Interleukin-19 induces angiogenesis in the absence of hypoxia by direct and indirect immune mechanisms


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Submitted 6 January 2016; accepted in final form 4 April 2016

Kako F, Gabunia K, Ray M, Kelemen SE, England RN, Kako B, Scalia RG, Autieri MV. Interleukin-19 induces angiogenesis in the absence of hypoxia by direct and indirect immune mechanisms. Am J Physiol Cell Physiol 310: C931–C941, 2016. First published April 6, 2016; doi:10.1152/ajpcell.00006.2016.—Neovascularization and inflammation are independent biological processes but are linked in response to injury. The role of inflammation-dampening cytokines in the regulation of angiogenesis remains to be clarified. The purpose of this work was to test the hypothesis that IL-19 can induce angiogenesis in the absence of tissue hypoxia and to identify potential mechanisms. Using the aortic ring model of angiogenesis, we found significantly reduced sprouting capacity in aortic rings from IL-19−/− compared with wild-type mice. Using an in vivo assay, we found that IL-19−/− mice respond to vascular endothelial growth factor (VEGF) significantly less than wild-type mice and demonstrate decreased capillary formation in Matrigel plugs. IL-19 signals through the IL-20 receptor complex, and IL-19 induces IL-20 receptor subunit expression in aortic rings and cultured human vascular smooth muscle cells, but not endothelial cells, in a peroxisome proliferator-activated receptor-γ-dependent mechanism. IL-19 activates STAT3, and IL-19 angiogenic activity in aortic rings is STAT3-dependent. Using a quantitative RT-PCR screening assay, we determined that IL-19 has direct prosprangiogenic effects on aortic rings by inducing angiogenic gene expression. M2 macrophages participate in angiogenesis, and IL-19 has indirect angiogenic effects, as IL-19-stimulated bone marrow-derived macrophages secrete prosprangiogenic factors that induce greater sprouting of aortic rings than unstimulated controls. Using a quantitative RT-PCR screen, we determined that IL-19 induces expression of angiogenic cytokines in bone marrow-derived macrophages. Together, these data suggest that IL-19 can promote angiogenesis in the absence of hypoxia by at least two distinct mechanisms: 1) direct effects on vascular cells and 2) indirect effects by stimulation of macrophages.

angiogenesis; cytokines; endothelial cell; macrophage; vascular smooth muscle

ANGIOGENESIS, THE GROWTH of new blood vessels from preexisting blood vessels, is a dynamic, complex, and tightly regulated process that involves numerous cell types and soluble factors. Activation of endothelial cells (EC) by autocrine or paracrine factors initiates migration, proliferation, and polarization of EC. The order and extent of activation of each of these processes need to be tightly regulated, as they are also part of many pathophysiological processes other than angiogenesis, such as inflammation, permeability, and wound healing (7, 24).

Although inflammation and neovascularization are distinct processes, they are inexorably linked. Cytokines and their receptors common to leukocytes and EC link inflammatory and angiogenic processes: inflammatory cytokines participate in angiogenesis, and their contribution to generation of new vessels is necessary.

While it is generally accepted that proinflammatory cytokines increase angiogenesis, angiogenic effects of purported anti-inflammatory cytokines on EC are less clear. IL-10 is associated with VEGF downregulation and reduction of fibroblast growth factor (FGF)- and VEGF-induced proliferation of microvascular EC and is antiangiogenic (38). While IL-4 has been shown to inhibit VEGF production and reduce vascularization, it can also induce migration and tubelike structure formation in EC (13, 23, 43). IL-13 attenuates EC tube formation, and IL-20 has both pro- and antiangiogenic effects (16, 18, 30, 41). Anti-inflammatory modalities that not only limit tissue damage, but also increase capillary density, collateral formation, and perfusion, have the potential to salvage ischemic tissue. Thus it is important to identify and characterize molecules that participate in these processes, as they could lead to new therapies for tissue repair and neovascularization.

We previously reported prosprangiogenic effects of IL-19, a purported anti-inflammatory interleukin (11, 12). IL-19 is considered to be a member of the IL-10 subfamily, which includes IL-20, IL-22, and IL-24 (31, 34). Studies in our laboratory have suggested important functions for IL-19 outside the immune system. IL-19 is unique from other IL-10 family members, in that it is expressed by resident vascular cells, EC, and vascular smooth muscle cells (VSMC), suggesting the potential for IL-19 autocrine and paracrine prosprangiogenic effects (20, 40). In a previous study we reported that IL-19 was expressed in angiogenic tissue and had proliferative and promigratory effects on cultured human EC (20). In a more recent study we determined that IL-19 regulated neovascularization in the murine hindlimb ischemia model (35). In this study we also found that IL-19 could target and polarize the macrophage to the M2, prosprangiogenic phenotype, but we did not assay a direct causal effect of IL-19-stimulated macrophages on the angiogenic process. While use of the hindlimb ischemia model is an important in vivo platform on which to study angiogenesis, limitations inherent to any in vivo system, such as inflammation and hypoxia, make it difficult to discriminate between primary responses of EC and paracrine effects of immune cells. Furthermore, in the hindlimb ischemia model, blood flow recovery and vasculogenesis are thought to be driven by tissue hypoxia.

