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ABSTRACT

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We examined whether ALCAR act 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 showed 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-angiogenesis and anti-inflammatory properties and might be attractive candidate for cancer angioprevention.

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ABSTRACT

Carnitines play an important role in the energy exchange in cells, 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 than carnitine 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 act 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 showed 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-angiogenesis and anti-inflammatory properties and might be attractive candidate for cancer angioprevention.

Keywords:
Angiogenesis, L-acetyl-carnitine (ALCAR), chemoprevention, angioprevention, VEGF/VEGFR2, migration/invasion.

38 **List of abbreviations**

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ALCAR	*Acetyl – L – Carnitine*
ANGPT1	*Angiopoietin-1*
BPH	*Benign Prostatic Hyperplasia*
CPT1A	*Carnitine Palmitoyl Transferase 1*
CPT1C	*Carnitine O – Palmitoyl Transferase 1*
CXCR4	*C-X-C chemokine receptor type 4*
FAO	*Fatty Acid Oxidation*
FGF2	*Fibroblast Growth Factor 2*
Hif-1α	*Hypoxia- inducible Factor 1-alpha*
HUVECs	*Human Umbilical Vein Endothelial Cells*
ICAM-1	*Intracellular Cell Adhesion Molecule – 1*
CCL2	*MCP-1; Monocyte Chemoattractant Protein-1*
NF-κB	*Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells*
p-38 MAPK	*Cytokinin Specific Binding Protein*
PCa	*Prostate Cancer*
PECAM-1	*Platelet and Endothelial Cell Adhesion Molecule-1*
ERK 1/2	*Extracellular signal – Regulated protein Kinases 1 and 2*
PLCγ1	*Phospholipase C, gamma 1*
FAK	*Focal Adhesion Kinase*
Src	*Proto-Oncogene Tyrosine – Protein Kinase*
SACI	*Surface – Activated Chemical Ionization*
SANIST	*Rapid mass spectrometric SACI/ESI data acquisition and elaboration platform*
CXCL12	*SDF-1; Stromal Cell-Derived Factor-1*
THP-1	*Leukemic Monocyte*
TNFα	*Tumor Necrosis Factor α*
VCAM-1	*Vascular Cell Adhesion Molecule – 1*
VEGF	*Vascular Endothelial Growth Factor*
VEGFR2	*Vascular Endothelial Growth Factor Receptor 2*

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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 roadway 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 (β-hydroxy-γ-N-trimethylaminobutyric acid) is a naturally occurring quaternary ammonium compound and its derivatives acetyl-L-carnitine and propionyl-L-carnitine, are essential for lipid energy metabolism within the mitochondria, contributing to the transport of long-chain acyl CoA into the mitochondrial matrix where the enzymes for β-oxidation are located. Other roles for carnitine include buffering of branched-chain amino acid metabolism, removal of excess acyl groups, and peroxisomal fatty acid oxidation [18, 32]. Carnitine deficiency have 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 as 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], and carnitines showed 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 angioprevention 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 largely available in 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, and it downregulates VEGF and VEGFR2 and key down-stream protein kinases, including pTyr397-FAK, pTyr416-Src, p-38 MAPK, and p-Ser1248-PLCγ1. 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 inhibits the CXCL12/CXCR4 axis. We also found that ALCAR reduced the TNFα-induced adhesion of macrophages (THP-1) to an endothelial cell monolayer and inhibited inflammatory angiogenesis by inhibiting NF-κB activation and reduced the expression of ICAM-1. These results were consistent with *in vivo* data in the matrigel sponge assay, where we show an inhibition of angiogenesis and inflammation by substantially and significantly lowers endothelial cell 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. 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™, Lonza) supplemented with endothelial cell growth medium (EGM™SingleQuots™, Lonza), 10% Fetal Bovine Serum (FBS), 2mM L-glutamine, 100 U/mL penicillin and 100 µg/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, 2mM L-glutamine, 1% non-essential amino acids, 100 U/mL penicillin and 100 µg/mL streptomycin. The human monocytic cell line (THP-1) from ATCC were cultured in cell suspension in RPMI 1640 supplemented with 10% FBS, 2mM L-glutamine, 0.5mM β-mercaptoethanol, 100 U/mL penicillin and 100 µg/mL streptomycin. Peripheral blood mononuclear cells (PBMCs) were obtained by whole blood samples subjected to a density gradient stratification and maintained at 10^6 cells/mL density in RPMI 1640 supplemented with 10% FBS 2mM L-glutamine, 100 UI/mL recombinant human IL2 (R&D), 100 U/ml penicillin and 100 µg/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 on EBM2 complete medium and when 80% confluent were serum starved overnight. A 24-well plate, pre-chilled at -20°C, was carefully filled with 300µL of liquid matrigel (BD Biosciences) per well at 4°C and then polymerized for 1h at 37°C. 5×10^4 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 HUVEC cells either at basal level or activated with TNFα (10 ng/ml) or incubated in a hypoxic chamber (Eppendorf, Germany) at an atmosphere of 1% O_2, 94% N_2, 5% CO_2 at 37°C for 72 h, while the control cells were incubated in an atmosphere of 21% O_2 and 5% CO_2 at 37°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, $3x10^3$ cells were seeded on 4-

well chamber slides, pre-coated with 2 µg/ml fibronectin (Sigma Aldrich), for 45 min at 37°C, 5% CO_2. Following 90 mins of incubation, medium was removed, cells 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 HUVEC cells either at basal level or activated with TNFα (10 ng/ml), as indicated.

2.3.3 Migration and invasion

A modified Boyden Chamber, as described in [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 µm pore-size polycarbonate filters, pre-coated with matrigel (1 mg/ml, BD) for chemoinvasion assay and with collagen IV (50 µg/ml, Sigma Aldrich) for chemotaxis assay [2, 5] were used as the interface between the two chamber compartments. Following 6h (chemotaxis) or 24h (chemoinvasion) of incubation at 37°C in 5% CO_2, 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 HUVEC cells either at basal level or activated with TNFα (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 (25µg) 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 hour and then incubated overnight at 4°C with the following primary antibodies: anti-VEGFR2, anti-HIF-1α, anti-NF-κB p65, anti-p-38 MAPK, anti-p-Src, anti-FAK, anti-p-PLCγ1 (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 peroxidase-linked anti-rabbit IgG or anti-mouse IgG (GE Healthcare Life science) diluted at 1:3000 for 1 hour 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 TNFα (10 ng/ml) as specifically indicated.

2.6 Flow cytometry for cytokine detection

The effects of ALCAR in modulating 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 hrs. Following treatment, cells ($3x10^5$ 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 minutes at 4°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 #REA248, 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 TNFα, 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,), 2ng/ml TNFα (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 mL. 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 3Rs 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 1mg/mL CollagenaseII (Sigma Aldrich) for 30 minutes at 37°C, 5% CO_2. The cell suspension obtained was analyzed by multicolor flow cytometry for the detection of endothelial cells and macrophages.

2.8 Flow cytometry for endothelial cells and macrophage detection in vivo

The cell suspension obtained from the excised matrigel plugs was used to determine the infiltration of endothelial cells and macrophages. $3x10^5$ cells per tube were stained for 30 minutes at 4°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 according to physical parameters (FSC/SSC). Endothelial cells were identified as $CD31^+CD45^-CD3^-F4/80^-$ cells and macrophages as $CD45^+CD3^-CD31^-F4/80^+$ 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 ± 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 Figure 1A) and MTT assay (Supplemental Figure 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 Figure 1A-C). We found that ALCAR exhibited little impact on the proliferation of normal cells at the highest concentration (10 mM), while showed a significantly higher effect on the proliferation of endothelial cells (Supplemental Figure 1A-C). ALCAR might exert activities on angiogenesis by selectively targeting endothelial cells. Based on data obtained from cell proliferation assay, detection of apoptosis and cell cycle arrest (Supplemental Figure 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 (Figure 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 morphogenesis *in vitro*.

