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ORIGINAL PAPER

Diminished vasculogenesis under inflammatory conditions is mediated by Activin A

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Abstract

Severe inflammatory stress often leads to vessel rarefaction and fibrosis, resulting in limited tissue recovery. However, signaling pathways mediating these processes are not completely understood. Patients with ischemic and inflammatory conditions have increased systemic Activin A level, which frequently correlates with the severity of pathology. Yet, Activin A's contribution to disease progression, specifically to vascular homeostasis and remodeling, is not well defined. This study investigated vasculogenesis in an inflammatory environment with an emphasis on Activin A's role. Exposure of endothelial cells (EC) and perivascular cells (adipose stromal cells, ASC) to inflammatory stimuli (represented by blood mononuclear cells from healthy donors activated with lipopolysaccharide, aPBMC) dramatically decreased EC tubulogenesis or caused vessel rarefaction compared to control co-cultures, concurrent with increased Activin A secretion. Both EC and ASC upregulated Inhibin Ba mRNA and Activin A secretion in response to aPBMC or their secretome. We identified TNFα (in EC) and IL-1β (in EC and ASC) as the exclusive inflammatory factors, present in aPBMC secretome, responsible for induction of Activin A. Similar to ASC, brain and placental pericytes upregulated Activin A in response to aPBMC and IL-1β, but not TNFα. Both these cytokines individually diminished EC tubulogenesis. Blocking Activin A with neutralizing IgG mitigated detrimental effects of aPBMC or TNFα/IL-1β on tubulogenesis in vitro and vessel formation in vivo. This study delineates the signaling pathway through which inflammatory cells have a detrimental effect on vessel formation and homeostasis, and highlights the central role of Activin A in this process. Transitory interference with Activin A during early phases of inflammatory or ischemic insult, with neutralizing antibodies or scavengers, may benefit vasculature preservation and overall tissue recovery.

Keywords Inflammation · Vasculogenesis · Activin A · Pericytes · Endothelial Cell · TNFα/IL-1β

Abbreviations

EC	Endothelial cell
CBEC	Cord-blood endothelial cell
HUVEC	Human umbilical vein endothelial cell
HMVEC	Human cardiac microvascular endothelial cell
ASC	Adipose stromal cells
PBMC	Peripheral blood mononuclear cells
aPBMC	Activated peripheral blood mononuclear cells
TNFα	Tumor necrosis factor alpha
IL-1β	Interleukin-1 beta
IFNγ	Interferon gamma
IL-6	Interleukin 6
LPS	Lipopolysaccharide
FST	Follistatin
FSTL3	Follistatin-like 3

Introduction

Diseases associated with vascular pathologies, such as myocardial infarction, brain insult, kidney diseases, heart failure, and critical limb ischemia, are the leading causes of morbidity and mortality in economically developed countries. Worldwide, approximately 153 million people are affected by ischemic heart diseases, 120 million by peripheral vascular diseases, and 67 million by ischemic stroke [1]. Many patients are unable to re-establish vascular networks sufficiently to compensate for the loss of the original blood supply, leading to progressive loss of function, tissue fibrosis, and eventually organ failure. Therefore, improving tissue revascularization has been a major focus of therapeutic

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interventions. Unfortunately, current interventions based on local delivery of angiogenic proteins, genes, or cells have not produced the desired effects. This is likely due to incomplete understanding of vascular cell responses to systemic and local pathological processes, including hypoxia, inflammation, and local spikes in reactive oxygen species [2, 3].

Studies have shown that hypoxia stimulates expression of factors that promote endothelial cell (EC) survival, proliferation, and tubulogenesis. However, these weak reparative responses are overcome by strong pathological processes, driven by increased numbers and activities of inflammatory cells at the site of injury, the net result of which is an anti-angiogenic and pro-fibrotic environment [4–8]. While limited inflammation benefits reparative processes, prolonged inflammatory stress is detrimental [9–12]. As such, chronic systemic inflammation is associated with increased risk of heart attack and stroke, and anti-inflammatory therapies reduce cardiovascular events [13, 14].

Many studies have revealed that ischemia and inflammation are associated with elevated Activin A that often correlates with disease severity [15–20]. Systemic Activin A is elevated in patients with pulmonary diseases (twofold) [21], atherosclerosis (40%) [15], myocardial infarction (80%) [22, 23], dilated cardiomyopathy [24], heart failure [25] with preserved ejection fraction [21, 26], kidney diseases [27–29], and pre-eclampsia (tenfold) [30]. The effects of Activin A on organ recovery are contradictory and, most likely, tissue dependent: it is neuroprotective of ischemic brain [31, 32] but promotes disease progression in ischemic kidney [33, 34]. Opposite effects were reported for Activin A in the immune system, in modulation of inflammation [35], and in ischemic hearts [22, 36]. While many ischemia- and inflammation-associated pathologies are accompanied by significant upregulation of Activin A, the role of Activin A in vessel homeostasis and remodeling in the inflammatory environment has not been addressed.

Activin A, a homodimer of Inhibin Ba and member of the TGFβ family of pleiotropic factors, is secreted by many cell types. It signals through type I receptor ALK4 and type II receptors ACVR IIA and IIB, and its activity is endogenously inhibited by follistatin and follistatin-like 3. The role of Activin A in vessel formation, maintenance, and remodeling is poorly understood, and the mechanisms and physiological implications of its induction in ischemic tissues are understudied. Several groups have shown that Activin A modulates vasculogenesis and angiogenesis; however, its contribution to these processes is also contradictory [37–42]. We have recently shown that interactions between endothelial and perivascular cells result in upregulation of Activin A expression. We postulated that expression of Activin A is necessary for the formation of mature vessels, but only at appropriate stages of vessel formation because we noted that premature expression of Activin A diminishes the efficiency of vasculogenesis due to its anti-angiogenic/angiostatic activities.

Independent of its effects on vasculogenesis and angiogenesis, Activin A also displays context-dependent pro- or anti-inflammatory [35] and fibrotic activities [43]. Some studies suggest that Activin A initiates the inflammatory cascade [44, 45], but others report its anti-inflammatory effects. It has been firmly established that inflammation affects vessel homeostasis and remodeling; however, how inflammation affects expression of Activin A in endothelial, mural (pericytes and smooth muscle), and perivascular stromal/mesenchymal cells has not been explored.

This study addressed the effects of activated peripheral blood mononuclear cells (aPBMC) on expression of Activin A and key members of its signaling pathway in vascular cells in order to define the role of Activin A in the impaired vasculo-/angiogenesis that occurs during inflammation mediated by inflammatory cells.

Materials and methods

Cell preparations

Procedures for adipose tissue and cord-blood collection were approved by the Indiana University School of Medicine IRB. Human adipose stromal cells (ASC) and cord-blood endothelial cells were isolated and expanded as previously described [46, 47] and used at passages 3–6. Human umbilical vein endothelial cells (HUVEC) and human microvascular endothelial cells (HMVEC) were purchased from Lonza, and human brain vascular pericytes were purchased from IX Biotechnologies. Placental pericytes were gift from Dr. Gonzalez (Yale University). Procedures for collecting blood were approved by the University of Florida IRB. Human peripheral blood mononuclear cells (PBMC) were isolated from whole blood of healthy donors (25–35 year old, both genders). Blood was drawn into CPT tubes prefilled with Ficoll Hypaque solution (BD Biosciences), and PBMC were separated by density gradient centrifugation at 1500 g for 20 min. Isolated PBMC were re-suspended in EBM-2/5%FBS media (Lonza) and were used in experiments untreated, or activated with 100 ng/ml lipopolysaccharide (LPS, Sigma) for 15 min at room temperature [48], or cryopreserved.

