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ORIGINAL PAPER

In vitro modeling of endothelial interaction with macrophages and pericytes demonstrates Notch signaling function in the vascular microenvironment

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Abstract Angiogenesis is regulated by complex interactions between endothelial cells and support cells of the vascular microenvironment, such as tissue myeloid cells and vascular mural cells. Multicellular interactions during angiogenesis are difficult to study in animals and challenging in a reductive setting. We incorporated stromal cells into an established bead-based capillary sprouting assay to develop assays that faithfully reproduce major steps of vessel sprouting and maturation. We observed that macrophages enhance angiogenesis, increasing the number and length of endothelial sprouts, a property we have dubbed "angiotrophism." We found that polarizing macrophages toward a pro-inflammatory profile further increased their angiotrophic stimulation of vessel sprouting, and this increase was dependent on macrophage Notch signaling. To study endothelial/pericyte interactions, we added vascular pericytes directly to the bead-bound endothelial monolayer. These pericytes formed close associations with the endothelial sprouts, causing increased sprout number and vessel caliber. We found that Jagged1 expression and Notch signaling are essential for the growth of both endothelial cells and pericytes and may function in their interaction. We observed that combining endothelial cells with both macrophages and pericytes in the same sprouting assay has multiplicative effects on sprouting. These results significantly improve bead-capillary sprouting assays and provide an enhanced method for modeling interactions between the endothelium and the vascular microenvironment. Achieving this in a reductive in vitro setting represents a significant step toward a better understanding of the cellular elements that contribute to the formation of mature vasculature.

Keywords Notch · In vitro · Endothelial cell · Pericyte · Macrophage

Introduction

In both developmental and pathological phenomena, the formation of mature and functional blood vessel networks relies upon the interaction between endothelial cells and the stromal cells that surround them in the vascular microenvironment. These interactions are particularly relevant in anti-angiogenic disease therapy. The interaction of anti-angiogenic agents with vascular support cells has recently become a focus of interest, as macrophages and perivascular cells play indispensible roles in blood vessel growth and organization [1–4].

Macrophages are myeloid cells, derived from the bone marrow, which exist both in tissue resident states and as migratory cells [5]. Though macrophages were first described in their role as phagocytic mediators of passive immunity, it is now appreciated that macrophages can adopt a wide variety of context-specific phenotypes that

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allow them to function in diverse physiological as well as pathological processes [6]. Though the role of macrophages in the vasculature has been studied extensively in the context of tumorigenesis [6, 7], they have also been implicated in physiological angiogenesis. In angiogenesis, macrophages often take on an angiotrophic role: that is, they foster the growth of vessel networks directly and indirectly, through physical involvement in the process and by the secretion of angiogenic factors. Microglia, neuronal myeloid cells closely related to macrophages, can influence sprouting in a mouse aortic ring model of angiogenesis [2]. Microglia have also been implicated in vivo in the facilitation of anastomosis between nascent vessels [1] and have been found to limit angiogenesis by the expression of inhibitory VEGFR-1 in a vascular bed-specific fashion [3].

Perivascular cells are stromal cells that interact closely with the abluminal surface of blood vessels to influence a wide range of vessel parameters. Different types of perivascular cells interact with different caliber vessels: Vascular smooth muscle cells (VSMCs) interact with larger caliber vessels, while pericytes form close attachments with smaller caliber capillaries [4]. VSMCs and pericytes are distinct cell types, and the exact differences between them, and indeed the exact definition of a pericyte, have been the focus of considerable research [4, 8]. Broadly, perivascular cells support vascular growth by forming close attachments with nascent vessels to promote vessel maturation, quiescence, and patency. VSMCs surrounding larger vessels and additionally regulate vessel tone [4].

The Notch receptors and their ligands are a family of well-conserved proteins that allow direct signaling between neighboring cells, and play roles in a wide array of physiological and pathological processes [9]. Notch signaling functions in several angiogenic mechanisms, most notably controlling the differentiation between endothelial tip- and stalk-cell identities [10]. More recently, Notch has been implicated in the interaction between endothelial cells and both macrophages and perivascular cells. In macrophages, Notch signaling has been found to be important for recruitment to sites of active angiogenesis in both developmental and pathological settings, and Notch signaling has been detected in macrophages at the sites of imminent or recent vessel anastomosis, suggesting a role in this process [11, 12]. In perivascular cells, Notch signaling between the endothelium and smooth muscle cells has been shown to control VSMC differentiation and in influencing the intercellular adhesion between the endothelium and VSMC [13]. A study in zebrafish also pointed to a comparable role for Notch3 signaling in pericytes [14], but so far the field remains understudied.

Given the complexity of the interactions that lead to the formation of vascular networks, and the great potential utility of understanding these relationships, there is a pressing need for the development of reductive in vitro systems that faithfully recapitulate the angiogenic process. A wide variety of methods have been developed to model elements of angiogenesis, such as endothelial proliferation, migration, and network formation [15]. Few of these assays faithfully re-create angiogenesis in a multi-cellular setting [16]. Some of the more successful in vitro models for angiogenesis are bead-based capillary sprouting assays using three-dimensional matrices. In one form of this assay, endothelial cells are bound to micro-carrier beads and embedded in a fibrin gel, where they sprout to form lumenized vessels in response to cues from a fibroblast feeder layer [17, 18]. This assay is typically focused on the endothelium, and while some recent studies have used the assay to describe interactions between endothelial cells and VSMCs [13], the potential of this assay as a tool for modeling the relationships between endothelial and vascular support cells remains largely untapped.

Methods

Cell culture

L929 fibroblasts were acquired from ATCC and maintained in DMEM (Gibco) 4.5 g/dL glucose + 10 % HI-FBS + 1× Penicillin/Streptomycin. L929 fibroblasts were used solely in the creation of conditioned medium for use in derivation of bone marrow macrophages.

LADMAC were acquired from ATCC and maintained in EMEM (ATCC) + 10 % HI-FBS + 1× Penn/Strep.

Human umbilical vein endothelial cells (HUVEC) were isolated from human tissue according to the established protocol [19]. Cells were maintained on collagen I (Corning)-coated plates in EGM2 (Lonza).

EOC2 microglia were purchased from ATCC and were maintained in DMEM (Gibco) 4.5 g/dl glucose + 10 % HI-FBS + 1× Penn/Strep, supplemented with 20 % LADMAC-conditioned medium as a source of M-CSF.

Bone marrow macrophages (BMM) were derived from bone marrow harvested from mouse femurs and tibias, according to the established protocol [11]. Briefly, bone marrow was isolated and cultured on untreated petri dishes (Falcon) in RPMI (Gibco) + 10 % HI-FBS supplemented with 20 % L929-conditioned medium as a source of M-CSF with media changes every 2 days. Over the course of a week, the bone marrow differentiates to form a monoculture of f4/80+ macrophages, as other cells do not adhere to the plastic and are washed off.

Human brain vascular pericytes were acquired from ScienCell. Cells were maintained on 1 % gelatin

(Millipore)-coated plates in DMEM (Gibco) 1 g/dL glucose + 10 % HI-FBS + 1× Penn/Strep.

D551 human skin fibroblasts were purchased from ATCC and maintained in EMEM (Gibco) + 10 % HI-FBS + 1× Penn/Strep. Medium was changed to EGM2 24 h prior to inclusion in capillary sprouting assay.

293T were acquired from (ATCC) and maintained in IMDM (Gibco) + 10 % HI-FBS + 1× Penn/Strep.