A useful ex vivo model of angiogenesis is the aortic ring assay, a three-dimensional organ-culture system (39). Sprout-
IL-19 induces angiogenesis by multiple mechanisms

Aortic ring assay. The aortic ring assay was carried out as we described previously (20) using wild-type or IL-19−/− C57BL/6 mice (8–10 wk old) according to standard protocols (3). Briefly, thoracic aortas were excised from the mice, and periadventitial tissues were removed. Aortas were cut into 1-mm rings, rinsed five times with PBS and delivered subcutaneously as a single plug to C57BL/6 wild-type or IL-19−/− mice. After 9 days, the plug was recovered and processed for immunohistochemistry, and microvessels, defined as CD31-positive structures surrounding a lumen, were identified by immunohistochemistry using platelet endothelial cell adhesion molecule (PECAM) antibody. Vessel density was expressed as number of vessels per square millimeter in more than six independent fields from more than four plugs. Four different mice were used per experimental condition, with the experiment performed three times. All animal procedures were approved by the Temple University Animal Care and Use Committee.

Immunohistochemistry. Matrigel plugs were recovered from mice and processed for immunohistochemistry as described elsewhere (20). Briefly, paraffin-embedded sections were incubated with anti-PECAM1 antibody (Neo Markers, San Diego, CA) at a concentration of 4 µg/ml and then with biotinylated secondary antibody (1:200 dilution) followed by avidin-biotin-peroxidase complex in a Vectastain Elite kit (both from Vector Labs, Burlingame, CA). The reaction product was visualized, with 3,3′-diaminobenzidine (Vector Labs) used as the chromogenic substrate, producing a reddish-brown stain. The sections were counterstained with hematoxylin and photographed. Whole-mount immunohistochemistry of aortic rings was performed using the thin-gel method as described elsewhere (43). Briefly, aortic rings were explanted and cultured as described above on Nunc chamber slides. After 7 days, gels containing explants were fixed in 10% neutral buffered formalin, washed in PBS, and then in 0.25% Triton X-100, and blocked with 5% rabbit serum. Tissue was fixed in 10% neutral buffered formalin, washed in PBS and then in 0.25% Triton X-100, and blocked with 5% rabbit serum. Tissue was then processed for immunohistochemistry, and microvessels, defined as CD31-positive structures surrounding a lumen, were identified by immunohistochemistry using platelet endothelial cell adhesion molecule (PECAM) antibody. Vessel density was expressed as number of vessels per square millimeter in more than six independent fields from more than four plugs. Four different mice were used per experimental condition, with the experiment performed three times. All animal procedures were approved by the Temple University Animal Care and Use Committee.

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Fig. 1. IL-19 promotes endothelial cell (EC) microvessel formation in mouse aortic rings. A: sectioned thoracic aortas from wild-type and IL-19−/− mice were cultured in triplicate in growth factor-reduced Matrigel in the presence or absence of IL-19, vascular endothelial growth factor (VEGF), or both, and microvessel outgrowth was photographed after 7 days. B: densitometric quantitation of the ratio of outgrowth area to tissue area (microvessel sprouting) in VEGF-treated EC. C: densitometric quantitation of the ratio of outgrowth area to tissue area (microvessel sprouting) in IL-19-stimulated EC. Values were obtained from 3 independent experiments. *P < 0.05, IL-19−/− [knockout (KO)] vs. wild-type. **P < 0.01 vs. unstimulated.
to stained explants. Images were captured with an Olympus THX-100 microscope and photographed at \( \times 20 \) magnification.

**Cells and culture.** Primary human microvascular EC and coronary artery VSMC were obtained as cryopreserved secondary culture from Lonza and used at passages 3–5. EC were grown in 5% fetal calf serum (FCS) and VSMC in 10% FCS. For proliferation, 5,000 primary human EC or 10,000 VSMC were seeded into 24-well plates. After adhesion, the medium was replaced with serum-reduced (0.5% FCS) basal medium, and samples were supplemented with 50% conditioned medium from bone marrow-derived macrophages (BMDM). At 4 days after seeding, cells were counted in the presence of Trypan blue using a standard hemocytometer as described elsewhere (35).

**Conditioned medium from bone marrow-derived macrophages (BMDM).** At 4 days after seeding, cells were counted in the presence of Trypan blue using a standard hemocytometer as described elsewhere (35). A second STAT3 inhibitor III, WP1066, was used at a concentration of 5.0 \( \mu \text{M} \) (19). Each inhibitor was added 30 min prior to addition of IL-19, as we described elsewhere (10).