3.2 ALCAR reduces oxidative stress in hypoxic conditions

ALCAR and others carnitine acyl esters effectively protect from oxidative damage [84] by acetyling 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 hours. We found that ALCAR reduced significantly the production of superoxides in the mitochondria of HUVE cells (Supplemental Figure 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

Adhesion to the extracellular matrix, migration and invasion are key steps in the angiogenesis and 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 (Figure 2A) in a dose dependent manner. Accordingly, ALCAR at 10 mM significantly interfered with HUVEC migration (P=0.0201) on collagen IV (Figure 2B) and invasion (P<0.0001) through a matrix layer, upon FBS as chemoattractant, according to published methods [2, 5] (Figure 2C), again 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 Figure S3). CXCR4 and its ligand, CXCL12, known as key regulators in pro-angiogenic migratory phenotype [42, 50], were significantly downregulated by ALCAR at mRNA ($P<0.0001$; Figure 2D) and protein levels ($P=0.031$ and $P=0.0010$; Figure 2D-E). CCL2, that acts on mononuclear cells and indirectly acts on endothelial cells to sustain inflammatory angiogenesis [38], was also inhibited by ALCAR ($P<0.0001$) (Figure 2E).

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 (Figure 3A). This was confirmed by flow cytometry analysis (Figure 3B) and by western blot (Figure 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 levels 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 Figure S3) and protein phosphorylation level (pTyr397-FAK) (Figure 3D). The downstream pathways and VEGFR2 signaling intermediates pTyr416-Src, p-38 MAPK and p-Ser1248-PLCγ1 were also inhibited by ALCAR at 1 and 10mM (Figure 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-1α), 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) (Figure 4A). This was accompanied by a downregulation of VEGF protein level, reflecting the HIF-1α trend, while VEGFR2 regulation seems not to be dependent on hypoxia (Figure 4), but was downregulated by ALCAR.

3.6 ALCAR inhibits the activation of the NF-κB signaling pathway by TNFα

Inflammation and angiogenesis are closely related events contributing to tumor insurgence and progression and NF-κB activation is considered a master inflammatory-regulator of angiogenesis [36]. Activation of NF-κB requires the degradation of inhibitor kappa B (IκB-α) 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-κB subunits. We explored the effect of ALCAR pre-treatment on NF-κB signaling on endothelial cells activated with TNFα, using functional angiogenesis assays, immunofluorescence and western blotting. Endothelial cells exposed to a cocktail of TNFα, 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 (Figure 5). Treatment with ALCAR also blocked the translocation of NF-κB p65 into nucleus after the addition of TNFα (Figure 6A) and inhibited the phosphorylation of NF-κB (Figure 6B). This was associated with decreased VEGFR2, FAK, PECAM-1, P-selectin and ICAM-1 at the transcription level, in a dose dependent manner (Figure 7A). Data from the modulated transcripts were confirmed at the protein level by flow cytometry (Figure 7B) and western blot (Figure 7C). We found that pre-treatment with ALCAR followed by TNFα 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 (TNFα) activated endothelial layer. We found that pre-treatment of HUVE cells with 10 mM ALCAR resulted in significantly (P=0.0013) lowered number of adhered THP-1 cells on the endothelial cell layer (Figure 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 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 (Figure 9A). In addition, F4/80 FACS analysis showed a decreased population of macrophages were recruited into the matrigel plugs upon treatment (Figure 9B-C). These results demonstrated that ALCAR was able to limit VEGF and TNFα (VTH)-induced endothelial cell and macrophage recruitments, confirming the results observed *in vitro.*

4. DISCUSSION

Angiogenesis and inflammation are necessary and complementary processes to support tumor insurgence and progression [4, 6]. With this knowledge, 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]. Based on this knowledge, many efforts have been addressed in the identification of diverse agents that target angiogenesis in a preventive approach (angioprevention) and interception [6], for repurposing (such as metformin, aspirin) or using phytochemicals [6],

Carnitine, a micronutrient derived from an amino acid, is found in almost all cells of the body, and is involved in energy metabolism [33], transport of long-chain fatty acids across the membranes of mitochondria in muscle cells, and β-oxidation and the transport of fatty acids out of the mitochondria [33]. Carnitine supplementation has been largely reported to be beneficial in 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 sample from PCa patients as compared to those from individuals with BPH [3]. This finding suggests a potential protective role of carnitine against progression to 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 angiopreventive agent in two relevant microenvironment settings; hypoxia and inflammation. We also unveil the molecular mechanisms involved. We first identified a dose range of ALCAR to be potentially employed in anti-angiogenesis and angiopreventive settings. Carnitine supplements present in 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 sufficient able to reduce HUVEC 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 heathy 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 suppress 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-I/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 a 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 of 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-κB [31]. Expression of genes encoding inflammatory cytokines, adhesion molecules, enzymes and angiogenic factors are regulated by NF-κB activation [43, 59]. We showed that pre-treatment of HUVEC with ALCAR reduced TNFα mediated angiogenesis by decreasing p-NF-κB 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-κB plays a crucial role in the macrophage infiltration, also interconnected with angiogenesis that predisposes individuals towards developing cancer metastasis [35]. Targeting NF-κB 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.

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. Beside 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, suggesting a potential employment of ALCAR as a possible dietary supplement in the prevention of tumor and

inflammatory angiogenesis to be used as chemo/angioprevention approaches in subjects at high risk to develop cancer.