Mouse husbandry

BMMs were derived from mice with the following genotypes: $LysM^{cre/cre}$; $DNMAML\text{-}GFP^{+/+}$ (functionally wild type), $LysM^{cre/cre}$; $DNMAML\text{-}GFP^{fl/+}$, and $LysM^{cre/+}$; $EYFP^{YFP/+}$. LysMcre mice were purchased from the Jackson Laboratory [20]. DNMAML-GFP mice were a kind gift from Warren Pear via Boris Reizis [21]. R26-EYFP mice were purchased from the Jackson Laboratory [22]. Prior to killing, mice were housed in the barrier facility at the Irving Cancer Research Center at Columbia University Medical Center according to the institutional guidelines. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Labeling of macrophages for capillary sprouting assay

Bone marrow-derived macrophages were labeled using Vybrant Dio cell-labeling solution (Molecular Probes) according to the manufacturer's instructions. Briefly, BMM were resuspended in serum-free RPMI, incubated with the labeling solution for 20 min at 37 °C, and washed twice with serum-free RPMI before being introduced to the fibrin for the capillary sprouting assay.

Macrophage polarization

Prior to inclusion in co-culture, macrophages were treated for 24 h with either LPS (100 ng/mL) (Calbiotech) and recombinant murine interferon-gamma (100 U/mL) (Peprotech) or recombinant murine interleukin 4 (5 ng/mL) (R&D Systems). Factors were removed, and cells were washed three times with sterile PBS prior to cell isolation and inclusion in the co-culture. In order to examine whether the possibility of LPS carry over into the capillary sprouting assay may affect endothelial sprouting in the absence of macrophages, a capillary sprouting assay was performed in which EGM2 containing LPS (0.1, 1.0, and 10 ng/mL) was replaced each day of the assay.

Lentiviral infection

To allow expression of fluorescent markers, the DNMAML-GFP construct, and shRNA knockdowns, HUVEC and HBVP were infected via a third-generation lentiviral gene delivery system, as described previously [15]. Briefly, 293T cells were co-transfected via $CaPO_4$ with the core lentiviral plasmids along with either one or two lentiviral expression vectors containing genes of interest. The transfected cells produced virus-containing media, which was filtered and placed onto target cells. Target gene plasmids used were pCCL-GFP, pCCL-RFP, pCCL-DNMAML-GFP, and pLKO-based knockdown constructs for Jagged1 and Dll4, as well as a scrambled pLKO control. The pCCL-DNMAML construct was a kind gift of Andrew Weng. Further plasmid information is available upon request.

Western blot

In order to confirm Jagged1 and Dll4 knockdowns, total cell lysates from HUVECs infected with lentiviral knockdown constructs were isolated in Tris–triton lysis buffer (10 mM Tris, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 % Triton X-100, 10 % glycerol, 0.1 % SDS, 0.5 % deoxycholate, pH 7.4) containing Halt™ protease and phosphate inhibitor cocktail (Thermo Scientific). Western blots were performed by 10 % SDS-PAGE, transferred to nitrocellulose, blocked with 3 % BSA, probed with antibodies to Dll4 (Santa Cruz Biotechnology, SC-18639) or alpha-tubulin (Sigma T6074) as a loading control. HRP-conjugated secondary antibodies were used with ECL (GE Lifesciences) to detect bands on X-ray film using a BioRad developer.

Proliferation assay

To measure the growth rate of endothelial cells, cells were seeded in triplicates at 5×10^3 cells per well in 24-well plates coated with type I collagen. Cell number was assessed after cells settle down (4 h after seeding) and at day 4 with the Cell Counting Kit-8 assay (CK04-11; Dojindo Molecular Technologies, Inc., Gaithersburg, MD).

cDNA library creation and quantitative RT-PCR

mRNA was isolated from HUVEC, BMM, and HBVP via the RNEasy kit (Qiagen), and cDNA libraries were generated via the Verso cDNA synthesis kit (Fischer Scientific), all according to the manufacturer specifications. Samples were then assayed for mRNA expression macrophage polarity markers iNOS and arginase or for Jagged1 using SYBR Green (Applied Biosystems) in an Applied

Biosystems 7300 Real-Time PCR System. Target gene primer sequences are available upon request.

Flow cytometry

BMM were washed in PCN buffer. Samples were assessed on a BD FACSCaliber. No antibodies were used, as the purpose was to assess the inherent fluorescence of the DNMAML-GFP construct.

Capillary sprouting assay

The capillary sprouting assay has been described previously [17], though the present study introduces substantive variations in the protocol. HUVEC were incubated with Cytodex 3 beads (Sigma Aldrich) in EGM2 at a ratio of 400 cells per bead for 4 h with periodic agitation. This ratio is a calculated excess—not all HUVEC will bind to a bead, but this ratio allows complete monolayer coverage of all beads within 4 h. The beads were plated overnight on TC-treated plates in EGM2 to allow loose HUVEC to settle to the bottom. The following day, the beads were removed from the plate, washed in EGM2, and resuspended in a 1× PBS solution containing 3 mg/mL fibrinogen (Sigma Aldrich) and 0.15TI U/mL aprotinin (Sigma Aldrich). This solution was mixed with 0.625 U/mL thrombin (Sigma Aldrich) in a 24-well plate as a concentration of 150 beads in 500 µl fibrinogen solution per well and left for 25 min to polymerize. Wells were then covered with 1 mL of EGM2 containing 1e5 D551 cells per mL, which settled to form a monolayer on top of the fibrin clot.

To include myeloid cells in the assay, the myeloid cells were added to the fibrinogen solution before the inclusion of the HUVEC-coated beads. The myeloid cells were added at a concentration of 1e4 cells per well.

To include pericytes in the assay, HUVEC were incubated as normal with Cytodex beads and then washed thoroughly with EGM2. HBVP were then added to the mixture at a ratio of 400 HBVP per bead and incubated for a further 4 h with periodic agitation. As before, this ratio is a calculated excess, and only a small proportion of HBVP binds to the HUVEC-coated beads. The beads were then plated overnight, and the rest of the assay completed as normal. Schematics of the expanded capillary sprouting assays are provided (Online Resource 1).

The capillary sprouting assay matured between experimental days 4 and 6, with day 4 typically showing early vessel formation and day 6 exhibiting a matured plexus. There was some variation in growth tempo between separate experiments, which is internally controlled within individual experiments. Data presented highlighted the point of maximal difference between experimental groups, and the experimental day is noted in all figures.

Quantification

All capillary sprouting assays were performed in triplicate. Sprout number and length were tabulated from five low-power (5×) images from each well, for a total of between 60 and 100 beads per group. To quantify sprout length, the number of sprouts greater than 100 µm in length (approximately half the diameter of a bead) was normalized to the total number of sprouts. Sprout width was calculated from five mid-power (10×) images from each group. Only connecting, non-terminal sprouts were counted, and the value was expressed as the number of sprouts >40 µm in width, normalized to the total number of sprouts counted. Each experiment was performed at least three times (except where noted), and figures displayed here represent a single typical iteration of those experiments.

Results

The presence of myeloid cells accelerated sprouting in an in vitro model of angiogenesis

The complex process of sprouting angiogenesis is well modeled in vitro by a bead-capillary sprouting assay, in which human umbilical vein endothelial cells (HUVECs) are bound to collagen-/dextran-coated beads. When embedded in a fibrin clot, these HUVECs sprout to form capillary-like structures in response to cues from a fibroblast feeder layer [17, 23, 24]. We have improved the visibility of the endothelial cells by lentivirally infecting HUVEC to express a red fluorescent protein (RFP; Fig. 1a), which does not detectably alter the growth dynamics of the assay (data not shown).

To assess the contribution of myeloid cells to angiogenesis, we incorporated either EOC2 immortalized microglia or primary bone marrow-derived macrophages (BMM) into the capillary sprouting model of angiogenesis. Myeloid cells were mixed into the fibrinogen, such that they were evenly spaced throughout the resulting polymerized matrix (Online Resource 1, Fig. 1a). Our visual assessments suggested that macrophages remained stationary within the gel, and were not observed to migrate relative to the endothelial beads.