EC-ASC vasculogenic models

The two-dimensional vasculogenesis model used for the initial studies was established as previously described, with minor modifications [49]. 6×10^4 cells/cm^2 ASC and 1×10^4 cells/cm^2 EC were premixed and plated in EBM-2/5%FBS. 24 h later, some wells were augmented with either untreated or LPS-activated PBMC (6×10^4 cells/cm^2) and cultured

for 2 days, followed by media exchange to EBM-2/5%FBS alone. In a subset of tests, on day 1 after plating of EC-ASC co-cultures, media were augmented with 10 ng/ml TNFα and 10 ng/ml IL-1β with or without either Activin A neutralizing IgG (RnD, MAB3381) or isotype control (RnD, MAB002) IgG for 2 days and incubated for an additional 3 days in control media. Another subset of EC-ASC co-cultures was incubated in DMEM/F12 media containing bovine serum albumin (0.25%, Sigma), ITS Media Supplement (Sigma), bFGF (5 ng/ml, RnD Systems), and SDF-1 (4 ng/ml, RnD Systems), and exposed to inflammatory cell secretome, cytokines, and IgG. To reveal EC-vascular cords, co-cultures were fixed in methanol (− 20 °C, 5 min) and subsequently incubated with biotinylated Ulex Europaeus Agglutinin I (Vector labs) for 1 h and Streptavidin Alexa 488 (Invitrogen) for 30 min. Automatically acquired images of fluorescently labeled EC-cords (9 images, 30% of the well surface) were collected and the combined total lengths of vascular cords calculated using MetaMorph software (Molecular Devices, Downingtown, PA) as previously described [49].

The three-dimensional vasculogenesis co-culture model was established by combining DsRed-expressing EC (6×10^4), ASC (1.5×10^4), and aPBMC (3×10^4) in 60 µl fibrinogen (final concentration 2.5–5 mg/ml) and plating in 96-well plates containing 3 µl thrombin (10U/ml) per well. In addition, subsets of mixtures were augmented with either Activin A IgG or isotype IgG (10 µg/ml). Polymerized gels were overlaid with EBM-2/5%FBS ± Activin A/isotype control IgG (10 µg/ml). Images of developing networks were acquired using fluorescent Nikon Ti microscope.

EC, ASC, and pericyte co-cultures with PBMC

EC, ASC, or pericytes, plated at 6×10^4 cells/cm^2 in EBM-2/5%FBS, were exposed to equal amounts of non-activated or activated PBMC 24 h after plating.

In a subset of tests, EC and PBMC or ASC and PBMC were incubated in the same wells but physically separated by 0.4 µm-pore-diameter Costar® Transwell® Permeable Supports (Corning Inc.) to prevent direct contact. In addition, EC + aPBMC and ASC + aPBMC co-cultures were incubated in the presence of either IL-1β neutralizing IgG (3 µg/ml) and TNFα neutralizing IgG (0.5 µg/ml) or matching isotype control IgG (all from RnD Systems) for 24 h. In parallel, EC monocultures were subjected to the following: (1) individually to TNFα, IL-1β, IFNγ, and IL-6 (all at 10 ng/ml; Gemini); (2) 50% secretome of unmodified (PBMC-S) or activated PBMC (aPBMC-S); both either unmodified or pre-treated with either IL-1β (3 µg/ml) and TNFα neutralizing IgG (0.5 µg/ml) or isotype control IgG for 1 h at 37 °C. PBMC-S (the conditioned media collected from PBMC cultures) were generated by incubating 10^6 PBMC (or aPBMC) per 1 ml of EBM-2/5%FBS for 24 h, followed by removal of floating cells and debris by media centrifugation at 500 g for 5 min. Media collected from PBMC and EC mono- and co-cultures were evaluated for Activin A, FST, and $TGF_{\beta1}$ using enzyme-linked immunosorbent assays kits from RnD Systems.

RNA isolation and RT-qPCR

Total RNA was isolated from cells using NucleoSpin RNA II kit (Macherey–Nagel) and reverse transcribed to cDNA using iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad). qPCR reactions were performed using TaqMan Fast Advanced Master mix and TaqMan probes purchased from Thermo Fisher Scientific, on the QuantStudio 3 Real-Time PCR System (Applied Biosystems). Expression of β-actin was used for normalization and was unaffected by treatments. Gene expression levels were analyzed using QuantStudio™ Design & Analysis Software.

SDS-PAGE and Immunoblotting

Cells were lysed with RIPA buffer-containing protease and phosphatase inhibitors (Thermo Fisher Scientific). The lysates were then clarified, and protein concentration was determined by Bradford assay. Samples were resolved on 12% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad), transferred onto PVDF membranes (Bio-Rad), blocked in 5% Milk/TBS, and probed for SMAD 2,3 (1:1000, cat#8685), pSMAD2 (1:500, cat#3108), and β-actin (1:5000, cat#3700). Secondary antibodies were either HRP-conjugated anti-rabbit IgG (1:3000, cat#7074) or anti-mouse IgG (1:3000, cat#7076). All antibodies were from Cell Signaling Technologies. The bands were visualized with SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific), and imaged on Chemi-Doc Imaging system (Bio-Rad).

Analysis of vascularization of subcutaneous implants

Animals were cared for in accordance with guidelines published by the National Institutes of Health, and the study procedures were approved by the University of Florida Institutional Animal Care and Use Committee. 1.6×10^6 vascular cells (EC and ASC mixed 1:1) alone or with either PBMC or aPBMC (at 8×10^5) were re-suspended in 100 µl of PBS/thrombin (6 U/ml) and combined with 300 µl of a semisolid matrix composed of 1.5 mg/ml collagen, 50 µg/ml fibronectin, 5 mg/ml fibrinogen, 10% FBS, and 25 mM HEPES with pH 7.5 (adjusted with 1N NaOH). In a subset of tests, cell/matrices were augmented with Activin A neutralizing IgG or mouse isotype control IgG (both at 10 µg/ml). Immediately after combination, the mixtures were subcutaneously

injected on the back and flanks of immune-compromised Nude mice (The Jackson laboratories). Implants were harvested 7–9 days later, preserved with 10% formalin, paraffin embedded, and sectioned. Human EC-derived vessels were revealed by staining thin sections of implants with anti-human CD31 IgG (Invitrogen), followed by incubation with biotinylated horse anti-mouse IgG, VECTASTAIN® ABC Reagent (both Vector), and DAB substrate (Sigma) sequentially. Nuclei were visualized by hematoxylin (Sigma) counterstaining.

Rat Aorta assay

Aortas and blood were obtained from Sprague–Dawley young adult male rats. Rat PBMC were immediately isolated and activated as described above for human cells. Aortas were sectioned into 2-mm-long rings, placed into DMEM/F12 media, and overlaid with either non-activated or LPS-activated PBMC and incubated for 24 h. Then, conditioned media were collected and assessed for Activin A by ELISA.