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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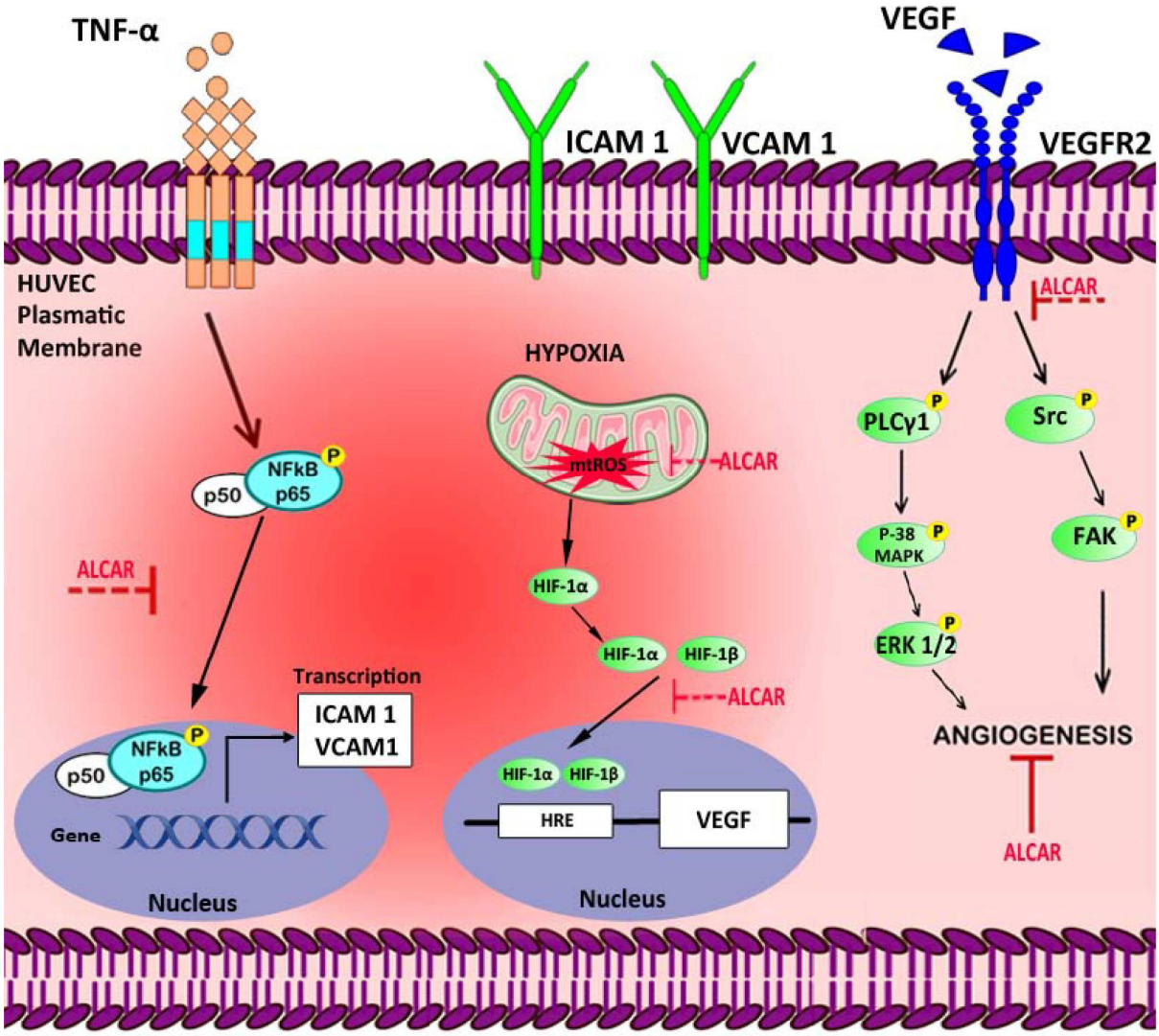
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ALCAR
P-38
MAPK
FAK
HIF-1α
ERK 1/2