**Western blotting.** Protein extracts from aortic rings were made as described elsewhere (35), separated by SDS-PAGE, transferred to a nitrocellulose membrane, incubated with a 1:3,000 dilution of phosphorylated (Tyr705) STAT3 antibody (Cell Signaling Technology), and a 1:8,000 dilution of secondary antibody. For Western blotting of IL-20 receptors 1 and 2 (IL-20R1 and IL-20R2; Santa Cruz Biotechnology) were used at a 1:3,000 dilution. For detection of gene expression in aortic rings and BMDM using SYBR green, the murine angiogenesis RT2 Profiler PCR array (SABiosciences) was used according to the manufacturer’s instructions and as we described previously (35). The following primer pairs were purchased from Integrated DNA Technologies (Coralville, IA): 5'-GCAAGACACT-GAGCAAGAG (forward) and 5'-GGGTCGAGATGAAAATTTG (reverse) for mouse GAPDH, 5'-TCTTCTCAGATCCGTATCGT (forward) and 5'-TCAGTGGTTCAAGACTTCTG (reverse) for mouse IL-20R1, 5'-TGTCGCGATTTCTTTG (forward) and 5'-CACAGCCCAAAAGAGATGCT (reverse) for mouse IL-20R2, 5'-TG-GCTTCCACTGTTATTTG (forward) and 5'-CCTGGTCATCATGTTTAG (reverse) for human IL-20R1, and 5'-GAGCAGCT-GATCCAGTCCG (forward) and 5'-TCTTCTCCGTCAAGATCGCC (reverse) for human PPARγ.

**BMDM and conditioned medium.** For generation of BMDM, mouse femurs and tibiae were flushed with sterile DMEM. After lysis of red blood cells, total bone marrow cells were plated at a density of 3.5 \( \times 10^6 \) cells per 10-cm petri dish in 10 ml of macrophage growth medium.

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![Image](https://example.com/image1.png)  
**Fig. 2.** IL-19 augments capillary invasion of Matrigel plugs in vivo. A: wild-type or IL-19-deficient (IL-19 KO) mice were injected subcutaneously with 0.5 ml of Matrigel premixed with PBS (control), VEGF, or IL-19. After 9 days, Matrigel plugs were harvested and processed for immunofluorescence [platelet endothelial cell adhesion molecule 1 (PECAM1) staining]. Image is representative of 4 plugs from 4 different mice. Magnification \( \times 600 \) (plugs) and \( \times 400 \) (capsule). B: quantification of data from representative photomicrographs of PECAM1-positive cells surrounding a lumen in the plug and capsule. HPF, high-power field. **P < 0.01 vs. PBS (control).
[complete DMEM with 10% FBS and 100 ng/ml macrophage colony-stimulating factor (PeproTech)] and allowed to differentiate for 5–7 days. Cells were fed an additional 5 ml of growth medium on day 3. On day 7, cells were lifted with VERSENSE 1 X solution (GIBCO) at 37°C and replated in 12- or 6-well plates [1 × 10⁶ cells ml per well (12-well) or 2 × 10⁶ cells per 3 ml well (6-well)] in macrophage complete medium (DMEM + 10% FBS). BMDM were then incubated with 100 ng/ml IL-19 for 24 h. Medium was removed, cells were washed three times with PBS, fresh medium was supplied, and cells were incubated for 24 h to generate conditioned medium.

Statistical analysis. Results are expressed as means ± SE. Differences between groups were evaluated using ANOVA, with the Newman-Keuls method to evaluate differences between individual mean values and paired t-tests where appropriate. Differences were considered significant at P < 0.05.

RESULTS

Sprouting in aortic rings from IL-19−/− mice is reduced in response to VEGF. To determine if deletion of IL-19 reduced angiogenesis, we used the aortic ring assay, in which EC sprout from preexisting vessels to form new structures. Mouse thoracic aortas from wild-type and IL-19−/− mice were sectioned, placed onto growth factor-reduced Matrigel, and incubated in medium containing VEGF. Sprouting from rings was analyzed daily and photographed on day 7, and outgrowth area was quantitated by image analysis. Microvessel outgrowth was significantly greater in aortic rings from wild-type mice than in those explanted from IL-19−/− mice (5.31 ± 0.55 vs. 3.61 ± 0.44, P < 0.05; Fig. 1, A and B). Aortic rings from IL-19−/− mice did, however, respond to IL-19 stimulation and exhibited significantly greater microvessel outgrowth than unstimulated rings and in an amount comparable to wild-type rings (1.76 ± 0.20 vs. 3.85 ± 0.38, P < 0.01; Fig. 1, A and C).

