found that treatment with ALCAR at 1 and 10 mM (corresponding to a dosage of 2 and 20 mg/kg, respectively) significantly reduced endothelial (CD31 $^{+}$  cells) content (Fig. 9A). In addition, ALCAR was able to limit F4/80 $^{+}$  macrophages recruitment into the matrigel plug, *in vivo*. (Fig. 9B–C). These results demonstrated that ALCAR was able to limit VEGF and TNF- $\alpha$  (VTH)-induced endothelial cells and macrophages *in vivo* recruitment, confirming the results observed *in vitro* (see Fig. 10).

#### 4. Discussion

Angiogenesis and inflammation are necessary and complementary processes to support tumor insurgence and progression [4,6]. Upon this assessment, several anti-angiogenic agents have been developed and employed in the clinic to be combined with standard chemotherapy. There is a growing interest in identifying novel active compounds from natural sources [1,4,6,10] in relation to their biological properties and potential health benefits. These compounds have been investigated based on their anti-proliferative, anti-oxidant and anti-angiogenic properties, and their ability to target both malignant transformed cells and the surrounding microenvironment [1,4,6,10]. Hence many efforts have been addressed in the identification of diverse agents that target angiogenesis in a preventive approach (angio-prevention) and interception [6], for repurposing (such as metformin, aspirin) or using phytochemicals [6].

Carnitine, a micronutrient derived from amino acids lysine and

methionine, is found in almost all body cells, and is involved in energy metabolism [33], transport of long-chain fatty acids across the mitochondrial membranes in muscle cells,  $\beta$ -oxidation and the transport of fatty acids out of the mitochondria [33]. Carnitine supplementation has been largely reported to be beneficial for patients with primary and secondary carnitine deficiencies, mostly including chronic inflammatory diseases, such as diabetes, cardiovascular disorders and cancer [32]. Anti-inflammatory, antioxidant and free radical scavenging properties of ALCAR, as well as its stabilizing effects on mitochondrial membrane, have been reported [9,15,30,57,71].

Using novel highly sensitive mass spectrometry approach, based on Surface-Activated Chemical Ionization (SACI) with an Electrospray Ionization (ESI) source and bioinformatics analyses (SANIST platform) for prostate cancer (PCa) biomarker discovery, we found that 3 molecules from the carnitine family (decanoyl-l-Carnitine, octanoyl-l-carnitine and 5-cis-tetradecenoyl carnitine) were significantly decreased in serum samples from PCa patients, compared to those from individuals with BPH [3]. This finding suggests a potential protective role of carnitine against progression of PCa, and we investigated whether these properties may act on angiogenesis and inflammation, two relevant hallmarks of cancer. Most of the studies on the effects of carnitine on cancer and angiogenesis are focused on carnitine transporters (CPT1 and CPT2) [45,70,79], rather than on carnitine itself.

Here, we demonstrated for the first time that ALCAR acts as an anti-angiogenic and angio-preventive agent in two relevant micro-environment settings: hypoxia and inflammation (see Fig. 10). We also unveiled the molecular mechanisms involved. We first

identified a dose range of ALCAR to be potentially employed in anti-angiogenic and angiopreventive settings. Carnitine supplements currently on the market cover the dosage of 2 g/day; we found that administration of ALCAR at 1 and 10 mM, corresponding to 2 and 20 mg/kg respectively, was able to reduce HUVE cell proliferation without having toxicity. ALCAR selected concentrations showed no effects on other normal cells proliferation, such as fibroblasts and peripheral blood mononuclear cells from healthy volunteers.

We investigated the ability of ALCAR to limit key functional steps of angiogenesis induction, such as endothelial cell adhesion, migration, invasion and formation of capillary like structures. We found that ALCAR was able to inhibit these key processes in HUVE cells. ALCAR is able to maintain the transition of mitochondrial membrane potential and suppresses the induction of reactive oxygen species (ROS). It is recognized that ROS acts as signaling molecule in endothelial cells, and can support angiogenesis through VEGF expression or VEGF receptors, mainly VEGFR2 (Flk-1/KDR), and angiopoietin-1/Tie-2 receptors [23]. VEGF-VEGFRs signals constitute the most important signaling pathways in tumor angiogenesis [7,72]. Among VEGFRs, VEGFR2 is the major receptor, which mediates the angiogenic activity of VEGF via different signaling pathways including MAPK family and Src-FAK complex. Molecularly, we found the functional alteration observed in HUVEC exposed to ALCAR was associated to ALCAR ability to target the VEGF-VEGFR2 axis, whose reduction was observed both at transcript and protein levels.