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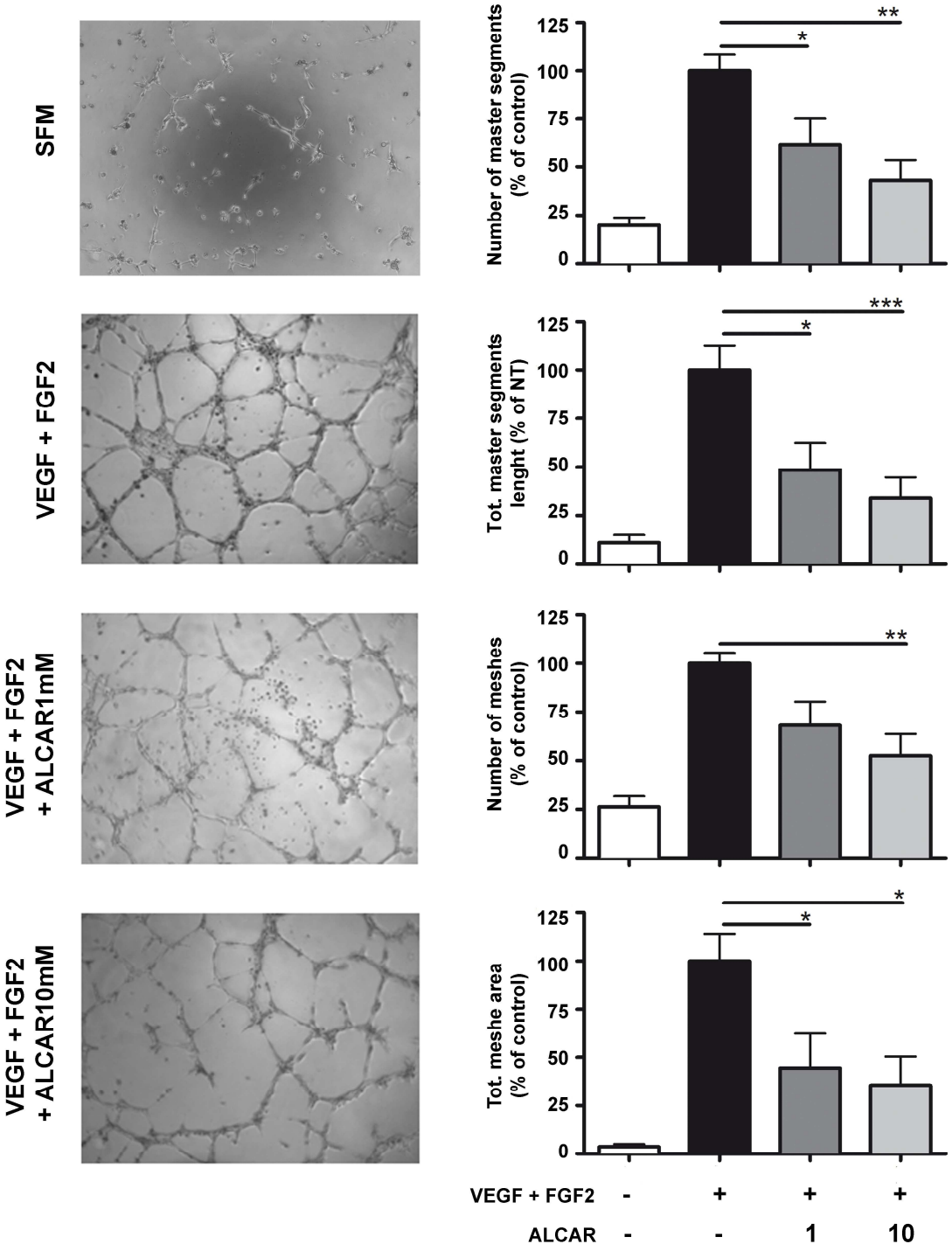
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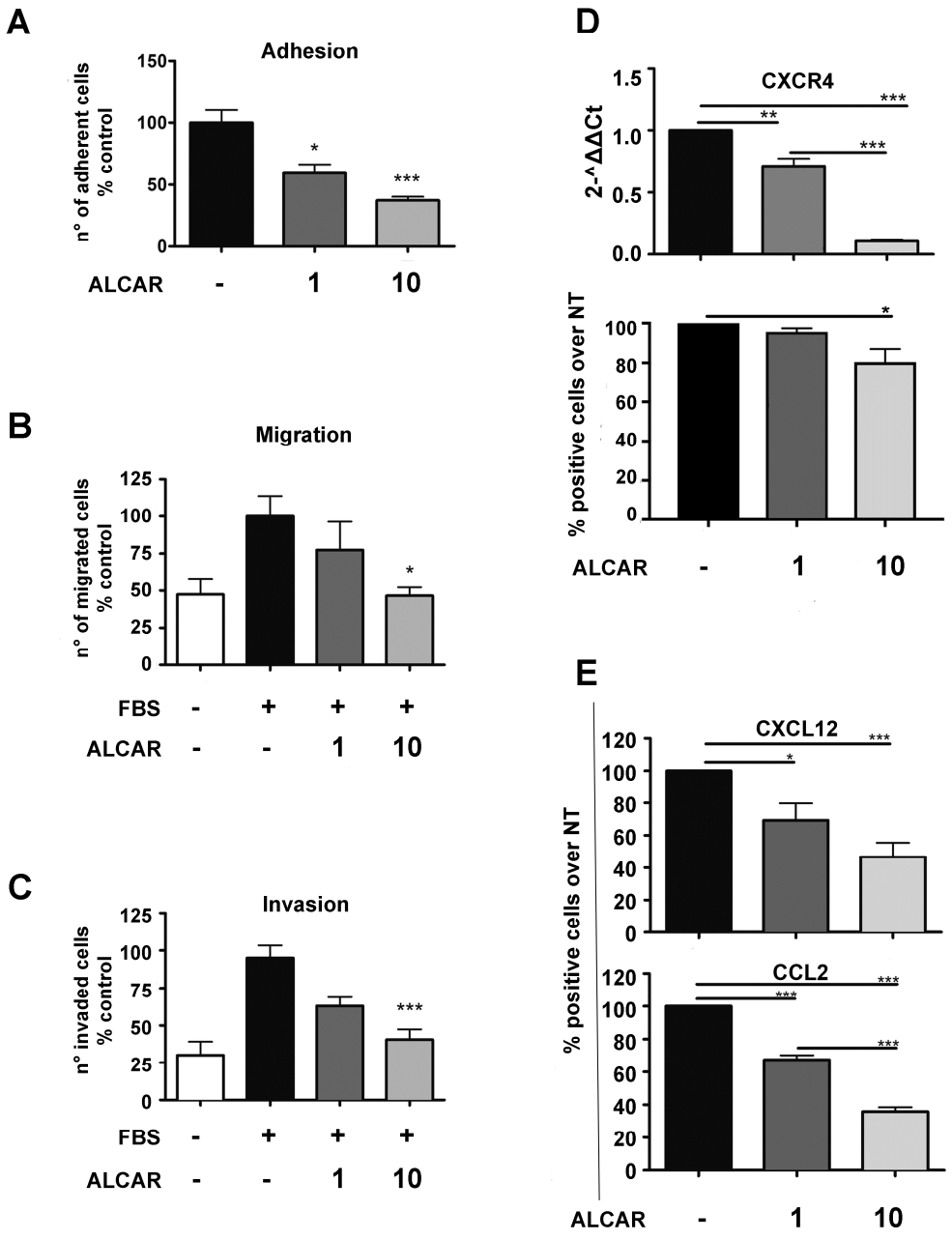
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0