IL-19 increases angiogenesis in vivo. We utilized the Matrigel plug assay to determine if the absence of IL-19 was detrimental for microvessel formation in vivo. For these experiments, 0.5 ml of growth-factor free Matrigel was mixed with 200 ng of VEGF or PBS control and delivered as a single plug subcutaneously to wild-type or IL-19−/− mice. After 9 days, the plug was recovered, and microvessels, defined as CD31-positive structures surrounding a lumen, were determined by immunohistochemistry. Significantly more PECAM1-positive structures surrounding a lumen were contained in Matrigel plugs from wild-type than IL-19−/− mice (4.1 ± 0.84 vs. 1.53 ± 0.29, P < 0.01; Fig. 2, A and B). Most striking was the large amount

Fig. 3. Differential expression of IL-20 receptor subunits (IL-20R1 and IL-20R2) in IL-19-stimulated aortic rings. A: IL-20R2, but not IL-20R1, mRNA expression is induced by IL-19 stimulation in isolated aortic rings. B: differential expression of IL-20R1 and IL-20R2 in cultured human endothelial cells (EC) and vascular smooth muscle cells (VSMC). IL-20R1 is not induced by IL-19 in cultured EC and VSMC. C: IL-20R2 is expressed at higher levels in VSMC than EC, and IL-19 is able to increase IL-20R2 mRNA in VSMC, but not EC. D: representative immunoblot showing no change in IL-20R1 and IL-20R2 protein expression in IL-19-stimulated human EC. E: representative immunoblot showing significant increase in IL-20R2, but not IL-20R1, protein expression in IL-19-stimulated human VSMC. F: densitometric analysis of IL-20R1 expression in human EC and VSMC. G: densitometric analysis of IL-20R2 protein expression in human EC and VSMC. Values were calculated from data obtained from ≥3 different Western blots. *P < 0.05, **P < 0.01.
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IL-19 does not have its own named receptor but, rather, signals through the IL-20 heterodimeric receptor complex (32). To determine if these receptors are expressed and regulated in aortic rings, RNA was extracted from control and IL-19-stimulated rings. Figure 3A shows expression of IL-20R1 and IL-20R2 murine aortic rings but significantly more IL-20R2 expression in IL-19-stimulated than control rings (1.00 ± 0.07 vs. 2.24 ± 0.08, \( P < 0.001 \)). The major cell types in aortic rings are VSMC and EC, but IL-20 receptor expression has not been characterized in either cell type. We next quantified expression of each receptor chain in cultured human EC and VSMC. Similar to our observations in aortic rings, we saw no significant difference in basal levels of IL-20R1 mRNA or protein in EC and VSMC, nor did we observe an increase in IL-20R1 expression in response to IL-19 stimulation (Fig. 3, B, D, and F). In contrast, we observed significantly higher basal levels of the IL-20R2 chain mRNA in VSMC than EC (Fig. 3C). Importantly, we saw robust induction of the IL-20R2 chain mRNA and protein when VSMC were stimulated with IL-19 (Fig. 3, C, E, and G). This contrasts with EC, in which IL-19 did not affect the basal levels of the IL-20R2 chain. This suggests that IL-19 stimulation induces increased IL-20R2, but not IL-20R1, expression in VSMC, but not EC.

It was important to determine the molecular mechanisms responsible for the differential expression of IL-20R2. First, we determined the role of STAT3 in this process. Because IL-19 does not induce IL-20R2 expression in EC but strongly induces IL-20R2 expression in VSMC, we treated human VSMC with two different STAT inhibitors and IL-19. Figure 4A shows that neither of these inhibitors reduces the IL-19-driven increase in IL-20R2 mRNA expression, suggesting that STAT3 does not play a role in IL-19-driven IL-20R2 expression in VSMC.

We know from other studies in macrophages that IL-19 induces PPARγ expression and activation (10). The IL-20R2 promoter contains two PPARγ consensus elements; therefore, we quantitated IL-19-induced PPARγ expression in VSMC and EC. Figure 4B shows that IL-19 induced PPARγ mRNA expression in VSMC, but not EC (0.66 ± 0.1 vs. 1.17 ± 0.02 for EC and VSMC, respectively, \( P < 0.05 \)). We next transfected VSMC with PPARγ siRNA and found that IL-19 stimulation failed to induce IL-20R2 mRNA and protein expression, suggesting that IL-19 activation of PPARγ regulates IL-20R2 expression in VSMC (Fig. 4, C–E).
IL-19 angiogenic activity is mediated by STAT3 activation. The IL-20 receptor is known to activate STAT3 (12, 32). To ascertain potential molecular mechanisms for IL-19 angiogenic effects, we determined if IL-19 activated STAT3 in aortic rings. Protein was extracted from unstimulated control and rIL-19-stimulated rings, and lysates were immunoblotted with anti-phosphorylated (Tyr705) STAT3. B: densitometric quantitation of STAT3 phosphorylation in aortic rings. C: rings incubated with STAT inhibitors (Stattic and WP1066) show significantly less microvessel sprouting than controls. D: densitometric quantitation of ratio of area of microvessel sprouting to tissue area in response to IL-19. E: expression and inhibition of matrix metalloproteinase (MMP) proteins in aortic rings. Representative immunoblot shows MMP2, MMP9, and MMP14 protein expression in unstimulated and IL-19-stimulated aortic rings. F: densitometric quantitation of MMP expression in aortic rings. Values were calculated from data obtained from ≥3 Western blots. *P < 0.05, ***P < 0.001.