Statistical analysis

Data analysis was performed using Prism 8 (GraphPad). Results are expressed as mean ± SEM. Sample number (n) per experiment is noted in the figure legends. Each experimental set was repeated at least 3 times.

Statistical analysis was conducted using two-tailed *t* test or ANOVA with suitable tests for normality. For datasets with normal distribution, two-tailed unpaired Student's *t* test was used for comparison between two groups, and one-way ANOVA with post hoc Tukey multiple comparisons was used for groups ≥ 3. A $p < 0.05$ was considered significant. No statistical method was used to calculate sample size, and no sample values were excluded during analysis.

Results

Activated PBMC limit EC vasculogenesis

EC-cultured atop ASC monolayers organize into dense networks of vascular cords (Fig. 1a). Introduction of LPS-activated PBMC to EC-ASC co-cultures, during days 1 to 3 of six days in culture, decreased vessel density by almost 50% (Fig. 1a), while non-activated PBMC had no such effect. The decrease in vessel density in co-cultures with aPBMC was accompanied by a significant decline (41.6%) in EC survival (Fig. 1b), but no change in ASC enumeration (not shown). This effect, at least partially, was attributed to decrease in EC tolerance to aPBMC—loss of cells in subconfluent monolayers of EC exposed to aPBMC for 4 days (Fig. 1c). These findings were further supported by the observations made with three-dimensional vasculogenic model where EC, ASC, and aPBMC were co-embedded in fibrin gel: at day 6 of incubation, the density of interconnected vascular cords in the gel with aPBMC was 25% lower compared to aPBMC-free gels (Fig. 1d, e). In addition to the detrimental effects on tubulogenesis during the initial phases of vessel organization, aPBMC induced-rarefaction of established vascular structures. Co-cultures exposed to aPBMC between days 6 and 9 of incubation resulted in a 75% decrease in vessel density compared to control cultures without aPBMC (Fig. 1f, g).

Inflammatory cells upregulate Activin A in EC and mural cells

Analysis of media conditioned by co-cultures of vasculogenic cells, when incubated alone or with aPBMC in two- and three-dimensional vasculogenesis models, revealed a substantial accumulation of Activin A in aPBMC-enriched co-cultures (Fig. 1h). Similarly, ex vivo rat aorta segments incubated with rat aPBMC secreted 3.5-fold more Activin A than vessels cultured either alone or with non-activated PBMC (Fig. 1i).

To test which vascular cells responded to aPBMC with increased Activin A secretion, aPBMC were applied to EC or ASC monolayers. Independent of both the source of EC used in this test (cord blood, cardiac microvasculature, and umbilical cord) and of Activin A secretion at baseline, aPBMC treatment led to 2.3–4.9-fold increase in Activin A accumulation in incubation media (Fig. 2a). A strong induction of Activin A was also observed when cord-blood-derived EC were treated with cell-free aPBMC secretome (Fig. 2b) or when EC and aPBMC were incubated in the same well, but spatially separated by 0.4 µm pore membrane (Fig. 2c); however, level of Activin A induction was lower than in direct co-cultures. No Activin A was detected in the media conditioned by aPBMC monocultures and EC + non-activated PBMC co-cultures (Fig. 2c). In parallel, slight increases in mRNA expressions of endogenous inhibitors of Activin A, follistatin, and follistatin-like-3 were observed in EC + aPBMC co-cultures (Fig. 2d).

Complementarily, ASC were exposed to either PBMC, or aPBMC, or their secretome for 24 h. Intact ASC had undetectable Activin A, but their exposure to aPBMC or aPBMC secretome resulted in a dramatic increase in Activin A secretion (Fig. 2e, f). When ASC and aPBMC were co-cultured with physical separation, a lower level of Activin A in incubation media was detected (Fig. 2g), as with EC + aPBMC. ASC + aPBMC co-cultures were also characterized by significant increase in follistatin mRNA expression and minor increase of follistatin-like-3 mRNA (Fig. 2h).

Extending these results, a similar upregulation or induction of Activin A was observed in placental and brain