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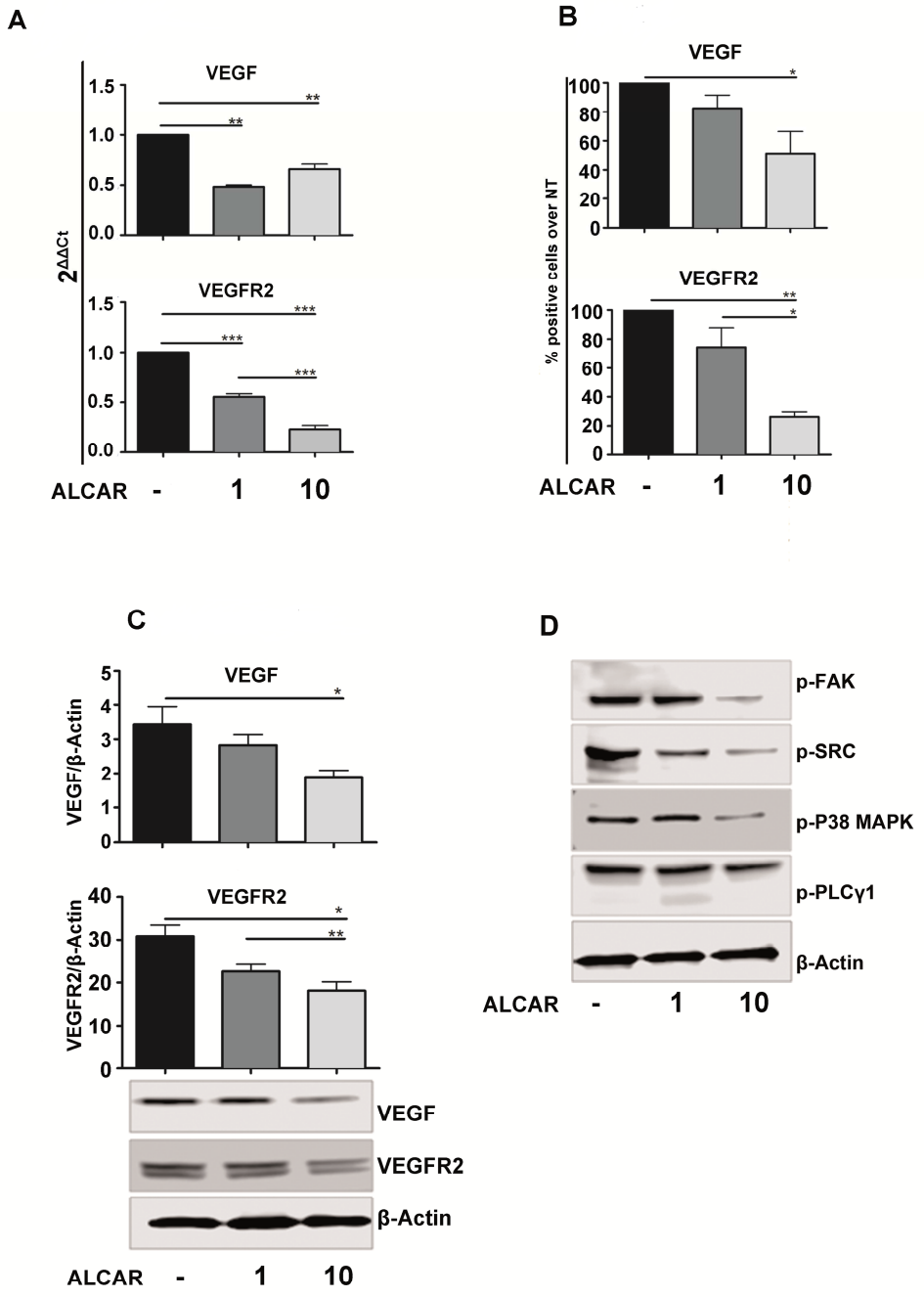
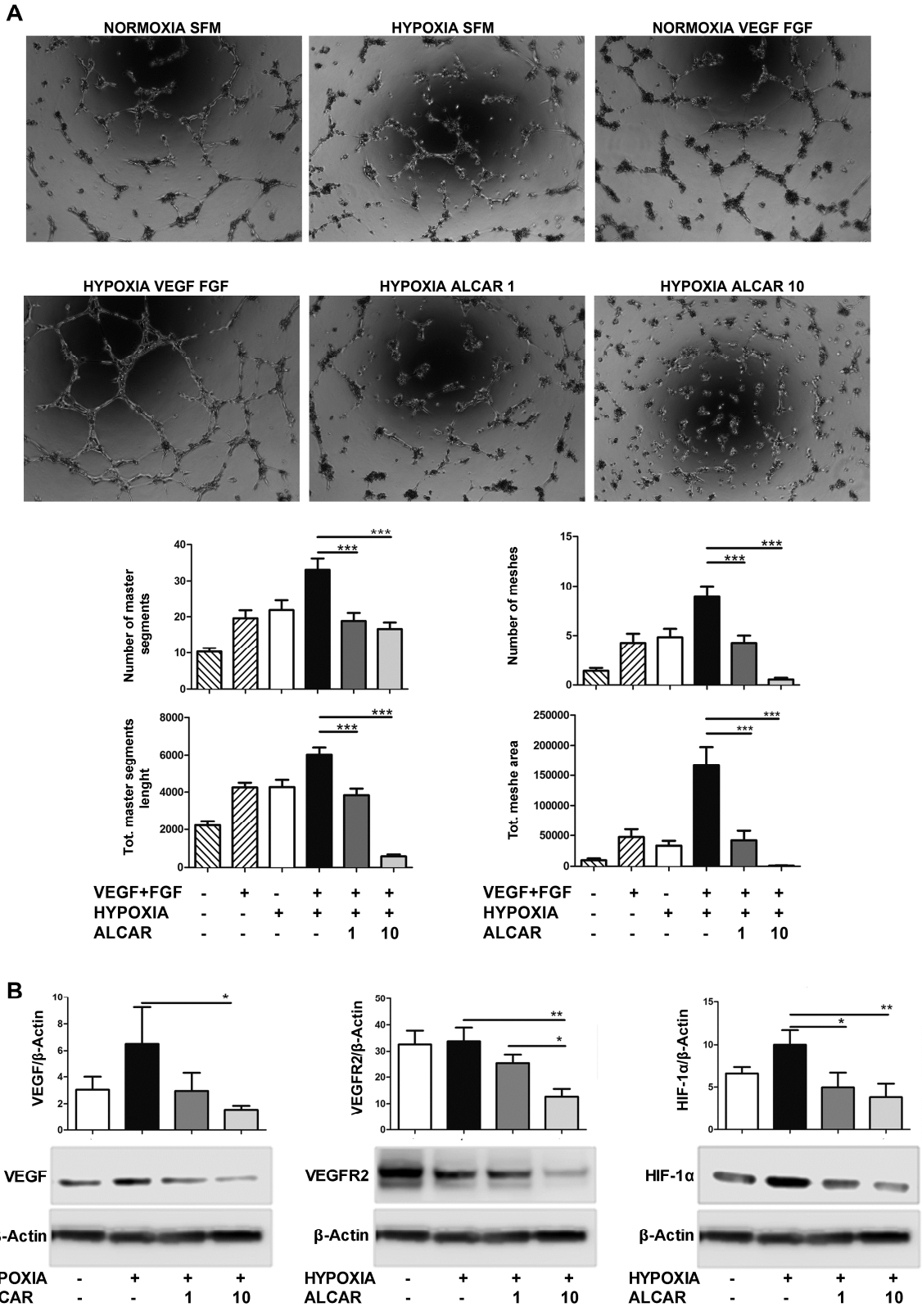
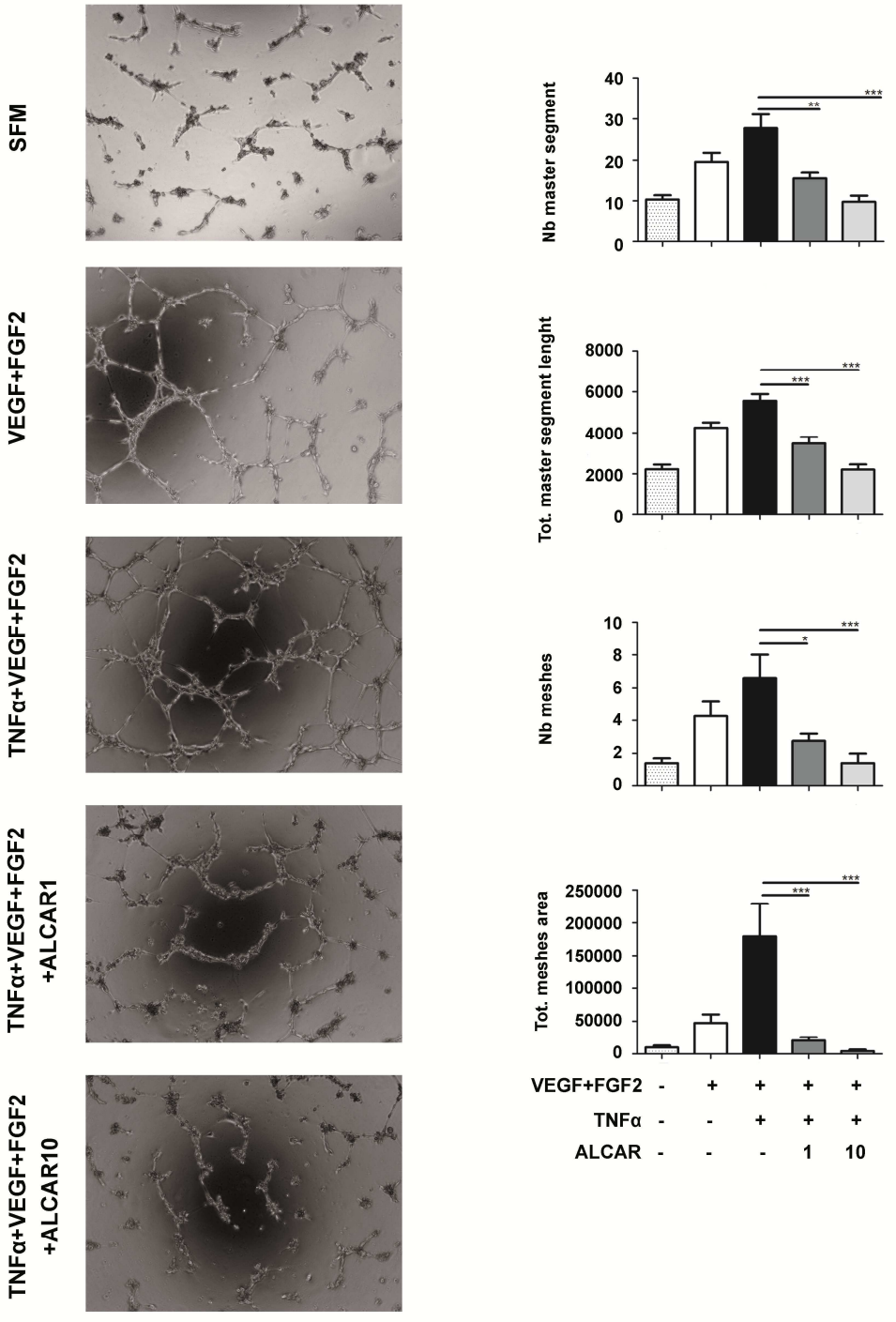
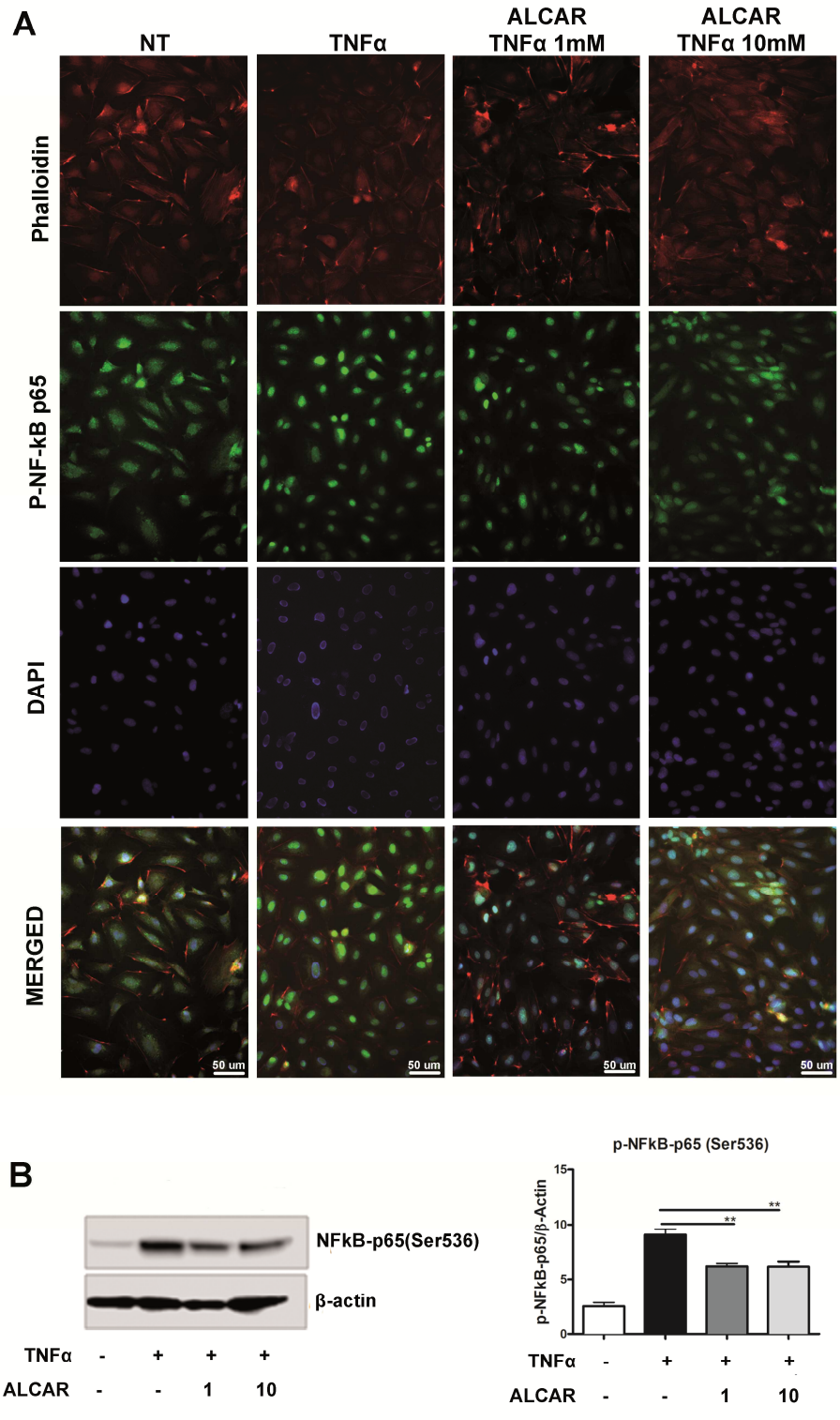
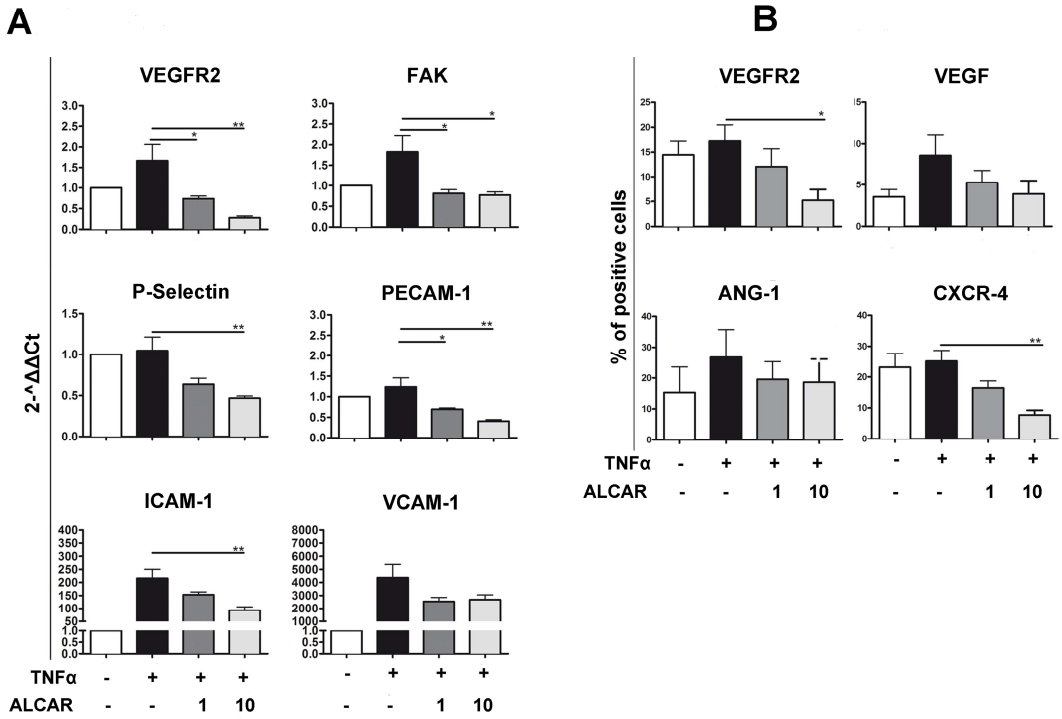
***
***
ALCAR - 1 10