IL-19 induces angiogenic gene expression in aortic rings. Angiogenesis in aortic cultures is regulated by communication between EC, smooth muscle cells, and other cell types and driven by angiogenic gene expression. A quantitative RT-PCR-based screening assay was used to test the hypothesis that IL-19 could directly induce expression of proangiogenic genes in isolated aortic rings. Of 96 possible angiogenesis-related genes, 13 were identified to have a role in angiogenesis and/or EC activation (Table 1), which increased ≥2.0-fold. This suggests that IL-19 treatment alone, in the absence of hypoxia or inflammation, can directly induce a gene expression pattern consistent with angiogenesis in isolated aortic rings.

Proteolysis is one of the earliest and most sustained activities involved in the formation of new blood vessels, and members of the MMP family are involved in many steps in the angiogenic process (15). It was striking that mRNAs for three different MMPs were induced by IL-19. To validate MMP protein expression from aortic rings and, also, to determine if these MMPs are targets of IL-19-induced STAT3 activation, we extracted protein from aortic rings treated with STAT3 inhibitors. Figure 5, E and F, shows increased MMP2, MMP9, and MMP14 expression in IL-19-treated compared with untreated aortic rings. Furthermore, expression of MMP14, but not MMP2 or MMP9, was reduced by the STAT3 inhibitor Stattic.

Conditioned medium from BMDM incubated with IL-19 is angiogenic. It is recognized that M2 macrophages have proangiogenic effects (4), and we previously showed that IL-19 induces a polarization to the M2 macrophage phenotype (35).
Table 1. Quantitative RT-PCR screening of genes associated with angiogenesis

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Name</th>
<th>Fold Change</th>
</tr>
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<tbody>
<tr>
<td>Coll1a1</td>
<td>Collagen, type XVIII, α1</td>
<td>3.34</td>
</tr>
<tr>
<td>Ephb2</td>
<td>Ephrin B2</td>
<td>4.16</td>
</tr>
<tr>
<td>Epas1</td>
<td>Endothelial PAS domain protein 1</td>
<td>1.99</td>
</tr>
<tr>
<td>Igf1</td>
<td>Insulin-like growth factor 1</td>
<td>3.11</td>
</tr>
<tr>
<td>Jag1</td>
<td>Jagged 1</td>
<td>2.28</td>
</tr>
<tr>
<td>Mmp14</td>
<td>Matrix metalloproteinase 14</td>
<td>3.83 (membrane-inserted)</td>
</tr>
<tr>
<td>Mmp2</td>
<td>Matrix metalloproteinase 2</td>
<td>3.97</td>
</tr>
<tr>
<td>Mmp9</td>
<td>Matrix metalloproteinase 9</td>
<td>2.60</td>
</tr>
<tr>
<td>Pecam1</td>
<td>Platelet endothelial cell adhesion molecule 1</td>
<td>15.13</td>
</tr>
<tr>
<td>Pgf</td>
<td>Placental growth factor</td>
<td>5.77</td>
</tr>
<tr>
<td>Plau</td>
<td>Plasminogen activator, urokinase</td>
<td>15.24</td>
</tr>
<tr>
<td>Slpr1</td>
<td>Sphingosine-1-phosphate receptor 1</td>
<td>6.45</td>
</tr>
<tr>
<td>Thbs2</td>
<td>Thrombospondin 2</td>
<td>2.99</td>
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RNA was extracted from 4 pooled aortic rings from wild-type mice treated with IL-19 or PBS for 7 days. RNA was reverse-transcribed, and target genes were amplified by quantitative PCR. Genes with fold change >2.0, P < 0.05, which meet criteria of a valid PCR result, are listed.

To test the hypothesis that macrophage factors may indirectly participate in IL-19-driven angiogenesis, BMDM were stimulated with IL-19 or saline. After 24 h, medium was removed, and cells were washed three times to remove any residual IL-19 and allowed to incubate a further 24 h, at which time conditioned medium was collected. Figure 6, A and B, shows that aortic rings incubated with conditioned medium from IL-19-stimulated macrophages develop a significantly larger sprout area than aortic rings incubated with medium from BMDM controls and aortic rings stimulated with VEGF alone (6.67 ± 0.84 vs. 4.295 ± 0.50 and 4.29 ± 0.52 for IL-19, VEGF, and PBS controls, respectively, P < 0.05). This suggests induction of soluble angiogenic factors in BMDM by IL-19.