BMM were able to influence the endothelial cells to augment the angiogenic growth occurring from beads, with increased number of endothelial sprouts, observable by day 3 of the assay (Fig. 1b) and persisting through day 5 (Fig. 1c, d). BMM inclusion caused an approximate 50 % increase in overall number of sprouts at day 5. After 5 days of assay, the sprouts were longer in BMM-containing wells, with a significantly greater proportion of sprouts longer than 200 µm when measured from the bead (Fig. 1c, e). There

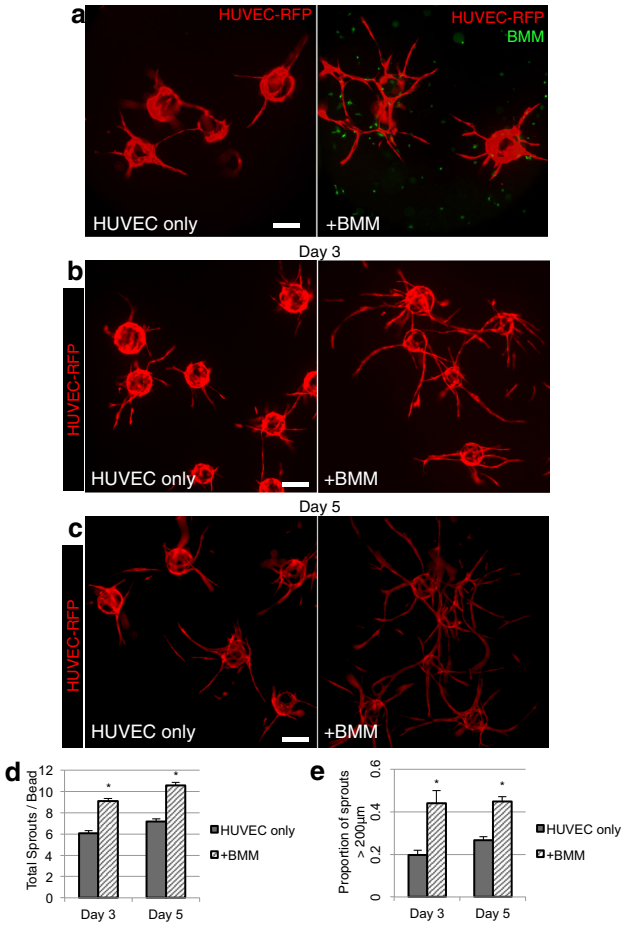

Fig. 1 Macrophage presence increases vascular sprouting. **a** Visualization of endothelial sprouts (*red*), with and without the inclusion of bone marrow macrophages (labeled in *green*). **b** BMM inclusion increases number and length of endothelial sprouts at early timepoint day 3. **c** Sprout number and length remain increased through late timepoint day 5. **d** Quantification of sprout number. **e** Quantification of frequency of longer (>200 μm) sprouts. Scale bars represent 200 μm. *Error bars* represent standard error. $*p < 0.05$

were no observed changes in branching, network formation, or lumen formation between experimental groups. EOC2 inclusion also increased sprout growth and length, though to a lesser degree than BMM (Online Resource 2a, b, c, d).

Inflammatory polarization of macrophages further increased their angiotrophic potential

Macrophages exhibit a wide range of context-specific phenotypes that allow them to participate in many physiological and pathological processes [5–7]. In vitro, macrophages can be activated "classically" or "alternatively" by treatment with specific factors and cytokines, and it is thought that this reflects one dimension of their differentiation capacity in vivo. We promoted macrophage classical polarization by pre-treatment of BMM with lipopolysaccharide (LPS) and interferon-gamma (IFNγ). To achieve alternate activation, BMM were treated with IL-4. Classical and alternative activation was confirmed by qPCR for polarity markers iNOS and arginase, respectively (Online Resource 3a, b).

LPS treatment of the capillary sprouting assay using endothelial cells alone did not affect angiogenesis (Online Resource 4a, b). LPS-/IFNγ-treated macrophages promoted angiogenesis to a greater degree than either the non-stimulated or IL-4-treated groups. By day 2 of the assay, the wells containing LPS-/IFNγ-treated BMM produced nearly double the number of initial sprouts compared to non-stimulated BMM (Fig. 2a, b). This increased sprouting was less profound, but still significant, by day 4, with LPS-/IFNγ-treated BMM inducing approximately 25 % more sprouts than non-stimulated BMM (Fig. 2a, b). However, LPS/IFNγ stimulation did not affect sprout length; when normalized to the total number of sprouts, LPS-/IFNγ-treated BMM induced the same proportion of longer sprouts as non-stimulated BMM (Fig. 2a, c).

IL-4 treatment did not alter the number of sprouts compared to non-stimulated macrophages (Fig. 2a–c). However, it did appear that the sprouts induced by IL-4-treated macrophages were less developed, with a smaller proportion of long sprouts (Fig. 2a, c).

Notch inhibition abrogated the angiotrophic advantage of inflammatory polarization

Notch signaling has been shown to function in macrophages, both as a regulator of macrophage polarization [25] and as a mediator of macrophage angiogenic functionality [11, 12]. To examine the involvement of Notch signaling in macrophage angiotrophism using the co-culture model, we used transgenic macrophages that express a dominant negative mastermind-like (DNMAML) construct. This construct binds to the Notch/CSL complex and prevents activation of canonical Notch targets, functioning as a dominant negative repressor of Notch signaling [21]. We confirmed expression of the GFP-tagged transgene via FACS analysis (Online Resource 5).

Non-stimulated DNMAML-expressing BMM increased endothelial sprouting in a fashion comparable to wild-type BMM (Fig. 3a, c). However, BMM-DNMAML cells stimulated with LPS/IFNγ did not promote a further increase in sprouting in response to this treatment, in contrast to wild-type BMM (Fig. 3b, d). Transcript analysis showed that the relative transcript levels of iNOS, a marker of the pro-inflammatory state, were approximately halved in LPS-/IFNγ-induced BMM-DNMAML compared to wild-type BMM (Fig. 3e). Thus, Notch inhibition tempered