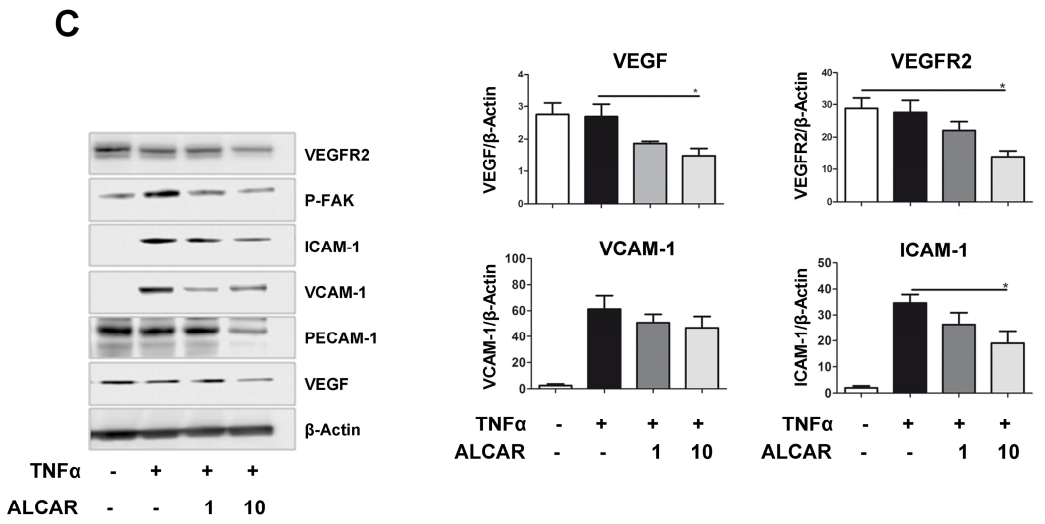

A
VEGF
VEGFR2
2ΔΔCt
1.5
1.0
0.5
0.0
**
***
ALCAR - 1 10
B
VEGF
VEGFR2
% positive cells over NT
100
80
60
40
20
0
*
**
ALCAR - 1 10
C
VEGF
VEGFR2
VEGF/β-Actin
VEGFR2/β-Actin
5
4
3
2
1
0
40
30
20
10
VEGF
VEGFR2
β-Actin
ALCAR - 1 10
D
p-FAK
p-SRC
p-P38 MAPK
p-PLCγ1
β-Actin
ALCAR - 1 10