To better mimic the scenario occurring in the tumor microenvironment during tumor angiogenesis [12], we also treated HUVE cells with ALCAR in hypoxic and pro-inflammatory conditions, where we found ALCAR effective in limiting endothelial tube formation. ALCAR acted on VEGF and VEGFR2 downstream signaling pathways, including MAPK family and the activated complex Src-FAK that mediate endothelial cell migration and survival [17,37,51,63]. We showed that the downregulation of SRC/FAK and MAPK family members by ALCAR was correlated with functionally reduced endothelial cell adhesion, migration and invasion in a dose dependent manner.

In the presence of pathological angiogenesis, at the sprouting tips of growing vessels, the CXCL12/CXCR4 axis and CCL2 play a fundamental role in endothelial cell invasion, mobilization/migration, extravasation, directional migration, homing, and cell survival [40,44,53]. We demonstrated that ALCAR, in an inflammatory microenvironment, inhibits protein expression levels of CCL2, CXCL12 and CXCR4. Additionally, transcripts levels of cell-associated surface proteins, such as PECAM-1, that are important drivers of cell migration [61,83], were also significantly reduced. The inhibition of CXCR4 is particularly relevant, because its expression is frequently upregulated and involvement in human cancer metastasis [11].

Targeting mediators and cellular effectors of inflammation and angiogenesis could lead to improved prevention and treatment of tumors. Inflammation has been recognized as a relevant hallmark of cancer and is related to angiogenesis [36]. During the angiogenic switch this interaction becomes more relevant, since inflammatory cells recruited into the tissues can also support angiogenesis by acquiring altered phenotypes and release pro-angiogenic factors [19,20].

The principal source of ROS, mitochondria, regulate innate immunity responses via two major pathways including either direct activation of inflammasome complexes or upregulation of redox-sensitive transcription factors such as NF- $\kappa$ B [31]. Expression of genes encoding inflammatory cytokines, adhesion molecules, enzymes and angiogenic factors are regulated by NF- $\kappa$ B activation [43,59]. We showed that pre-treatment of HUVEC with ALCAR reduced TNF- $\alpha$  mediated angiogenesis by decreasing p-NF- $\kappa$ B

translocation into the nucleus, consequently blocking the upregulation of chemokines and adhesion molecules involved in inflammatory response [27].

Macrophages are among the most abundant immune infiltrate in inflamed tissues and induce a pro-angiogenic environment [48,49]. In microenvironment of smoldering inflammation, NF- $\kappa$ B plays a crucial role in the macrophage infiltration, also interconnected with angiogenesis that predisposes individuals towards developing cancer metastasis [35]. Targeting NF- $\kappa$ B by ALCAR effectively blocked both endothelial and macrophage recruitment *in vivo* and lowered the expression of ICAM-1, supporting the hypothesis that ALCAR may directly and indirectly (by inhibiting inflammation) inhibit angiogenesis (Fig. 10).

Given the dietary antioxidants properties on preventing tumor angiogenesis by acting on oxidative stress-induced pathological angiogenesis we speculate that ALCAR inhibits inflammatory-induced angiogenesis possibly due to its antioxidant ability and stabilizing effects on mitochondria [8]. Our results highlight the anti-angiogenic and anti-inflammatory properties of ALCAR and allow the identification of major molecular pathways through which ALCAR inhibits angiogenesis. Besides the anti-angiogenic agents that have been clinically approved by the US FDA, our data showed that ALCAR downregulates angiogenesis by multiple and overlapping mechanisms of action. To our knowledge this is the first study demonstrating that ALCAR is anti-angiogenic in tumor-like conditions, suggesting a potential employment of ALCAR as a supplement in the prevention of cancer and inflammatory angiogenesis to be used as chemo/angioprevention approaches.

## Author contributions

AA, AB, DB and DMN conceived and designed the experiments. DB, AB, BB, MT performed the *in vitro* experiments. AB, DB, LM performed the *in vivo* experiments. AA, AB, DB performed the statistical analysis. AA, AB, DB, DMN wrote the paper.

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## Declarations of interest

None.

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## Appendix A Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.canlet.2018.04.018>.

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