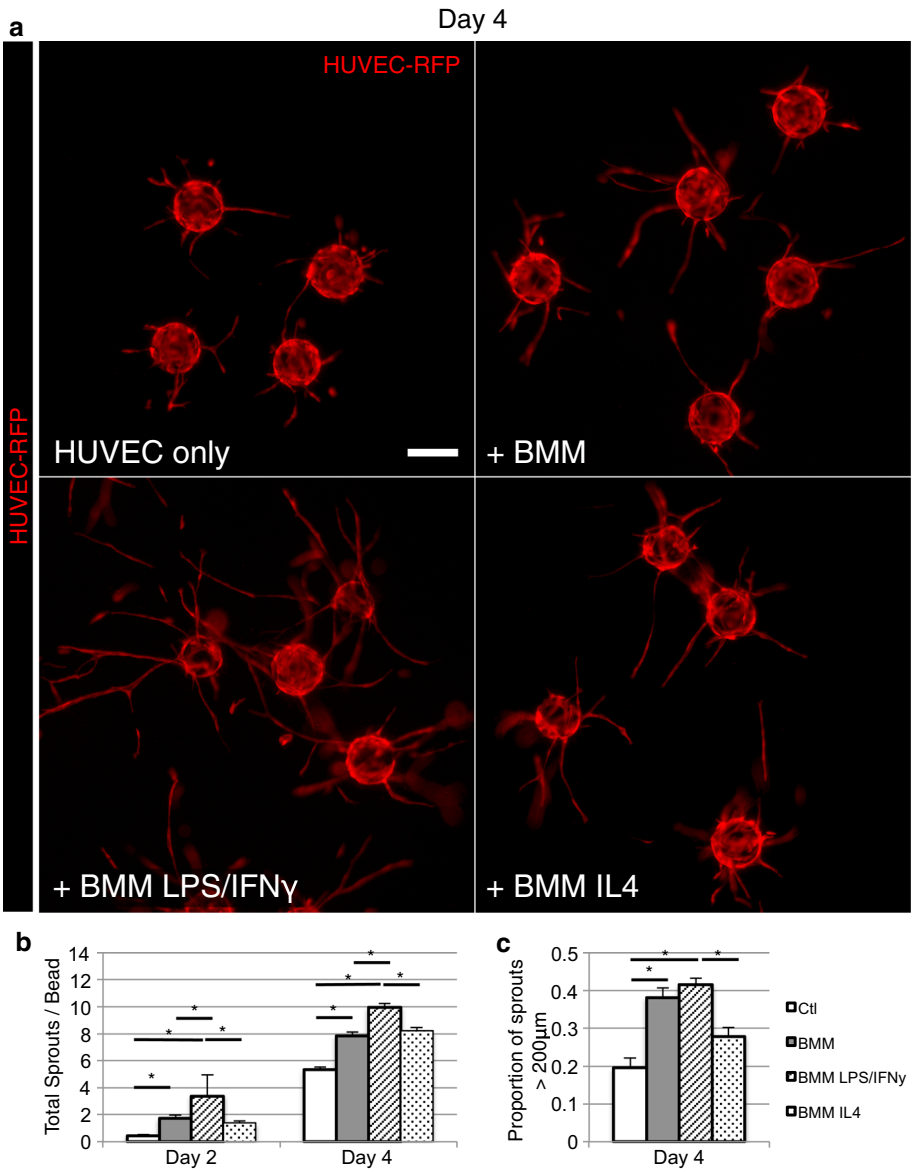

Fig. 2 Pro-inflammatory macrophages show greater angiotrophism. **a** LPS-/IFNγ-treated BMM cause increased sprouting in excess of that caused by unstimulated BMM, while IL-4-treated BMM do not. **b** Quantification of sprout number. **c** Quantification of frequency of longer sprouts. The difference in longer sprout frequency between unstimulated and LPS-/IFNγ-treated BMM is not significant. *Scale bars* represent 200 µm. *Error bars* represent standard error. *$p < 0.05$

the ability of LPS-/IFNγ-treated BMM to promote angiogenesis.

Pericyte and endothelial cell co-culture increased sprout stability and maturation in vitro

Endothelial cells rely on interactions with vascular smooth muscle cells (VSMCs) and pericytes to guide vessel growth and maturation. In small caliber capillaries, vascular pericytes are the physiologically relevant cell that participates in the angiogenic process. To study endothelial/pericyte interactions, we performed the capillary sprouting assay incorporating human brain vascular pericytes (HBVPs) [18]. In this variation in the assay, HUVEC-coated Cytodex beads were mixed with HBVPs and incubated together such that the HBVPs adhered to the outside of the endothelial monolayer already coating the beads. The beads were then embedded in a fibrin clot as normal.

HBVP co-culture with endothelial cells greatly altered the growth kinetics and dynamics of the capillary-like sprouting that occurred in the assay. In the presence of HBVP, sprouts grew faster and were clearly visible within 24 h of the start of the assay (Fig. 4a), unlike assays using only endothelial cells. By day 5, the difference became even more apparent, as the HBVP-containing wells formed a much more complex vascular network, with more sprouts and an increased proportion of mature, large caliber vessels (Fig. 4b, c). The pericytes formed close associations with the endothelial sprouts and can be seen in contact with the majority of sprouts (Fig. 4b). Some pericytes not associated with vessels were observed. As an alternative approach, pericytes were suspended in the fibrin gel, rather

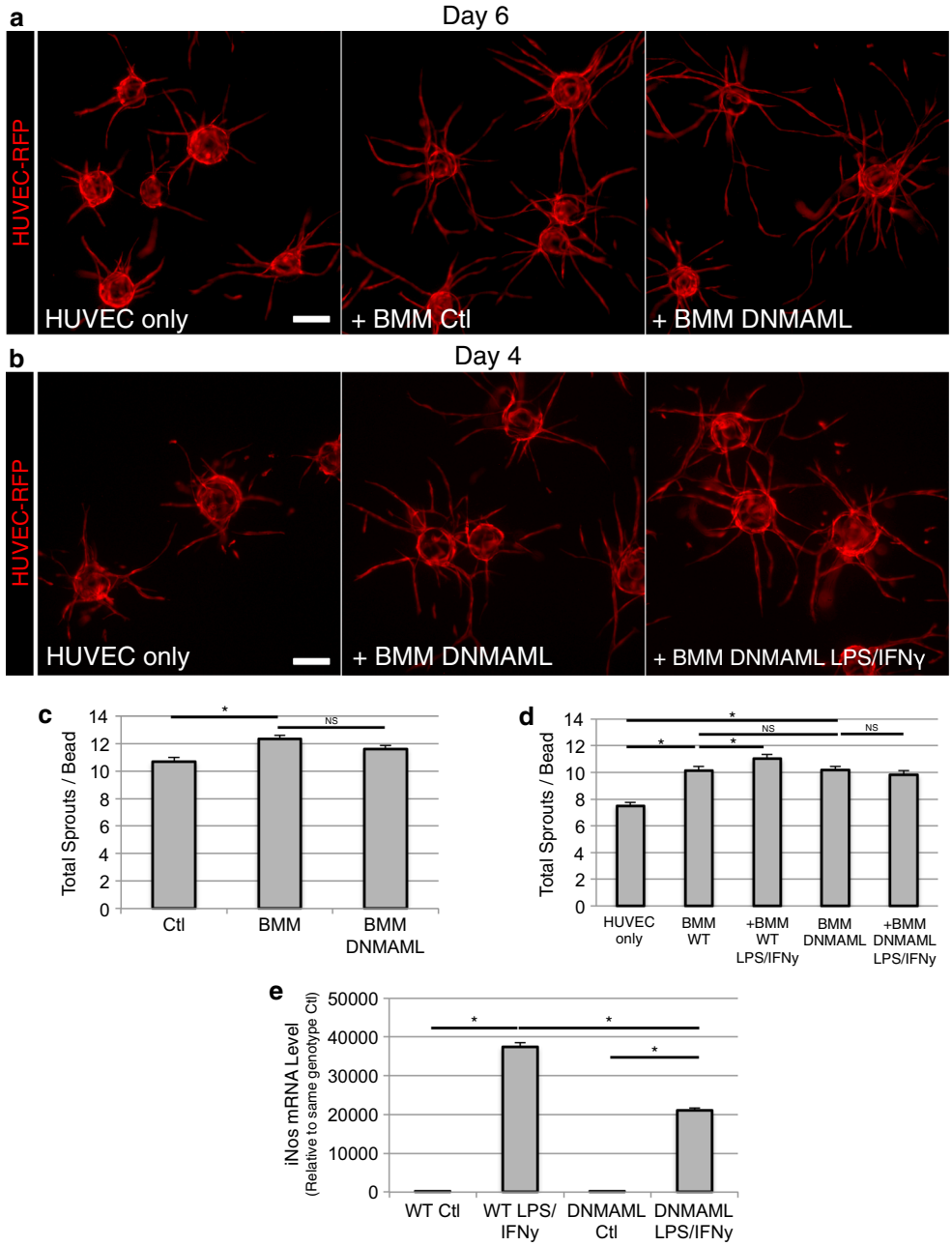

Fig. 3 Macrophage Notch signaling inhibition abrogates angiotrophic advantage of inflammatory polarization. **a** BMM expressing the Notch inhibitor DNMAML show similar angiotrophic properties to control BMM. **b** BMM-DNMAML do not show increased stimulation of angiogenesis when treated with LPS/IFNγ. **c** Quantification of sprout number in Fig. 3a. **d** Quantification of sprout number in Fig. 3b. **e** mRNA expression of inflammatory marker iNos is decreased in BMM expressing DNMAML. *Scale bars* represent 200 μm. *Error bars* represent standard error. $*p < 0.05$. This capillary sprouting experiment was performed twice; results shown represent a typical iteration

than bound directly to beads. In this approach, the association between endothelial cells and pericytes was much more limited than if they were both bound to the bead, and no change in the growth of the vessel networks was observed relative to endothelial cell-only assays (Online Resource 6).