A
NORMOXIA SFM
HYPOXIA SFM
NORMOXIA VEGF FGF
HYPOXIA VEGF FGF
HYPOXIA ALCAR 1
HYPOXIA ALCAR 10
Number of master segments
Number of meshes
Tot. master segments lenght
Tot. meshe area
VEGF+FGF - + - + + +
HYPOXIA - - + + + +
ALCAR - - - - 1 10
B
VEGF/β-Actin
VEGFR2/β-Actin
HIF-1α/β-Actin
VEGF
VEGFR2
HIF-1α
β-Actin
HYPOXIA - + + +
ALCAR - - 1 10


SFM
VEGF+FGF2
TNFα+VEGF+FGF2
TNFα+VEGF+FGF2 +ALCAR1
TNFα+VEGF+FGF2 +ALCAR10
Nb master segment
Tot. master segment lenght
Nb meshes
Tot. meshes area
VEGF+FGF2 - + + + +
TNFα - - + + +
ALCAR - - - 1 10


A
NT
TNFα
ALCAR TNFα 1mM
ALCAR TNFα 10mM
Phalloidin
P-NF-kB p65
DAPI
MERGED
50 um
B
NFkB-p65(Ser536)
β-actin
TNFα - + + +
ALCAR - - 1 10
p-NFkB-p65 (Ser536)
p-NFkB-p65/β-Actin
15
10
5
0
**
**
TNFα - + + +
ALCAR - - 1 10


A
VEGFR2
FAK
P-Selectin
PECAM-1
ICAM-1
VCAM-1
2-^ΔΔCt
TNFα - + + +
ALCAR - - 1 10
B
VEGFR2
VEGF
ANG-1
CXCR-4
% of positive cells
TNFα - + + +
ALCAR - - 1 10


C
VEGFR2
P-FAK
ICAM-1
VCAM-1
PECAM-1
VEGF
β-Actin
TNFα - + + +
ALCAR - - 1 10
VEGF
VEGFR2
VCAM-1
ICAM-1
VEGF/β-Actin
VEGFR2/β-Actin
VCAM-1/β-Actin
ICAM-1/β-Actin
TNFα - + + +
ALCAR - - 1 10

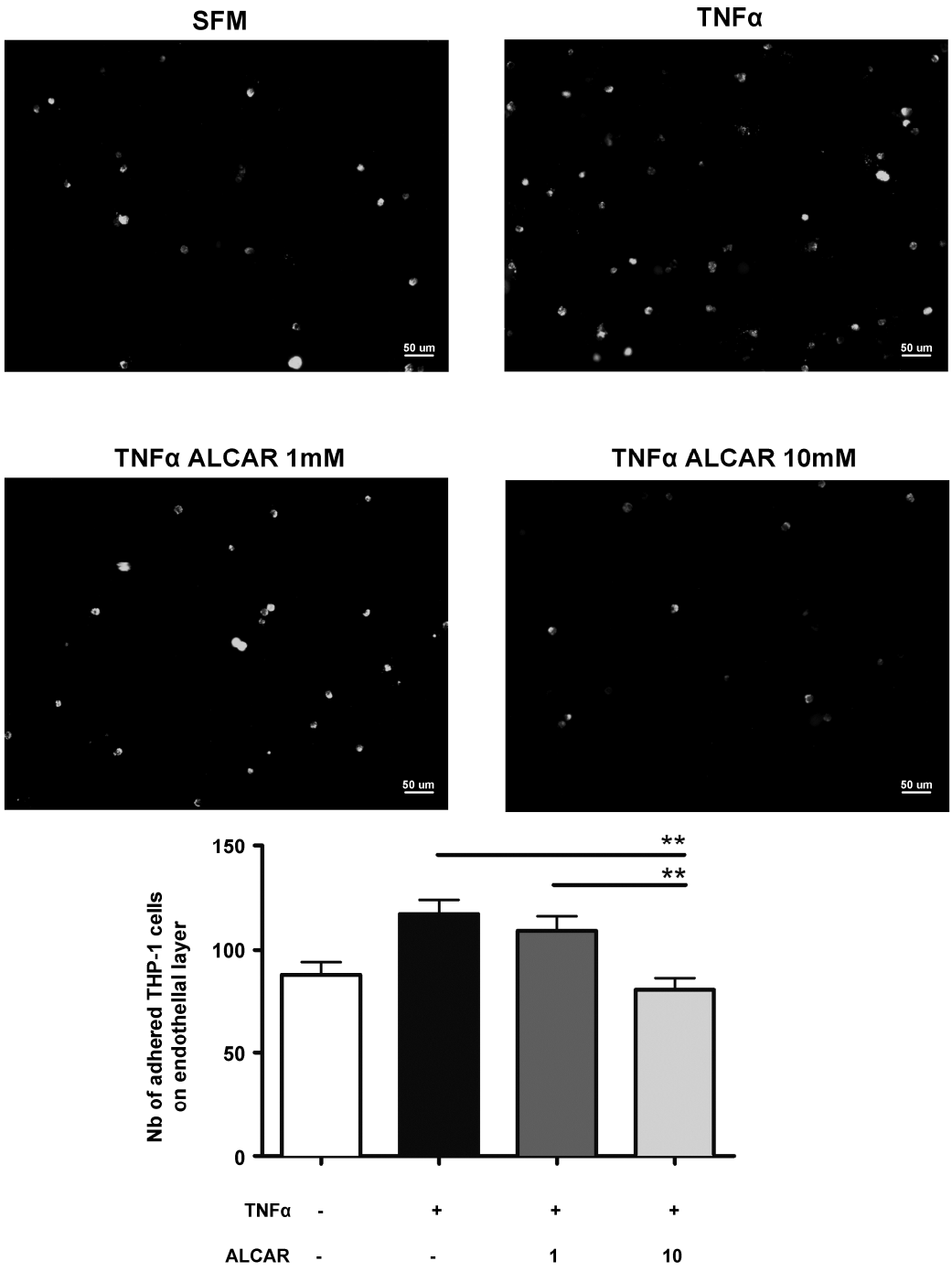

SFM
TNFα
TNFα ALCAR 1mM
TNFα ALCAR 10mM
50 um
50 um
50 um
50 um
Nb of adhered THP-1 cells on endothellal layer
150
100
50
0
**
**
TNFα - + + +
ALCAR - - 1 10

Figure legends

Figure 1: Effects of ALCAR on capillary-like structure formation of HUVE cells. HUVE cells were pretreated with ALCAR at 1 and 10mM for 24 hours or treated with vehicle alone, were then placed in 96-well plates coated with a layer of Matrigel (15×10^3 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 5X magnification and quantified by the Angiogenesis analyzer ImageJ tool kit. Pre-treatment with ALCAR at 1 and 10mM for 24 hours 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.

Figure 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 10mM. The gene expression of CXCR4 is quantified relatively to NT cells, normalized to the housekeeping gene, 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 ± SEM *p<0.05; **p<0.01; ***p<0.0001 as indicated by the bars (One-Way ANOVA).

Figure 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 10mM after 24h. 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-PLCγ.

Figure 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 24h resulted in significantly reduced ability to form capillary-like structures on matrigel compared to vehicle treated HUVE cells. Microphotographs were taken at 5X magnification, representative images are shown. Images were quantified by Angiogenesis analyzer ImageJ tool kit. Data are showed as Mean ± 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).

Figure 5: Effect of ALCAR on TNFα-induced capillary-like structure formation

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

Figure 6: ALCAR inhibits the TNFα-induced p65 phosphorylation and nuclear translocation

(A) HUVE cells were incubated with ALCAR 1 and 10mM for 6h and followed by TNFα (10 ng/ml) stimulation for 30 min. After fixation, cells were stained with specific anti-NFκB-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 20X magnification objective (Axio Observer A1, Zeiss, Germany). Scale bars: 50 µm. 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-NFκB-p65 (Ser536) in HUVE cells incubated with ALCAR 1 and 10 mM for 6h, followed by TNFα (10 ng/ml) stimulation for 30 min. The graphs show quantification of anti-NFκB-p65 (Ser536) indicating significantly differences (P< 0.0001) of ALCAR treated *vs* non-treated cells. Data are expressed as Mean ± SEM (n= 5 independent experiments).