To further elucidate a cellular mechanism for these effects, human EC and VSMC were incubated with conditioned medium from BMDM stimulated with IL-19 or PBS, as described above. After 4 days, cells were trypsinized and counted. Figure 6, C and D, shows that EC incubated with conditioned medium from IL-19-stimulated BMDM proliferate significantly more rapidly than control EC and EC stimulated with medium from BMDM incubated with PBS (6.50 ± 0.25 vs. 1.58 ± 1.08 and 3.25 ± 0.43 cells/mm² for IL-19-stimulated, control, and PBS-stimulated EC, respectively, P < 0.01 and P < 0.05). Interestingly, a similar pattern was seen in VSMC: VSMC cultured with conditioned medium from IL-19-stimulated BMDM proliferate significantly more rapidly than control and saline-stimulated VSMC (22.92 ± 1.04 vs. 2.66 ± 0.71 and 10.08 ± 1.15 cells/mm² for IL-19-stimulated, control, and PBS-stimulated VSMC, respectively, P < 0.01 and P < 0.001). To confirm EC and VSMC participation in this process, thin-mount immunohistochemistry of conditioned medium-treated aortic rings in which microvesSEL sprouts were coimmunostained with anti-PECAM1 and anti-smooth muscle (SM)-actin was performed. Figure 6E shows PECAM1 and SM-actin-positive cells in a microvesSEL sprout and suggests that IL-19 conditioned medium is sufficient to support EC and VSMC/mural cell differentiation and maturation into nascent vessels.

To identify the soluble factors produced by IL-19-stimulated BMDM that may support vascular cell proliferation, a quantitative RT-PCR-based screening assay was used to identify genes induced in BMDM by IL-19. RNA was extracted from BMDM treated with IL-19 for 24 h and compared with RNA extracted from saline-treated control BMDM. Table 2 lists mRNA expression levels for a number of soluble factors induced in BMDM by IL-19, mRNAs for chemokine (C-C motif) ligand 2 (CCL2), colony-stimulating factor 2 (CSF2), chemokine (C-X-C motif) ligand 2 (CXCL2), fibroblast growth factor 2 (FGF2), IL-3, IL-4, and IL-5 were significantly increased above baseline levels. Expression of these genes at the protein level was validated by protein microarray. Figure 7B shows that CCL2, IL-3, IL-4, and IL-5 protein was increased in IL-19-treated BMDM. We previously showed that IL-19 increases VEGF-A expression in BMDM (32). Many of these genes are known to be EC mitogens and angiogenic factors. Together, these data suggest that macrophage factors may indirectly participate in IL-19-driven angiogenesis.

DISCUSSION

The major novel findings of this study are as follows: 1) the absence of IL-19 reduces angiogenesis in the aortic ring and mouse Matrigel model; 2) IL-19 induces IL-20R2 expression in VSMC, but not EC; 3) IL-19 angiogenic activity is STAT3-dependent; 4) IL-19 alone, in the absence of hypoxia and inflammation, has direct proangiogenic effects on aortic rings; and 5) IL-19 has indirect angiogenic effects on aortic rings by inducing soluble angiogenic factors from macrophages.

The aortic ring assay allows characterization of IL-19 in the absence of the angiogenic drivers of inflammation and hypoxia. Other benefits of this model include its multicellular nature and the fact that EC were not preselected by passaging and, thus, are not in a biased proliferative state when explanted. We previously reported that the presence or absence of IL-19 regulates blood flow reperfusion in the hindlimb ischemia model, in which blood flow recovery is thought to be primarily driven by tissue hypoxia (35). Similar to our previous study, these results show significantly less sprouting in response to VEGF in aortic rings from IL-19−/− mice than control mice, but on this platform, angiogenesis occurred in the absence of tissue ischemia. Interestingly, rings from IL-19−/− mice did respond to IL-19 and demonstrated more sprouting capacity than unstimulated rings, suggesting that addition of IL-19 alone can induce microvessel growth. Similarly, in vivo, IL-19−/− mice responded poorly to VEGF-induced angiogenesis in the Matrigel plug assay. The ability of IL-19 to induce angiogenesis is unique among Th2 anti-inflammatory interleukins, which in general are antiangiogenic (13, 16, 23, 30, 38).