Inhibition of endothelial Jagged1 function prevented endothelial sprouting

To assess the role of Notch signaling in the interaction between endothelial cells and pericytes, we established Jagged1 knockdown endothelial cells using an shRNA

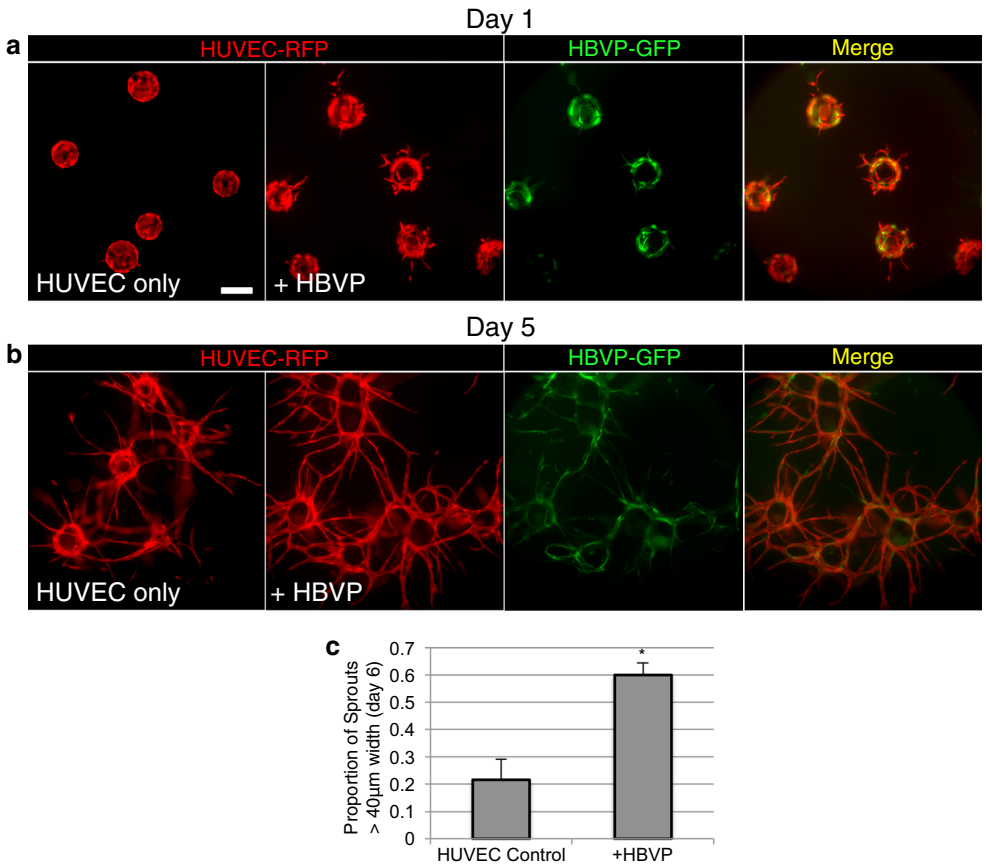

Fig. 4 Pericyte co-culture increases sprout number and vessel caliber. **a** Co-culture with human brain vascular pericytes (HBVP), labeled green, causes increased initial sprouting at early timepoint day 1. **b** Co-culture with HBVP leads to more complex vessel networks with wider vessel caliber by late timepoint day 5. **c** Quantification of sprout width. *Scale bars* represent 200 µm. *Error bars* represent standard error. $*p < 0.05$

construct to reduce Jagged1 expression in HUVEC, and knockdown was confirmed by Western blot (Online Resource 7). We found that Jagged1 knockdown almost completely abrogated sprouting in an endothelial monoculture bead-capillary sprouting assay (Fig. 5a). Co-culture of HUVEC-Jag1KD with pericytes was not able to significantly rescue this decreased sprouting, producing only scattered, scant vessels. (Figure 5a–c). These few vessels were noted to be slightly wider and showed limited association with the pericytes (Fig. 5a, d). Jagged1 knockdown also resulted in decreased HUVEC proliferation in monoculture (Online Resource 8). In contrast, we found that shRNA-mediated knockdown of Dll4 (Online Resource 9a) resulted in increased sprouting, both in endothelial cell-only sprouting assay and in an endothelial–pericyte co-culture assay (Online Resource 9b, c). Dll4 knockdown endothelial cells remained able to make extensive association with pericytes (Online Resource 9b).

Inhibition of endothelial Notch signaling decreased sprouting and partially disrupted endothelial/pericyte interactions

To further examine endothelial Notch function in co-culture assays, we expressed the DNMAML Notch inhibitory construct in HUVEC, which decreased endothelial sprouting in monoculture (Fig. 6a–c). Expression of DNMAML appeared to disrupt growth of endothelial–pericyte-containing sprouts, as co-culture of HUVEC-DNMAML with pericytes did not result in increased number or length of sprouts (Fig. 6a–c). However, the existing sprouts showed good association with pericytes and a roughly twofold increase in the presence of wide, mature sprouts, compared to the monoculture assay, similar to the increase in vessel width seen in wild-type co-culture (Fig. 6a, d). This suggests that at least some elements of the interaction between endothelial cells and pericytes remain intact when using endothelial cells expressing DNMAML.