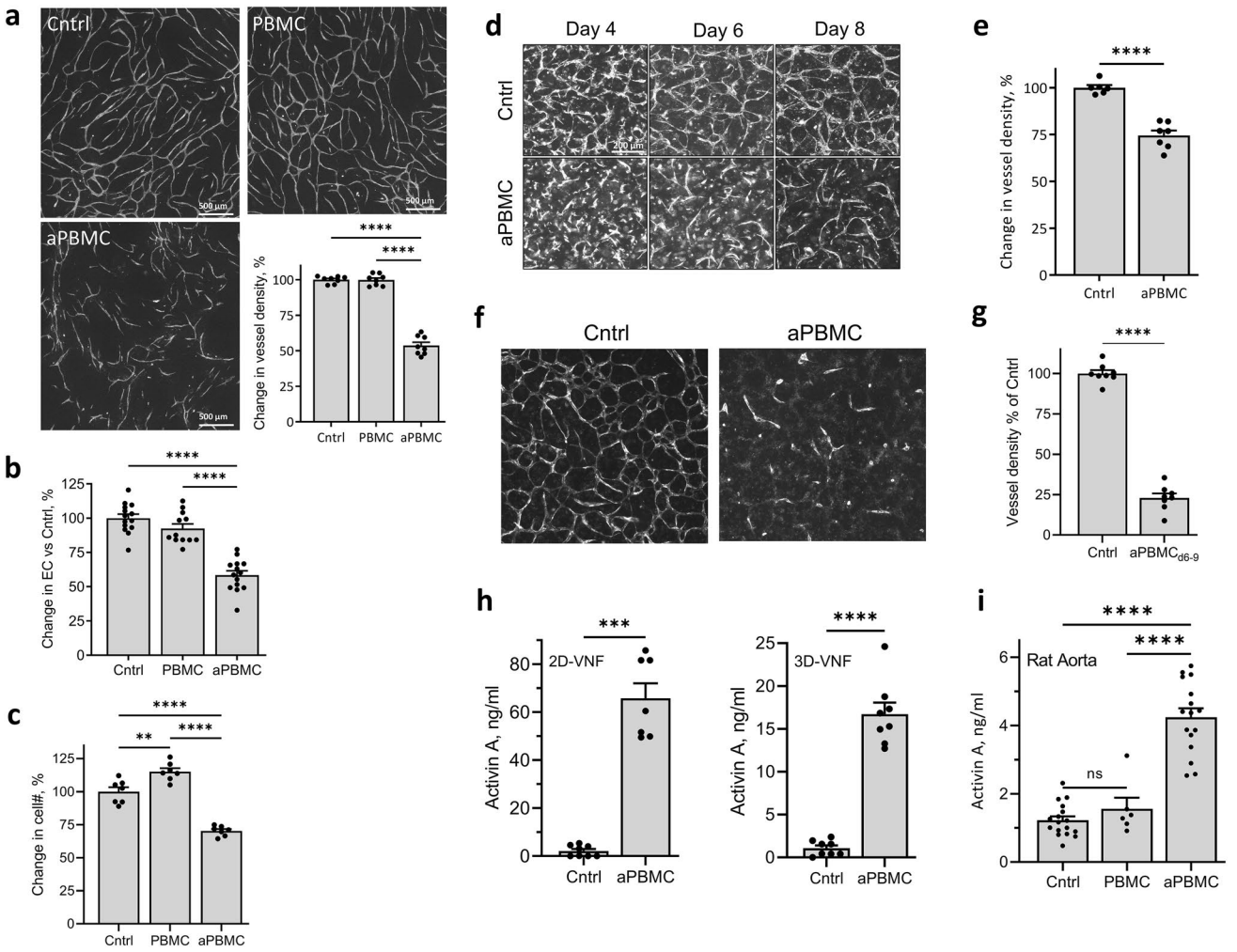

Fig. 1 Activated peripheral blood mononuclear cells (aPBMC) limit vessel formation, and upregulate Activin A secretion in 2D and 3D co-cultures of endothelial cells (EC) and adipose stem cells (ASC). **a**, Representative fluorescent images and relative quantitative analysis of the density of the vessels ($n=8$) established by EC + ASC co-cultures after 6 days of incubation. Co-cultures were incubated either alone, or in the presence of non-activated, or activated PBMC (aPBMC) for the first 3 days. Vessels were revealed by staining co-cultures with EC marker Ulex lectin (white). **b**, Relative numbers of extant EC detected in EC + ASC co-cultures incubated alone, with PBMC, or with aPBMC analyzed on day 6 of incubation ($n=12$–14). **c**, Cellular enumeration in EC monocultures after exposure to PBMC or aPBMC for 4 days ($n=3$). **d** and **e**, Representative fluorescent images (day 4, 6, and 8) (**d**) and relative quantitative analysis on day 6 (**e**) of vascular networks established by DsRed-expressing EC (white) when encapsulated with ASC in 3-dimensional fibrin gel and incubated either alone or in the presence of aPBMC for the first 3 days of incubation (n = 6–7). **f** and **g**, Representative fluorescent images (**f**) and relative quantitative analysis (**g**) on day 9 of the density of vessels established by DsRed-EC (white) when encapsulated with ASC in 3-dimensional fibrin gel and incubated either alone or in the presence of aPBMC between days 6 and 9 ($n=8$). **h**, Accumulation of Activin A in media conditioned for 24 h by EC + ASC co-cultures in 2-dimentional (2D-VNF) and 3-dimensional (3D-VNF) fibrin gel vasculogenic models while incubated with or without aPBMC ($n=8$). **i**, Accumulation of Activin A in the media conditioned by rat aorta segments while exposed to PBMC or aPBMC ($n=6$–17). ns—not significant, $^{**}p \le 0.01$, $^{***}p \le 0.001$, $^{****}p \le 0.0001$

pericytes, respectively, when exposed to aPBMC (Figure S1a).

To test whether exposure of EC and ASC to aPBMC induces Activin A canonical signaling pathway, media conditioned by either EC, or EC + PBMC co-culture, or EC + aPBMC co-culture were applied on EC for 1 h. Such approach ensured that naïve EC will be exposed to the factors secreted by PBMC (or aPBMC) and, EC in response to PBMC (or aPBMC). EC treated with EC + aPBMC secretome showed upregulated SMAD2 phosphorylation, whereas EC and EC + PBMC secretomes had no effect (Fig. 2i). Similar upregulation in SMAD2 phosphorylation was observed in ASC treated with media conditioned by ASC + aPBMC co-culture (Fig. 2j).