While IL-19 shares 20% amino acid identity with IL-10, it does not engage the IL-10 receptor but, rather, signals through the IL-20 heterodimeric receptor complex (9, 30). In contrast to IL-20, IL-19 requires the heterodimer of IL-20R1 and IL-20R2 to engage the receptor and activate STAT proteins (32, 34, 44). In one study examining 25 tissues, tissue-specific differences in expression of IL-20R1 and IL-20R2 were found, but neither VSMC nor EC were included in these studies (12, 21, 29, 44). While IL-20R1 is detected in many tissues, expression of IL-20R2 is much more restricted (29). With this in mind, we quantitated expression of each receptor chain in both human

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primary EC and VSMC, which constitute the majority of cell types in the isolated aortic ring. In both EC and VSMC we observed no difference in basal or IL-19-stimulated induction of IL-20R1 expression. In contrast, we observed significantly higher basal levels of the IL-20R2 chain in VSMC than EC. Furthermore, we saw robust induction of the IL-20R2 chain mRNA and protein in IL-19-stimulated VSMC, in contrast to EC, in which IL-19 did not increase the low basal levels of the IL-20R2 chain. It was important to determine why IL-19 induced IL-20R2 expression in VSMC, but not EC. First we determined that IL-19 activation of STAT3 was not involved in IL-20R2 expression, which is reasonable considering that the IL-20R2 promoter lacks STAT3 consensus elements. IL-19 induces PPARγ expression and activation in macrophages (10). PPARγ is an anti-inflammatory transcription factor that decreases the inflammatory response of many cardiovascular cells (14). Interestingly, IL-19 was able to increase PPARγ expression in VSMC, similar to macrophages, but not in EC. As the IL-20R2 promoter contains two consensus PPARγ elements, it was not surprising that siRNA knockdown of PPARγ reduced IL-20R2 expression in VSMC. While this may not be the only mechanism, we conclude that one potential mechanism for IL-19 differential regulation of IL-20R2 is induction of PPARγ in VSMC, but not EC.

Functional studies in lymphocytes indicate that IL-20R2 modulates the Th2 phenotypic switch and suggest that IL-20R2 signaling mediates the Th2 suppressive effect and decreases Th1 adaptive immune response, thus enabling Th2-like functions (42). It has been further noted that signaling through the IL-20R2 peptide inhibited the cutaneous inflammatory response and dampened production of proinflammatory cytokines, which is consistent with a reparative function (28). This implies that, at least in VSMC, IL-19 might amplify its own activity by upregulating IL-20R2 expression. Since IL-19 is proliferative for cultured EC, but not VSMC, this may also imply that induction of IL-20R2 in VSMC may mediate a suppressive effect on VSMC, but not EC (20, 40). Additional studies are necessary to determine if this differential regulation of the IL-20R2 chain may explain the suppressive effects of IL-19 on VSMC and the angiogenic effects of IL-19 on EC.

STAT3 is a well-characterized proangiogenic protein, and phosphorylation of STAT3 at Tyr705 leads to its dimerization, nuclear translocation, and target gene transcription (27). STAT3 activation is induced by several angiogenic factors, and
STAT3 activity also drives expression of angiogenic proteins (2, 6). In the present study we determined that IL-19 can activate STAT3 in aortic rings, which is consistent with our previous studies suggesting that IL-19 can activate STAT proteins in EC and VSMC (20, 40). This is noteworthy, in that although STAT3 activation is associated with neovascularization in hypoxic conditions such as tumorogenesis and ischemic situations such as hindlimb ischemia, there is little literature describing STAT3 activity in nonhypoxic angiogenic situations. Several STAT3 pharmacological inhibitors are available as anticancer therapeutics, and we utilized two different STAT3 inhibitors to determine that STAT3 activity mediated IL-19 proangiogenic effects in aortic rings, as STAT inhibitors significantly inhibited IL-19-driven sprouting. An interesting observation is that STAT3 proangiogenic activity is mainly attributed to its induction of VEGF expression (6); however, we did not observe a statistically significant increase in VEGF expression in IL-19-stimulated aortic rings. One explanation may be that STAT3-driven VEGF expression has been observed in hypoxic conditions and also required the activation of hypoxia-inducible factor (HIF)-1α, a key mediator of hypoxic responses. The aortic ring assay was not in a hypoxic environment, nor was HIF-1α expression induced by IL-19. This implies that IL-19 may stimulate an alternative, STAT3-mediated, VEGF-independent pathway to induce angiogenesis.

Angiogenesis is regulated by gene expression, and an approach to characterize direct angiogenic effects of IL-19 was to determine IL-19-driven induction of angiogenic gene expression. Addition of IL-19 to aortic rings induces a gene expression pattern consistent with angiogenesis. Several transcripts including cytokines and proteases were significantly increased above control levels. Interestingly, genes more typically associated with angiogenesis, such as the aforementioned VEGF and HIF-1α, were not increased above unstimulated levels in this system, again suggesting an alternative proangiogenic gene expression program induced by IL-19.