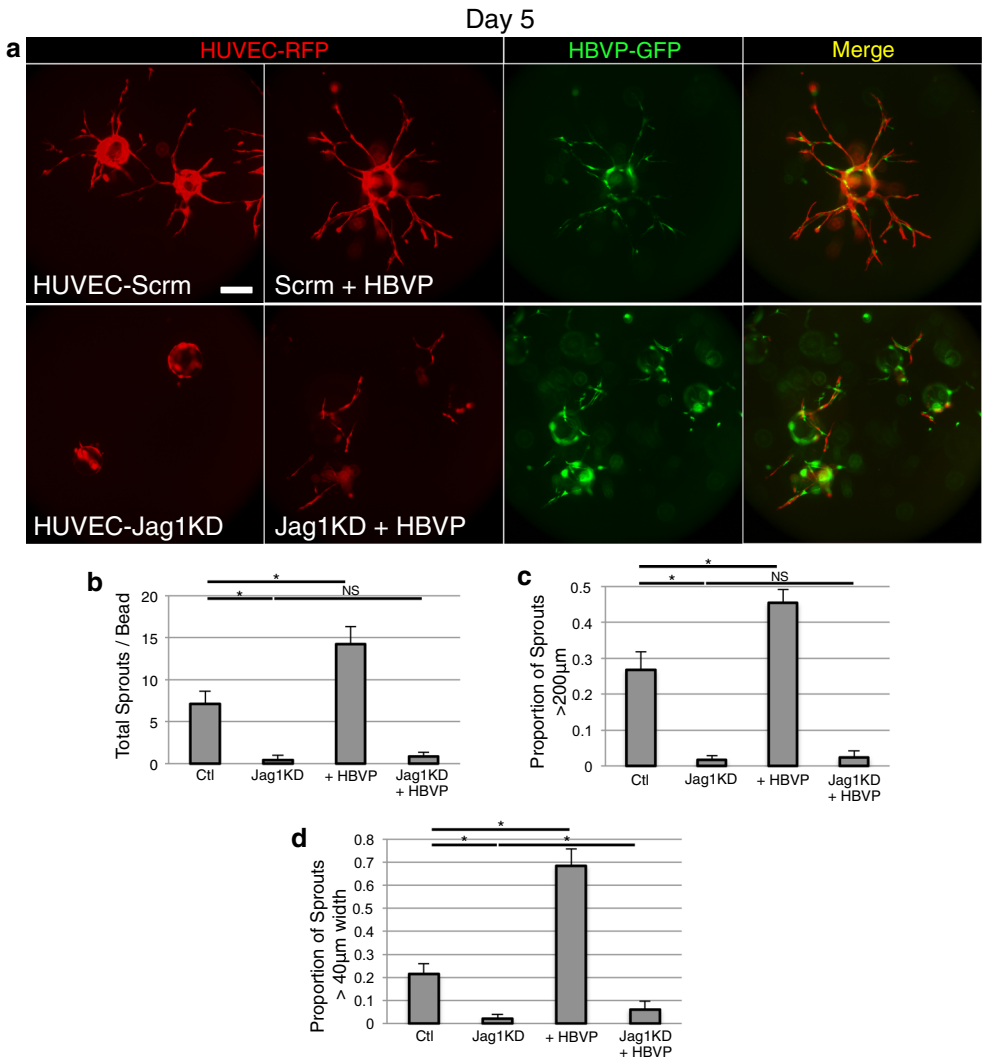

Fig. 5 Endothelial Jagged1 knockdown abrogates endothelial sprouting. **a** Endothelial shRNA-mediated Jagged1 knockdown causes failure of sprouting by day 5. Co-culture with wild-type pericytes causes a minimal increase in presence of small, disorganized sprouts, with some endothelial–pericyte association. **b** Quantification of number of sprouts. **c** Quantification of frequency of longer (>200 µm) sprouts. **d** Quantification of frequency of wide (>40 µm) vessels. *Scale bars* represent 200 µm. *Error bars* represent standard error. $*p < 0.05$

Pericyte Notch signaling was essential for cell survival and contribution to vessel maturation

We next employed the DNMAML construct to inhibit Notch signaling in pericytes and to determine whether pericyte Notch activity is necessary for growth of sprouts in the co-culture assay. DNMAML-expressing pericytes grew very poorly, and when co-cultured with endothelial cells they initially bound to the endothelial-coated beads (data not shown), but were not detectable by day 5 (Fig. 7a). Unsurprisingly, co-culture with these pericytes had minimal effects on endothelial sprouting or vessel maturation (Fig. 7a–d). Interestingly, co-culture with DNMAML-expressing pericytes actually decreased overall sprout length, resulting in only one-third the proportion of long sprouts compared to the endothelial monoculture assay (Fig. 7a, c).

Combination of macrophages and pericytes has multiplicative effects on angiogenesis

We tested whether the macrophage and pericyte co-cultures could be combined to create an even more accurate representation of the vascular microenvironment. We

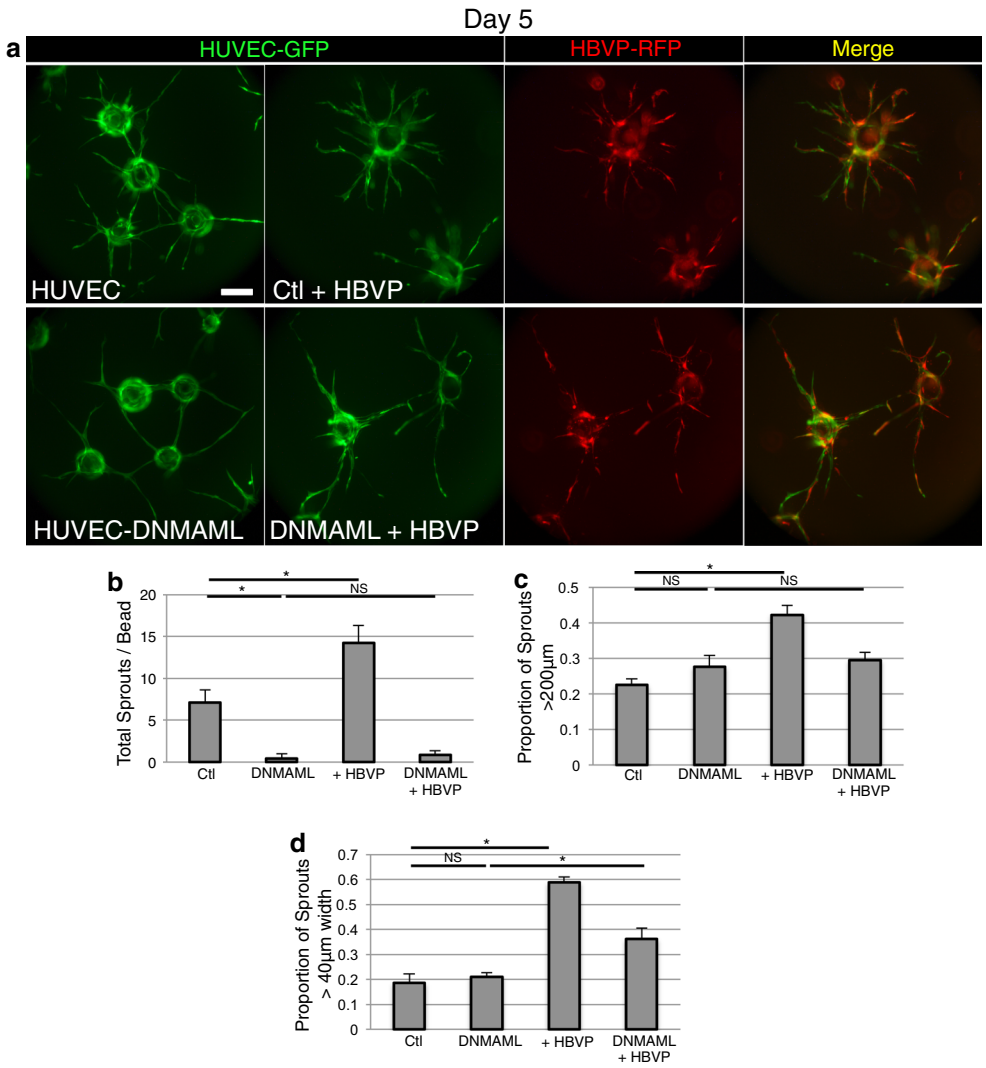

Fig. 6 Endothelial Notch receptor signaling inhibition decreases sprouting and partially disrupts endothelial–pericyte interaction. **a** Endothelial expression of DNMAML causes reduced sprouting. Addition of wild-type pericytes results in only a slight increase in sprouting and sprout length, much less than that caused by pericyte addition to control endothelial cells. However, pericyte co-culture does result in a >2-fold increase in wide, mature vessels, similar to control. **b** Quantification of number of sprouts. **c** Quantification of frequency of longer (>200 µm) sprouts**. d** Quantification of frequency of wide (>40 µm) vessels. *Scale bars* represent 200 µm. *Error bars* represent standard error. $*p < 0.05$

found that triple-cell cultures incorporating endothelial cells, macrophages, and pericytes grew well. These triple-cell assays exhibited a growth pattern suggesting non-redundant angiotrophic contribution from both types of stromal cells (Fig. 8a). The vasculature in triple-cell cultures exhibited more sprouts than addition of either BMM or HBVP individually (Fig. 8a, b), and the vessels formed were of comparable caliber to the HBVP co-cultures (Fig. 8a, c). Additionally, the percentage change in sprouting with the addition of macrophages (approximately 20 % in this iteration of the experiment) or pericytes (approximately 80 % in this iteration) was preserved when the two were combined. This suggests that the effects of macrophages and pericytes on the vasculature are multiplicative and are neither synergistic nor completely redundant.