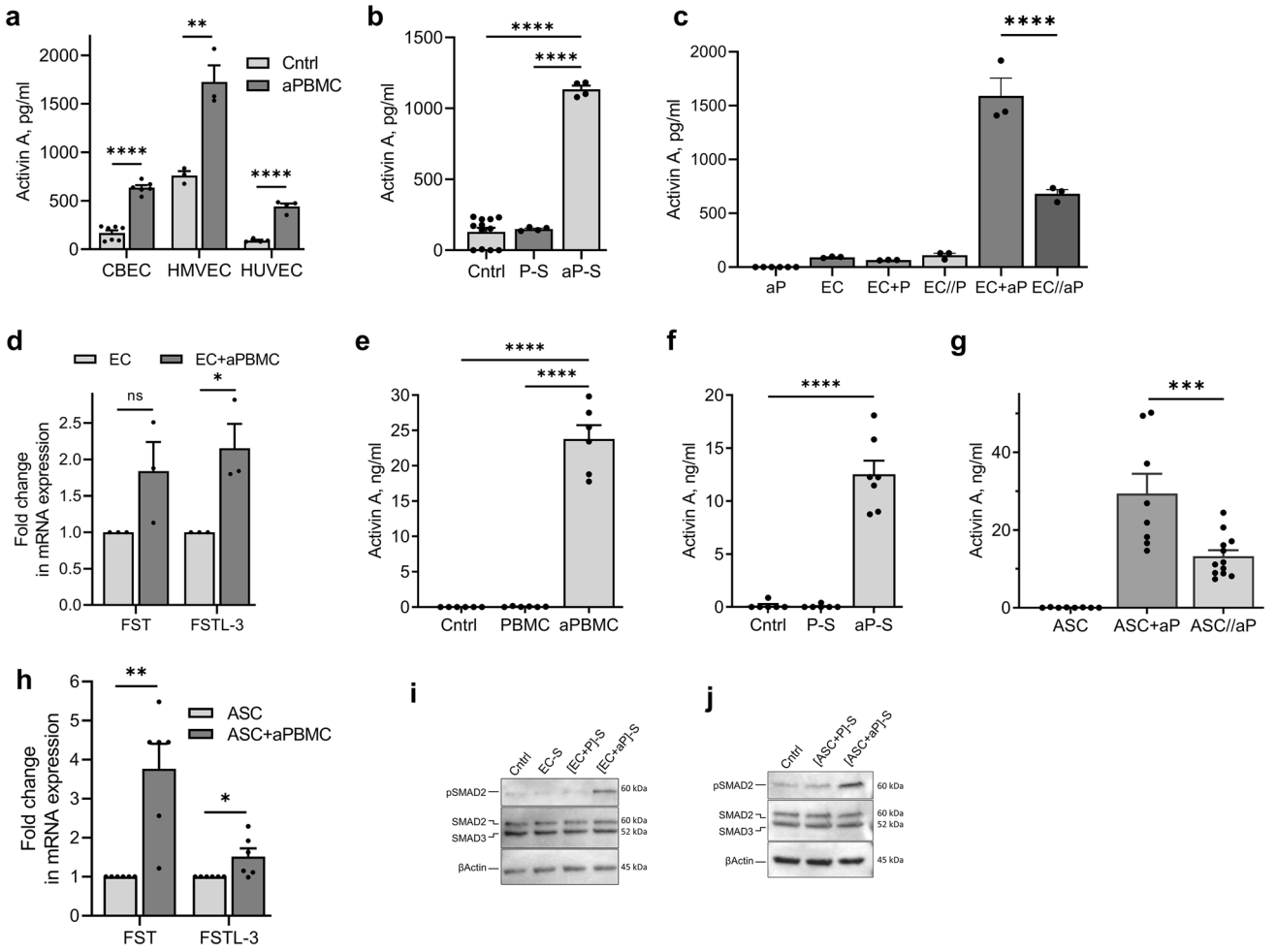

Fig. 2 Activated peripheral blood mononuclear cells (aPBMC) induce Activin A in both vascular cells (EC and ASC) through paracrine mechanism. **a** and **b**, Accumulation of Activin A in media conditioned by human endothelial cells (EC) isolated from cord blood (cbEC), cardiac microvasculature (HMVEC), and umbilical vein (HUVEC) when cultured alone or with aPBMC (**a**) ($n=3$–7) or aPBMC secretome (aPBMC-S) (**b**) ($n=4$). **c**, Accumulation of Activin A in media conditioned by aPBMC, cbEC, cbEC + PBMC (EC + P), cbEC + aPBMC (EC + aP) in direct co-cultures or when spatially separated by 0.4 µm pore size membrane (EC//P and EC//aP). **d,** Change in follistatin (FST) and follistatin-like-3 (FSTL-3) mRNA expression in cord-blood EC monocultures vs EC + aPBMC co-cultures ($n=3$). **e** and **f**, Accumulation of Activin A in media conditioned by ASC cultured alone or with aPBMC (**e**) ($n=6$) or with aPBMC-S (**f**) ($n=4$). **g,** Accumulation of Activin A in media conditioned by ASC, or by ASC + aPBMC either in direct co-cultures (ASC + aP) or when spatially separated (ASC//aP) ($n=8$–12). **h,** Change in follistatin (FST) and follistatin-like-3 (FSTL-3) mRNA expression in ASC monocultures vs ASC + aPBMC co-cultures ($n=6$). **i**, Immunoblot of SMAD2 and its phosphorylated form (pSMAD2) in intact EC (Cntrl) and EC treated with media conditioned by EC alone (EC-S), or EC with either PBMC ([EC + PBMC]-S), or aPBMC ([EC + aPBMC]-S) for 1 h. **j,** Immunoblot of SMAD2 and its phosphorylated form (pSMAD2) in intact ASC (Cntrl) and ASC treated with media conditioned by ASC with either PBMC ([ASC + PBMC]-S), or aPBMC ([ASC + aPBMC]-S) for 1 h. Expression of β-Actin was used to loading control. ns—not significant, $*p \le 0.05$, $**p \le 0.01$, $***p \le 0.001$, $****p \le 0.0001$

IL-1β and TNFα mediate aPBMC-induced Activin A secretion in vascular cells

To define which cytokine mediated aPBMC-induced Activin A expression, monocultures of EC and ASC were exposed to several cytokines for 24 h. TNFα and IL-1β upregulated Activin A secretion in EC, while IL-6 was ineffective, and IFNγ inhibited its secretion (Fig. 3a). In ASC and in placental and brain pericytes, only IL-1β induced Activin A expression (Fig. 3c and S1b). In complementary studies, the effect of aPBMC on EC was substantially decreased when cells were co-cultured in media augmented with neutralizing antibodies against either TNFα or IL-1β, and was fully eliminated when these IgGs were used in combination (Fig. 3b). Akin to that, media conditioned by ASC + aPBMC co-cultured in the presence of IL-1β IgG were free of Activin A (Fig. 3d).

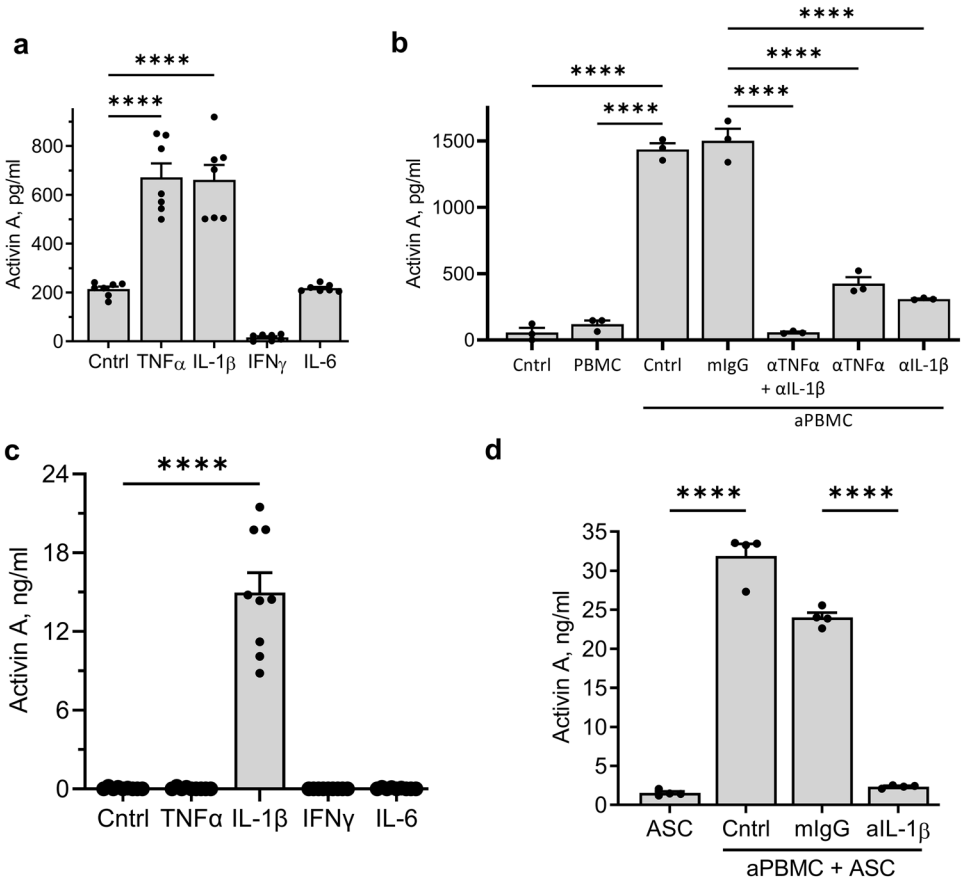

Fig. 3 Activated peripheral blood mononuclear cells (aPBMC)-induced Activin A secretion in vascular cells is mediated by TNFα and IL-1β. **a** and **c**, Accumulation of Activin A in media conditioned by cord-blood-derived endothelial cells (EC) (**a**) or adipose stromal cells (ASC) (**c**) when exposed to TNFα (10 ng/ml), IL-1β (10 ng/ml), IFNγ (10 ng/ml), or IL-6 (10 ng/ml) ($n=7$–9). **b**, Accumulation of Activin A in media conditioned by cord-blood-derived EC when exposed to PBMC, aPBMC alone or with neutralizing IgG to TNFα and IL-1β individually or in combination or with isotype control mouse IgG ($n=3$). **d**, Accumulation of Activin A in media conditioned by ASC when exposed to aPBMC alone or with either IL-1β IgG or control mouse IgG ($n=4$). ****$p \leq 0.0001$

Activin A is responsible for the diminished vasculogenesis caused by aPBMC

Exposure of EC-ASC co-cultures in the two-dimensional model system to TNFα or IL-1β (each at 10 ng/ml) for the first 3 days of incubation led to 66% and 37% decreases in vessel density, respectively (Fig. 4a). Analyses of the effect of these cytokines on survival of monocultures of EC revealed 20% and 45% reductions in cell survival after 3 days of exposure to TNFα and IL-1β, respectively (Fig. 4b); exposure of EC to the two cytokines in combination had no additional effect. This was further confirmed in the three-dimensional model in which co-cultures, subjected to TNFα/IL-1β for days 6–9 of incubation, showed a 65% decrease in vessel density (Fig. 4c, d).