Figure 7: ALCAR inhibits TNFα induced proangiogenic and NFκB-regulated pathways.

(A) qPCR analysis showing 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 24h and followed by TNFα (10 ng/ml) stimulation for 30 min as compared to non-treated cells. mRNA levels [2^ (-delta delta Ct)] were calculated relatively to TNFα-stimulated HUVE cells, normalized to the housekeeping gene, GAPDH (n= 4 independent experiments). (B) FACS analysis showing a downregulation of angiogenic markers (VEGF, VEGFR2 and ANGIOP-1) and invasion markers (CXCR4), expressed as percentage of positive cells over TNFα-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 ± SEM *p<0.05; **p<0.01; ***p<0.0001 *vs* TNFα-treated cells or as indicated by the bars (One-Way ANOVA).

Figure 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 HUVE cells with ALCAR and activation with TNFα (10 ng/ml), calcein AM-labelled THP-1 cells were added on a top of HUVEC monolayer and incubated for 3h. After washing to remove non-adherent monocytes, calcein-AM-labelled THP-1 cells (green) adhering to HUVECs were counted. Results are showed as Mean ± SEM *p<0.05; **p<0.01; ***p<0.0001 *vs* TNFα- activated cells as indicated by the bars (One-Way ANOVA).

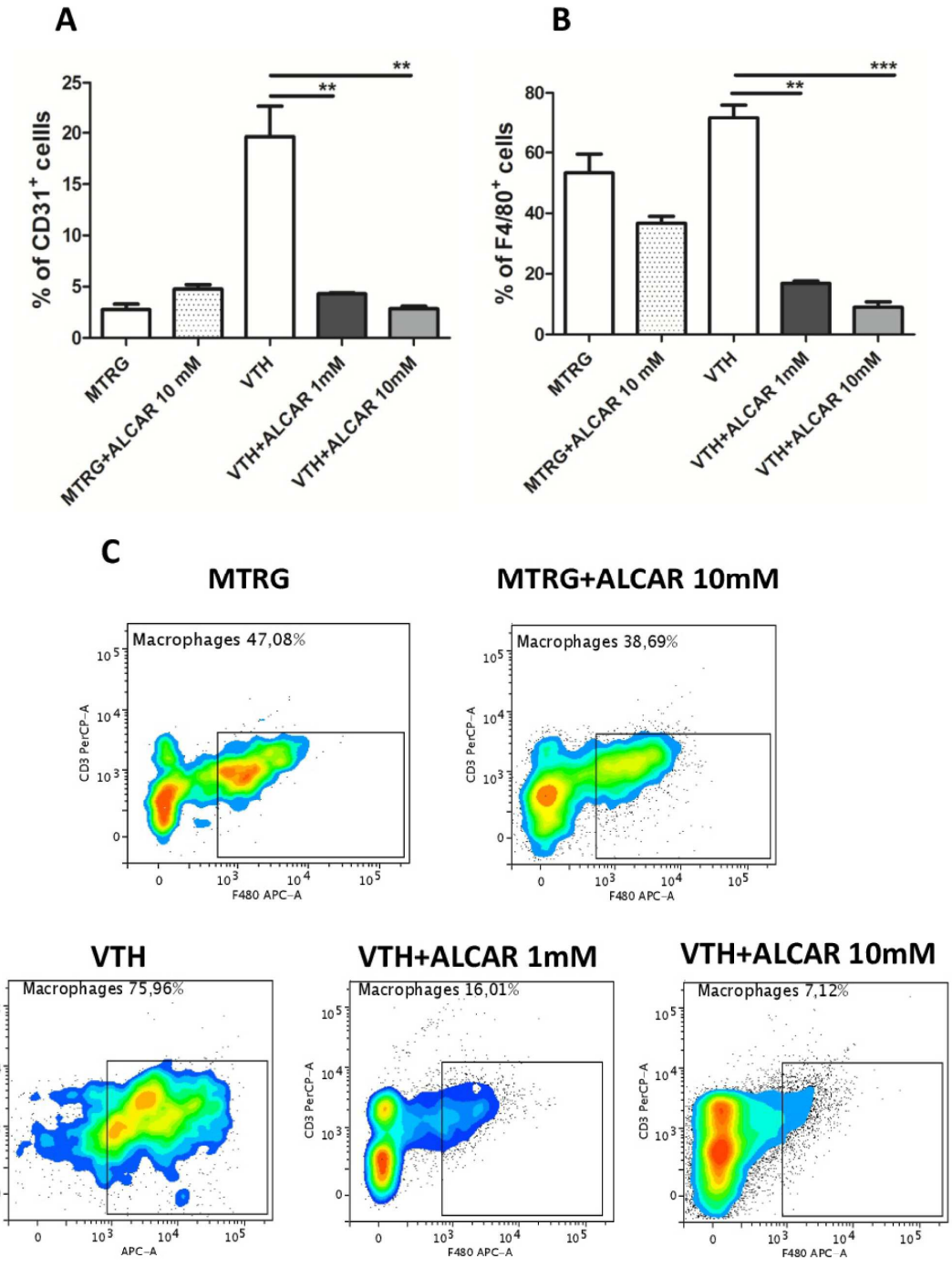
Figure 9: ALCAR inhibits angiogenesis *in vivo*

Flow cytometry analysis for endothelial cells and macrophage infiltration in the excised Matrigel plugs revealed ALCAR ability to inhibit VTH-induced A) $CD31^+$ endothelial cell infiltration and B) $CD45^+F4/80^+$ 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 ± SEM *p<0.05; **p<0.01 *vs* controls, One-Way ANOVA.

Figure 10: Schematic presentation of the anti-angiogenic signalling pathways regulated by ALCAR in HUVE cells

Proposed mechanism for the inhibition of angiogenesis by ALCAR: *i*) via inhibition of VEGF/VEGFR2-mediated signalling pathways; *ii*) via downregulation of HIF-1α and VEGF and *iii*) by blocking the activation of NF-κB, and downregulation of ICAM. Arrows indicate regulations by ALCAR treatment observed in our experiments.


A
% of CD31+ cellls
25
20
15
10
5
0
**
**
MTRG
MTRG+ALCAR 10 mM
VTH
VTH+ALCAR 1mM
VTH+ALCAR 10mM
B
% of F4/80+ cells
80
60
40
20
0
***
**
MTRG
MTRG+ALCAR 10 mM
VTH
VTH+ALCAR 1mM
VTH+ALCAR 10mM
C
MTRG
Macrophages 47,08%
MTRG+ALCAR 10mM
Macrophages 38,69%
VTH
Macrophages 75,96%
VTH+ALCAR 1mM
Macrophages 16,01%
VTH+ALCAR 10mM
Macrophages 7,12%
CD3 PerCP-A
F480 APC-A
PerCP-A
APC-A

Highlights.

- ALCAR targets VEGF/VEGFR2 axis and its downstream signaling intermediates.
- ALCAR blocks endothelial cell migration/invasion acting on CXCR4/ CXCL12 and CCL2.
- ALCAR downregulates inflammatory induced angiogenesis by inhibiting NF-κB and ICAM-1.
- ALCAR decrease endothelial cells and macrophage recruitment *in vivo.*