Discussion

We demonstrated methodological advancements of a well-established capillary sprouting assay that uses beads coated with endothelial cells. This advancement allowed for examination of the important interactions between endothelial cells and the stromal cells of the vascular microenvironment. Using these methods, we documented

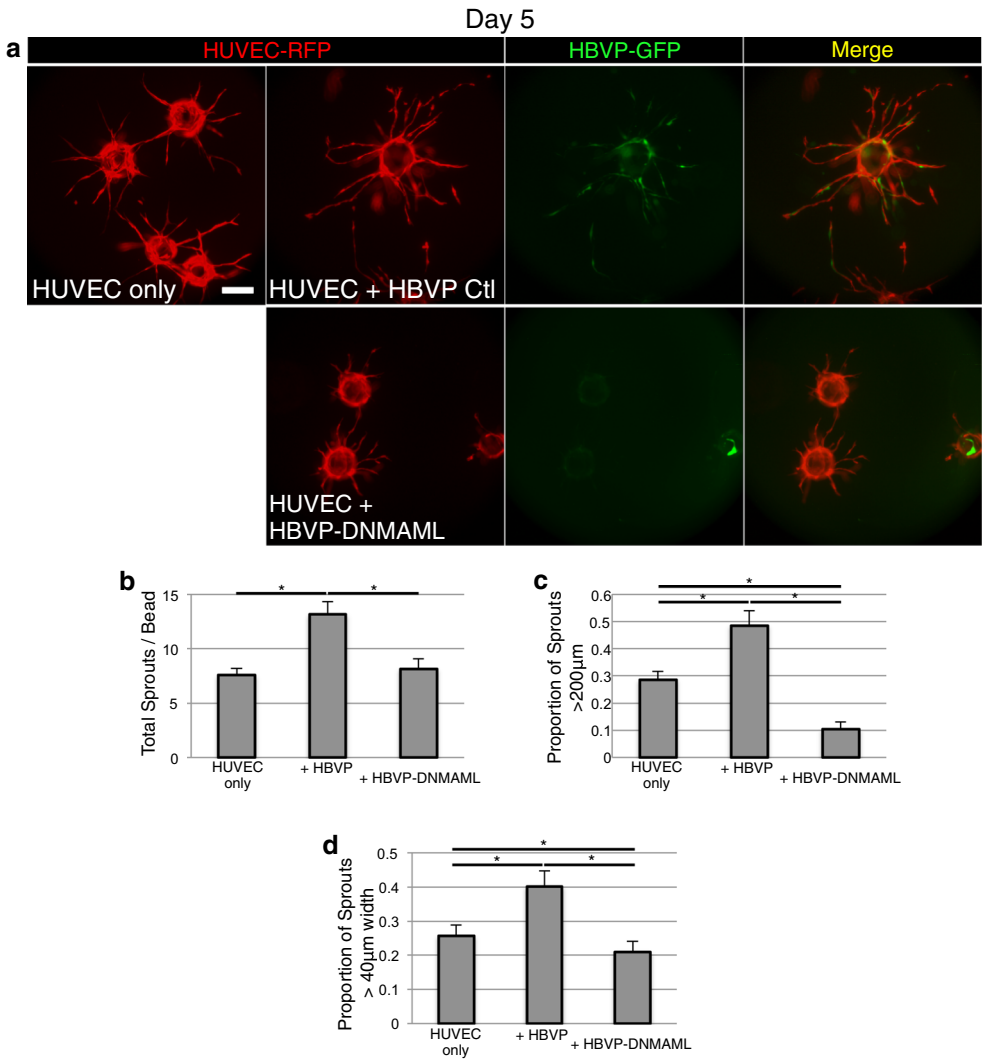

Fig. 7 Pericyte Notch receptor signaling inhibition causes pericyte death and prevents their angiotrophic contribution. **a** Expression of DNMAML in pericytes causes degeneration of pericytes from the culture by endpoint day 5. Endothelial sprouting and maturation are unaffected by addition of Notch-inhibited pericytes and show decreased sprout length. **b** Quantification of number of sprouts. **c** Quantification of frequency of longer (>200 µm) sprouts. **d** Quantification of frequency of wide (>40 µm) vessels. *Scale bars* represent 200 µm. *Error bars* represent standard error. *$p < 0.05$

the effect of macrophage and pericyte co-culture on endothelial sprouting and describe a role for Notch signaling in both types of interaction.

Inclusion of myeloid cells alongside endothelial-coated beads greatly increased the rate and extent of endothelial sprouting. The exact percentage increase in sprouting varies between approximately 20–50 % in different iterations of the experiment, but is always a statistically significant increase. This variability may be due in part to variability in the macrophages, which are derived from mouse bone marrow for every separate experiment and thus may vary slightly in their angiotrophic potential. For this reason, every experiment is rigorously internally controlled and replicated, and sprout growth is always compared within a single experimental iteration.

The pro-angiogenic character of myeloid cells has been documented previously in aortic ring explant studies [2], but the assay we utilized represents an even more reductive distillation of this interaction, as it incorporates only endothelial and myeloid cells. In addition, we show that polarizing the added macrophages impacted their ability to foster angiogenic sprouting. Notably, inflammatory polarization using LPS and interferon γ increased the angiotrophic character of the macrophages, meaning that they increased the ability of these macrophages to contribute to angiogenesis via the secretion of angiogenic factors. In

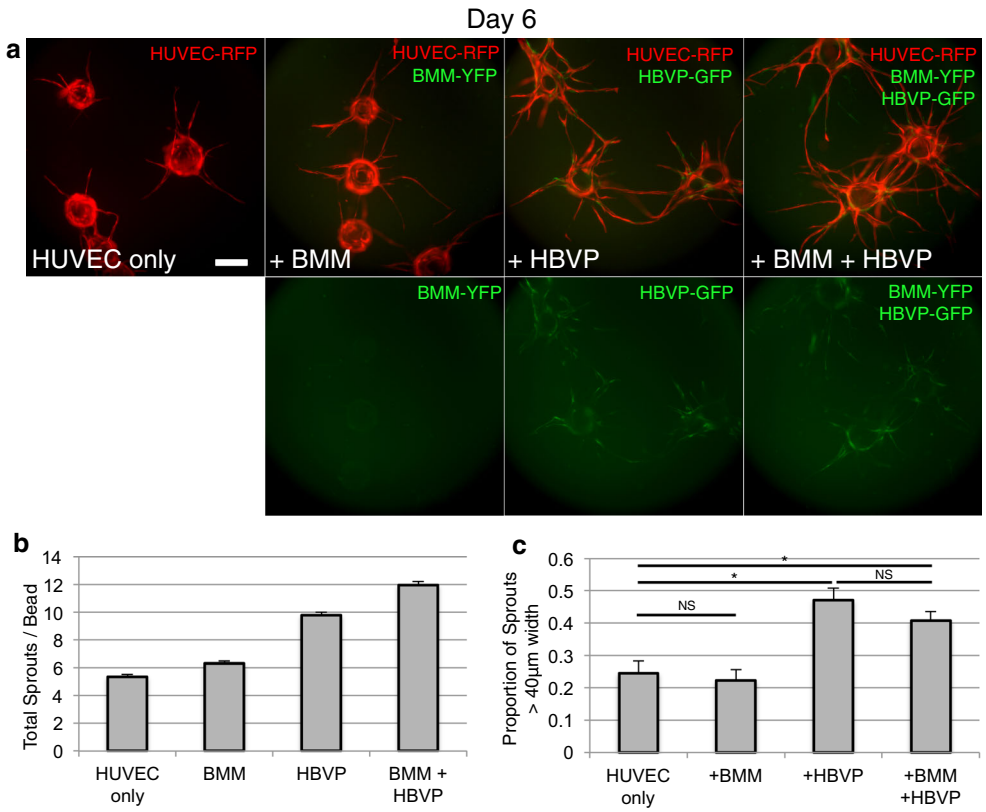

Fig. 8 Endothelial, macrophage, and pericyte triple culture shows multiplicative effects on angiogenesis. **a** Co-culture of HUVEC with BMM and HBVP shows increased sprouting compared to BMM or HBVP alone and maintains the increased vessel caliber seen in HBVP co-culture. BMM-YFP and HBVP-GFP both appear in the green channel and may be distinguished by morphology. **b** Quantification of sprout number at day 6, showing multiplicative increase in sprouting in the triple culture. All experimental groups are statistically significantly different ($p < 0.05$) from all other groups. **c** Quantification of vessel caliber. *Scale bars* represent 200 μm. *Error bars* represent standard error. *$p < 0.05$