To exclude the possibility that diminished tubulogenesis in response to inflammatory modulators could be mediated by the factors present in FBS, EC + ASC co-cultures were exposed to aPBMC or TNFα/IL-1β in basal media supplemented only with 0.25% BSA, ITS mixture, bFGF, and SDF-1. Tests conducted in serum-free media led to the same outcomes: a significant decrease in vessel formation in response to activated PBMC and TNFα/IL-1β, with no detrimental effect by non-activated PBMC, and a substantial accumulation of Activin A in incubation media of co-cultures exposed to inflammatory modulators (Figure S2).

To test whether this anti-angiogenic effect was due to Activin A, 2D co-cultures were treated with TNFα/IL-1β alone or in the presence of Activin A IgG. While inflammatory cytokines reduced vessel density by more than 50%, Activin A IgG eliminated their anti-angiogenic effect (Fig. 4e, f). Finally, Activin A IgG improved vascularization of fibrin gel implants containing EC, ASC, and aPBMC by 85% compared to gels with cells alone and with isotype control IgG (Fig. 4g).

In vivo collagen/fibrin implants

To extend in vitro observations to in vivo situation, EC, ASC, and either PBMC or aPBMC were encapsulated in a collagen/fibrin gel and subcutaneously implanted into Nude mice for 7 days. Gross examination of the implants revealed that those with EC and ASC alone or with non-activated PBMC were mostly bright red in color, whereas implants encapsulating aPBMC were paler, suggesting reduced perfusion (Fig. 5a). Indeed, the density of donor cell-derived functional vessels (human CD31 + with red blood cells filling their lumens) in thin sections of implants with aPBMC was substantially lower than in the sections without aPBMC

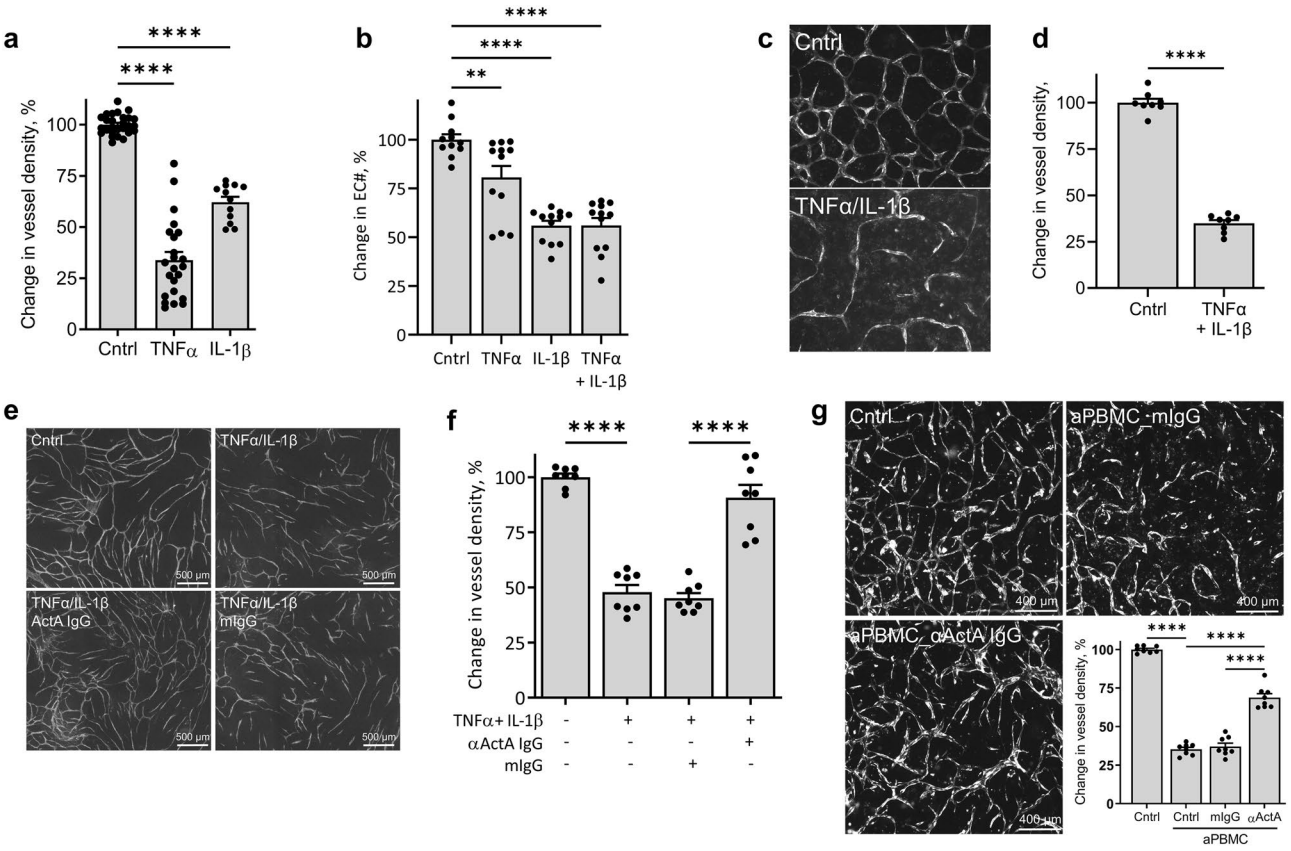

Fig. 4 Blocking Activin A activity improves impaired vasculogenesis induced by an inflammatory environment in vitro. **a**, Relative quantitative analysis of the vessel density in endothelial cells + adipose stromal cells (EC + ASC) co-cultures incubated in control media alone or supplemented with TNFα or IL-1β (each at 10 ng/ml) for the first 3 days of 6 day incubation (2D-VNF model). Vessels were revealed by staining co-cultures with EC marker Ulex lectin (white) ($n = 12–27$). **b,** Cellular enumeration in EC monocultures after exposure to TNFα or IL-1β either individually or in combination (each at 10 ng/ml) for 4 days ($n = 7–8$). **c** and **d**, Representative fluorescent images (**c**) and quantitative analysis (**d**) of the vessel density in co-cultures established by DsRed-expressing EC (white) encapsulated with ASC in fibrin gel and incubated either alone or in the presence of TNFα/IL-1β (each at 10 ng/ml) for the last 3 days of a 9-day incubation ($n = 8$). **e** and **f**, Representative fluorescent images (**e**) and relative quantitative analysis (**f**) of the vessel density in co-cultures established by EC + ASC incubated in control media alone, treated with TNFα/IL-1β or treated with these cytokines in combination with Activin A neutralizing IgG (αActA IgG) or isotype control mouse IgG (mIgG) for the first 3 days of a 6-day incubation ($n = 8$). Vessels were revealed by staining co-cultures with EC marker Ulex lectin (white). **g**, Representative fluorescent images and relative quantitative analysis of the vascular density in co-cultures established by DsRed-expressing EC (white) encapsulated with ASC alone or with ASC + aPBMC in fibrin gel and incubated in control media alone or with αActA or isotype control mouse IgG for the first 3 days of 9-day incubation ($n = 8$). $^{**}p \leq 0.01$, $^{****}p \leq 0.0001$

(Cntrl: 29.9 ± 5.5; PBMC: 21.6 ± 3.8; aPBMC:7.7 ± 1.9; vessels/mm2) (Fig. 5b, c). However, when aPBMC-containing implants were augmented with Activin A IgG, a 2.1-fold increase in the density of functional vessels was observed compared to the vessel density of implants with isotype control IgG (Fig. 5d and e).