contrast, treatment with alternative activator IL-4 did not enhance macrophage angiotrophism. Alternatively activated macrophages are held to play a substantial role in the development of tumor angiogenesis [6, 7]. Indeed, recent in vivo gel implantation studies have suggested that alternatively activated macrophages show additional angiotrophic character due to increased metalloproteinase activity, while inflammatory macrophages provided less pro-angiogenic stimulus [26]. It may be that chemical and structural differences between the collagen substrate of these in vivo experiments and the fibrin of the capillary sprouting assay alter the relative importance of metalloproteinases for the stimulation of angiogenesis. Additionally, the reductive nature of the bead-capillary sprouting assay may mean that direct pro-angiogenic effects of inflammatory macrophages are emphasized that may not be evident in the context of complex multicellular tissues. Though some studies have suggested that inflammatory polarized macrophages do not to contribute to angiogenesis [6, 7, 27], there is a growing body of evidence to indicate that inflammatory mediators can play a key pro-angiogenic role in certain contexts. A past study of in vitro angiogenesis employing mouse and rat aortic rings has demonstrated that treatment with TLR ligands such as LPS can recruit mural cells, cause macrophage transformation, and enhance angiogenesis in an NFκB-dependent fashion [28]. Additionally, it has been shown that secretion of pro-angiogenic factors such as VEGF-A by inflammatory macrophages represents an important part of the wound healing process [29]. It may be that similar functionality is being observed in this assay.

We observed that inhibition of macrophage Notch signaling was sufficient to abrogate the angiotrophic advantage of inflammatory polarization, such that Notch-inhibited, inflammatory polarized macrophages increased angiogenic sprouting at a rate comparable to non-stimulated cells. This is consistent with the role of Notch as a mediator of macrophage inflammatory polarization [25]. However, we observed that the up-regulation of the inflammatory marker iNOS, while stunted compared to wild type, was still substantially up-regulated in inflammatory polarized, Notch-inhibited macrophages. This

suggests that Notch signaling, in addition to its role as a gatekeeper of inflammatory polarity, may also be involved mechanistically in the observed increase in angiotrophism.

We examined the interaction between pericytes and endothelial cells in the bead-capillary sprouting assay. This relationship has previously been examined during vasculogenesis in collagen gels, where randomly seeded endothelial cells and pericytes interact to form vascular networks [18]. The role of vascular smooth muscle cells (VSMCs) in angiogenesis has been explored using the bead-capillary sprouting assay [13]. In these experiments, VSMCs were seeded evenly throughout the fibrin gel, such that they made limited but consistent contact with the endothelial sprouts [13]. Pericytes are perhaps better suited for the capillary sprouting assay than VSMCs, as they are known to interact with small caliber capillaries, which resemble the sprouts in the bead-capillary sprouting assay, while VSMCs interact with larger caliber vessels. Rather than seeding pericytes throughout the gel, we allowed pericytes to adhere directly to the bead-bound endothelial monolayer, which caused pericytes to migrate out from an existing monolayer, presumably along with endothelial cells, to form uniform close interactions with the maturing sprouts in a fashion that closely resembled observed in vivo behavior. Association with these pericytes drastically altered the morphology of the growing sprouts, leading to an increase in the number of sprouts, possibly due to decreased sprout regression compared to endothelial-only controls. Most significantly, pericyte-containing beads had a greater proportion of mature, wide vessels than the endothelial-only wells, and more lumens were visible. Thus, the pericytes in the co-cultures are fulfilling many of their described in vivo functions within the reductive, in vitro context of the capillary sprouting assay.

We examined the role of Notch ligand–receptor signaling in the interactions between endothelial cells and pericytes using co-culture assays. This proved to be challenging, as both Notch signaling and ligand expression were important for the growth of endothelial cells in monoculture bead-capillary sprouting assays. As a result, both Jagged1- and Notch-inhibited endothelial cells had significantly decreased sprouting. Indeed, the endothelial cells knocked down for Jagged1 failed to sprout at all, and while addition of pericytes may have caused a small increase in sprouting, it is difficult to draw any concrete conclusions about the role of endothelial Jagged1 in this process. The role of endothelial Dll4 and Notch signaling is well studied [10], and the results of our experiments employing both Dll4 knockdown and Notch receptor inhibition are in line with these established relationships. By contrast, the demonstrated inhibitory role for Jagged1 in angiogenesis is controversial, as others have proposed that Jagged1 may act chiefly as an antagonist of Dll4–Notch interactions [30], in which case one would not expect Jagged inhibition to have such a profound anti-angiogenic effect. However, our group has previously demonstrated that Jagged inhibition via a Notch1 soluble decoy causes decreased sprout formation in the bead-capillary sprouting assay [31], consistent with the results of the present study. This context-dependent differential role for Jagged1 in angiogenesis may be fundamentally dependent upon the glycosylation state of the Notch receptors, which may bias the receptor affinity toward Delta-like or Jagged ligands. In contrast, endothelial Notch inhibition via DNMAML caused only a modest decrease in sprouting, such that pericyte–endothelial interactions could still be assessed. In this setting, addition of pericytes did not have a strong effect on endothelial sprout formation or sprout length, suggesting that some elements of the endothelial–pericyte interaction may rely on intact Notch signaling function in endothelial cells. Interestingly, these co-cultures did show an increase in vessel width and maturity comparable to wild-type co-cultures. This suggests that the twofold contribution of pericytes to both initial sprouting and vessel maturation may occur via separate signaling modalities, and that endothelial Notch receptor signaling is only important in sprout initiation and lengthwise growth, and not in subsequent endothelial maturation.

We attempted to assay the effect of pericyte Notch signal inhibition on their contribution to endothelial sprouting and growth. Notch-inhibited pericytes grew very poorly, and when added to the co-cultures they failed to survive for the course of the experiment. Predictably, these degenerated pericytes failed to increase sprouting or vessel maturation in endothelial cells and actually stunted the growth of the sprouts. This is likely due to secondary effects from local resource consumption and cell death rather than to a specific modulation of endothelial–pericyte interactions. Future studies employing Notch receptor-specific knockdown may help to separate out this primary effect on pericytes and allow specific examination of the role of pericyte Notch in the interplay between pericytes and the endothelium.

Lastly, we combined the two co-culture techniques to create an assay that contained endothelial cells alongside both macrophages and pericytes. We found that wells containing BMM and HBVP showed evidence of the angiotrophic effect of both cell types, with increased sprouting and wide vessel caliber. The increase in sprout number was approximately 20 % over HBVP alone, which (in this iteration of the assay, at this timepoint) corresponded to the difference in sprouting between HUVEC alone and BMM co-culture. This increased sprouting suggested that the angiotrophic effects of combining BMM and HBVP are multiplicative rather than synergistic. The additive nature of the sprout increase may suggest non-

redundant roles for BMM and HBVP in the stimulation of angiogenic sprouting. It is possible that macrophages provide pro-angiogenic factors necessary for sprouting to occur, while a key function of pericytes is to stabilize nascent sprouts, decreasing their rate of regression and leading to increased overall sprout number. With the validation of triple co-culture, this assay is well placed to tease apart the disparate contributions of these different cell types.

Taken together, our studies extend the functionality of the bead-capillary sprouting assay to not only faithfully re-create endothelial sprouting and maturation, but also to model endothelial interactions with macrophages and pericytes within the context of angiogenesis. This allows for the exploration of not just the role of Notch signaling, as demonstrated here, but myriad other signaling modalities in a simplified setting that nonetheless captures many of the important elements of sprouting angiogenesis that have been historically difficult to re-create in vitro. These advancements therefore represent an important step toward a greater understanding of intercellular dynamics within the vascular microenvironment and potentially our ability to holistically understand and therapeutically manipulate the process of angiogenesis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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