Discussion

Many acute ischemic pathologies are associated with tissue inflammation that may progress to chronic conditions such as heart failure, chronic kidney disease, or critical limb ischemia if inflammation persists. Frequently, if not resolved promptly, inflammation causes tissue fibrosis and vascular rarefaction, leading to decline of organ function and eventually failure; which then can only be addressed by supporting therapy, organ transplant, or amputation. There is strong evidence that therapies limiting systemic inflammation slow progression of cardiovascular pathologies. It has been hypothesized that targeting inflammatory mediators or signaling pathways may prevent development of vascular pathologies and accelerate organ recovery after ischemic insult [50].

In this study, the effect of inflammation on the vascular system was addressed. Several studies have shown that

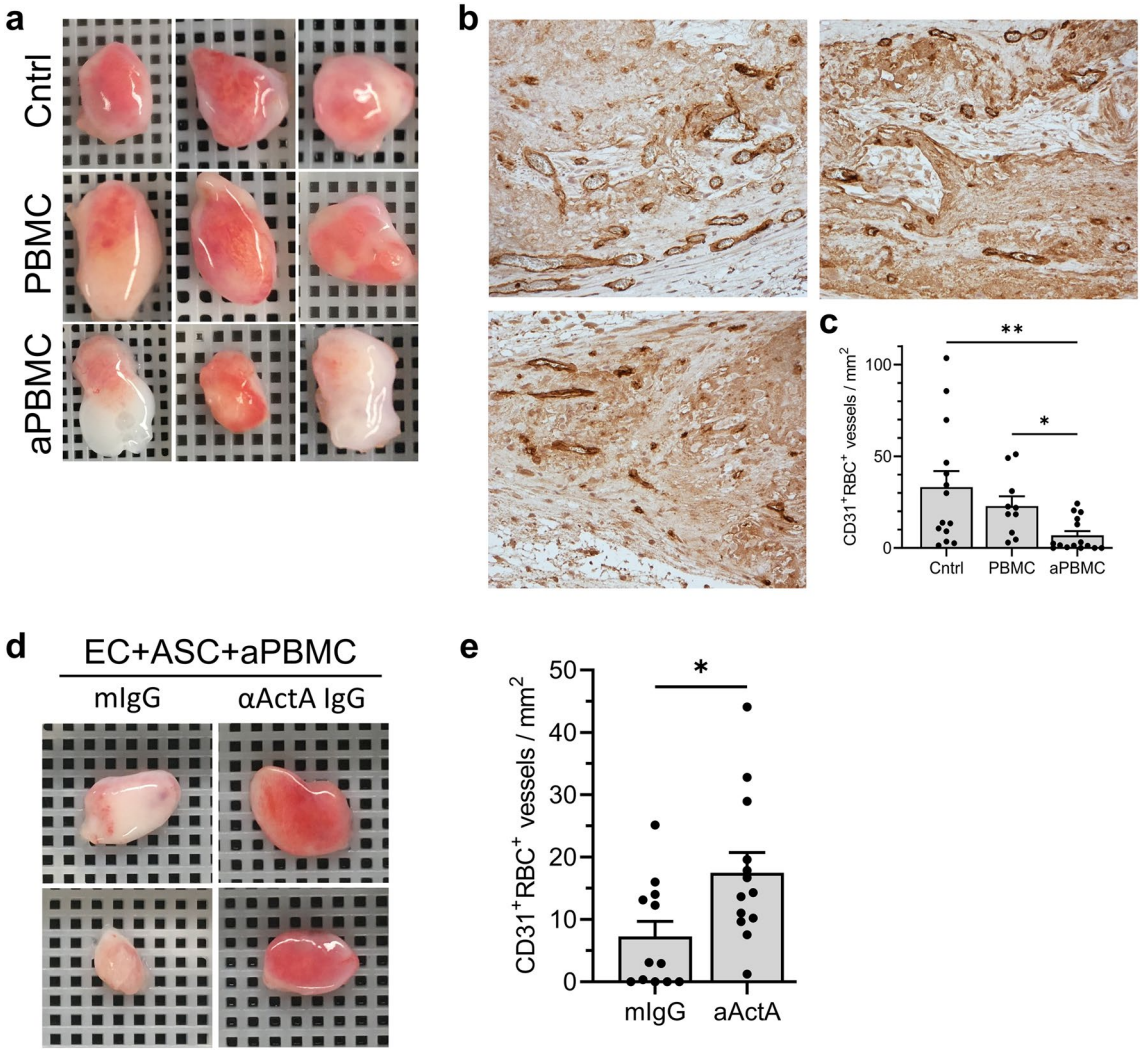

Fig. 5 Blocking Activin A activity improves impaired vasculogenesis in an inflammatory environment in vivo. **a**, Representative images of Col/Fn/Fg implants encapsulating EC with ASC alone (Cntrl) or additionally containing PBMC or aPBMC and harvested on day 7 post subcutaneous implantation. **b** and **c,** Representative images (**b**) and quantitative analysis (**c**) of the density of human CD31 + /RBC + vessels in thin sections of Cntrl, and either PBMC- or aPBMC-containing implants probed with human-specific CD31 IgG, followed by nuclei visualization with hematoxylin ($n = 10–15$). **d** and **e,** Representative images (**d**) and quantitative (**e**) analysis of functional (CD31 + /RBC +) vessels in Col/Fn/Fg implants encapsulating EC + ASC + aPBMC and augmented with either αActA or mIgG ($n = 12–13$). $^{*}p \leq 0.05$, $^{**}p \leq 0.01$

inflammation may lead to vascular rarefaction [51], but the precise mechanism is not well understood. We uncovered that TNFα and IL-1β, both secreted by LPS-activated PBMC [52, 53], promoted expression of Activin A in EC and perivascular cells, whereas expression of its endogenous inhibitors, follistatin and follistatin-like 3, were not substantially affected by activated PBMC. This new finding complements our prior discovery that severe ischemia caused a 12-fold increase in Activin A secretion from EC, via its regulation by HIF-1α and HIF-2α [54]. Since many pathologies are associated with both tissue ischemia and inflammation, local increases of Activin A in the vascular niche could be credited to both processes.

Multiple vascular and inflammatory disorders are accompanied by systemic upregulation of Activin A. However, the source of Activin A in these conditions as well as its contribution to the conditions' progression has not been fully explored. Activin A is a pleotropic factor that affects a variety of cells; this complicates the process of dissecting the mechanism of Activin A activity and effects. Interestingly, EC secrete low levels of Activin A at baseline, but substantially increase secretion in response to aPBMC, TNFα, or IL-1β, whereas pericyte progenitors showed no Activin A expression at baseline but dramatically increased expression when treated with aPBMC or IL-1β. Intact mature pericytes differed in their level of Activin A expression depending on

the source of these cells (brain vs placental); nonetheless in both cell types, there was a substantial increase in Activin A when exposed to aPBMC or IL-1β, but not to TNFα (Figure S1). While induction of Activin A by TNFα was previously reported for bone marrow cell lines [55], and by IL-1β in skin fibroblasts [56] and endometrial stromal cells [57], the current study is the first to show increased expression of Activin A in response to TNFα and IL-1β in primary endothelial and perivascular cells.

Expression of Activin A by EC or perivascular cells exposed to aPBMC secretome suggested that aPBMC paracrine activity was sufficient to induce Activin A. Interestingly, a lower level of Activin A was observed in the media conditioned by co-cultures of vascular cells and aPBMC without direct contact compared to its level in the media from direct co-cultures (Fig. 2c, g). This suggests that apart from paracrine signaling, juxtacrine signaling also could play a role in Activin A induction. However, taking into consideration that similar induction of Activin A was achieved by exposing EC to either aPBMC or aPBMC-S (Fig. 2a, b), we cannot exclude that the difference in Activin A expression in direct versus indirect co-cultures could be attributed to the rate of IL-1β and TNFα diffusion to the EC monolayer, which is not an issue when aPBMC are in contact or in close proximity to EC. Contribution of juxtracrine signaling to aPBMC-induced Activin A secretion in the vascular cells needs to be further explored in well-defined models.

We noticed that expression of Activin A, per cell, was significantly higher in aPBMC or IL-1β-stimulated ASC than in the three types of EC tested (Figs. 2a, e and 3a, c). Furthermore, stimulated placental pericytes showed secretion of Activin A similar to ASC, whereas brain pericytes showed a lower induction (Figure S1). The physiological meaning of such a difference requires further evaluation, and observations should be strengthened by screening responses of pericytes obtained from different tissues and multiple donors. Also, it is important to recognize that the ratio of EC to mural cells (pericytes/smooth muscle cells) differs between tissues and vessels of different diameter (progressively decreasing from capillaries to arteries and veins). This suggests that vessels of different calibers and from different tissues respond differently to an inflammatory environment with respect to Activin A secretion and its downstream effects. It is important to recognize that this initial study explored activities of vascular cells obtained from healthy donors. It is well known that aging, smoking, and diabetes modify bioactivities of vascular cells, and future studies are required to address whether these modifiers affect Activin A secretion by vascular cells in inflammatory environments. Interestingly, EC exposure to IFNγ eliminated baseline secretion of Activin A in EC (Fig. 3a) and blocked its expression in ASC treated with IL-1β (not shown), which is supported by prior observations in bone marrow fibroblasts [58]. Future studies should address whether such antagonistic effects of IL-1β and IFNγ have physiological roles.

A study by Dr. Davis' group has shown that both TNFα and IL-1β induce vessel rarefaction [51]. The current study extends these observations by demonstrating that the anti-angiogenic effect of these two cytokines is mediated by Activin A, and silencing its activity almost completely restored the ability of EC to organize into vascular structures (Fig. 4e, f). We have previously shown that Activin A is a key factor in the resolution phase of vessel formation, but its premature expression prevents normal vessel remodeling [59]. In accordance to that, detrimental effects of aPBMC on tubulogenesis in vitro were limited by application of Activin A neutralizing antibodies during the critical time window (i.e., the initial phase of network formation) (Fig. 4g), and such treatment improved vascularization of implants in vivo (Fig. 5d, e). Overall, these findings suggest that Activin A has an angiostatic effect on EC tubulogenesis. Interestingly, thrombin previously revealed as an additional rarefaction factor in a prior study [51] induces Activin A expression in perivascular cells [60]. The mechanism of the angiostatic effect of Activin A on EC requires further evaluation including whether Activin A mediates this directly or if its effects are indirect and mediated by activities of known endogenous inhibitors of revascularization, such as thrombospondin, vasohibin, IFNγ, angiostatin, and endostatin [61, 62].

This study also suggests that inflammatory cell-mediated inhibition of both de novo vessel formation (vasculogenesis; Fig. 1a) and rarefaction of established vessels (Fig. 1d–g), was, at least partially, attributable to increase in EC death (Fig. 1b, c) as noted when EC were exposed to inflammatory cells alone or with perivascular cells. EC by themselves have limited ability to organize into vascular structures and heavily rely on supporting cells (e.g., pericytes, smooth muscle cells, MSC, and fibroblasts) for that. These cells support EC tubulogenesis by paracrine and direct-contact mechanisms. We have previously shown that pre-treatment of pericyte progenitors with Activin A limits their ability to support EC organization into functional vessels [63]. Hence, we hypothesize that inflammatory cells, by modifying the perivascular cell secretome, limit their ability to support EC survival, proliferation, and tubulogenesis.

The finding that inflammatory cells induce Activin A through paracrine/endocrine mechanisms, suggests that systemic increases in TNFα or IL-1β may upregulate Activin A expression in remote organs, thus, limiting their ability to undergo vascular remodeling in response to acute injuries. To limit detrimental effects of inflammation on vessel rarefaction, several pharmacological compounds that may act at the level of ligand binding or target common downstream signaling pathways have been tested [51].

Prior preclinical studies suggested that attenuation of TNFα activity will mitigate progression of heart failure,

but subsequent clinical studies revealed that TNFα antagonism had no or an adverse effect [64], suggesting that blocking only TNFα activity is insufficient to prevent disease progression. The current study unveiled that Activin A represents an attractive therapeutic candidate to mitigate anti-angiogenic effects of inflammation. Several compounds that block Activin A, including fusion proteins of activin receptor type IIA extracellular domain (ACE-11) and activin receptor type IIB (ACE-031) were explored in multiple preclinical studies and clinical trials for non-vascular applications (ACE-11: NCT03496207; ACE-031: NCT01099761, NCT00952887). Systemic infusion of ACVRIIA fusion protein limited pulmonary hypertension, [65] and osteoporosis [66], and ACVRIIB fusion protein mitigated muscular dystrophy [67], fibrodysplasia ossificans progressive [68], and cancer cachexia [69]. While these compounds neutralize virtually all ligands that bind to corresponding receptors and do not have selectivity for Activin A; contributions of these ligands, other than Activin A, to vascular remodeling has not been shown, suggesting that temporal use of these compounds during acute phase of inflammation may be beneficial. Alternatively, to achieve a more targeted effect, Activin A IgG infusion could be used, as reported previously, to prevent cardiomyopathy [25]. Future studies are planned to explore the therapeutic effect of these compounds on tissue revascularization in ischemic and inflammatory conditions.

In conclusion, this study revealed that activated PBMC, through secretion of TNFα and IL-1β, induce dramatic increases in Activin A secretion from both of the primary vascular cells. Accumulation of Activin A in the vicinity of vessel formation or remodeling results in inefficient EC tubulogenesis or causes vessel rarefaction. Blocking Activin A improves vessel remodeling in the inflammatory environment. Overall, this suggests that systemic infusion of compounds that silence Activin A activity may improve organ preservation after ischemic insults, as well as success of cell therapies that are based on systemic infusion of endothelial progenitor cells [70, 71] or local injection of EC and mural cells mixtures for vascular ischemic indications.

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Data availability All data generated or analyzed during this study are included in this published article.

Declarations

Competing interests The authors have no competing interests to declare.

Ethical approval All procedures involving human participants were approved by the University of Florida Institutional Review Board (IRB201802558, IRB201800081). The study procedures involving animals were approved by the University of Florida Institutional Animal Care and Use Committee (Study# 202110176).

Consent to participate Informed consent was obtained from all individual participants in the study.

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