Proteolysis is one of the earliest and most sustained activities involved in the formation of new blood vessels. Members of the MMP family are involved in angiogenesis through proteolytic activation of growth factors and proteolytic degradation of the extracellular matrix, allowing EC migration and tube formation (15). It was technically challenging to collect intact aortic rings for protein analysis, but we confirmed IL-19 induction of MMP protein in aortic rings. We also determined that MMP14, but not MMP3 or MMP9, is reduced by the STAT3 inhibitor Stattic. This is a reasonable finding, in that MMP14 has three STAT3 recognition sites in its promoter region, compared with one for MMP2 and none for MMP9. This suggests that MMP14 may play a particularly important role in IL-19-driven aortic sprouting.

Similar to the in vivo scenario, angiogenesis in aortic cultures is regulated by paracrine and juxtacrine interactions and potential cooperation between EC, macrophages, and mural smooth muscle cells, as well as endogenously expressed growth factors and cytokines. We previously reported that IL-19 has mitogenic effects on EC (20). In contrast, IL-19 has growth-suppressive effects on VSMC (40). Our working hypothesis is that, in aortic rings or in vivo, IL-19 can stimulate juxtacrine expression of proliferative factors in EC and/or macrophages, which can then support VSMC growth and migration, leading to microvessel formation.

In vivo, macrophages participate in angiogenesis, as the M2, or alternatively activated, macrophages express several proangiogenic cytokines and are considered to promote wound repair and neovascularization (22, 25, 26). We reported that exposure of BMDM to IL-19 for 24 h can drive macrophages to the M2 phenotype, but we were not able to directly test if this polarization had proangiogenic effects (35). In the present study we determined that conditioned medium from macrophages incubated with IL-19 could induce significantly greater microvessel sprouting than medium from control unstimulated macrophages. To further characterize a potential mechanism for these effects, we observed that conditioned medium from IL-19-stimulated BMDM had a proliferative effect on cultured human EC and VSMC. Cell-specific two-color immunohistochemistry of sprouting aortic rings showed both PECAM1- and SM-

### Table 2. Quantitative RT-PCR screening of cytokine expression in IL-19-treated BMDM

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Name</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ccl2</td>
<td>Chemokine (C-C motif) ligand 2</td>
<td>3.81</td>
</tr>
<tr>
<td>Csf2</td>
<td>Colony-stimulating factor 2</td>
<td>7.01</td>
</tr>
<tr>
<td>Cxcl1</td>
<td>Chemokine (C-X-C motif) ligand 1</td>
<td>5.58</td>
</tr>
<tr>
<td>Fgf2</td>
<td>Fibroblast growth factor 2</td>
<td>3.81</td>
</tr>
<tr>
<td>IL-3</td>
<td>Interleukin-3</td>
<td>3.43</td>
</tr>
<tr>
<td>IL-4</td>
<td>Interleukin-4</td>
<td>8.51</td>
</tr>
<tr>
<td>IL-5</td>
<td>Interleukin-5</td>
<td>2.69</td>
</tr>
<tr>
<td>TGFβ2</td>
<td>Transforming growth factor, β2</td>
<td>2.25</td>
</tr>
<tr>
<td>VEGFA</td>
<td>Vascular endothelial growth factor A</td>
<td>2.54</td>
</tr>
</tbody>
</table>

RNA was extracted from bone marrow-derived macrophages (BMDM) from wild-type mice treated with IL-19 or PBS for 24 h. RNA was reverse-transcribed, and target genes were amplified by quantitative PCR. Genes with fold change >2.0, P < 0.05, which meet criteria of a valid PCR result, are listed.

Fig. 7. Protein expression in IL-19-stimulated BMDM. Protein was extracted from BMDM from wild-type mice treated with IL-19 or PBS for 24 h. A: protein abundance detected by protein array. B: densitometric quantitation of IL-19-induced protein. CCL2, chemokine (C-C motif) ligand 2; Csf2, colony-stimulating factor 2.
actin-positive cells in microvessel sprouts and supports this observation.

We hypothesized that addition of IL-19 to BMDM could induce BMDM to synthesize soluble factors, which would support vascular cell proliferation and maturation. To this end, we determined that IL-19-stimulated BMDM do synthesize mRNA and protein for numerous angiogenic factors. While it has been established that cytokines such as FGF2 and VEGF-A are potent proangiogenic factors, a number of other proangiogenic cytokines are also induced. IL-3 stimulates EC proliferation and tube formation (8). In addition to being an inducer of the M2 phenotype, IL-4 can induce migration and tubelike structure formation, activities consistent with angiogenesis, in EC (23, 43). CCL2 (monocyte chemoattractant protein-1) can induce VEGF-A expression and induce sprouting in aortic rings (17, 36). Antibody neutralization of CXCXL1 was found to reduce EC migration and proliferation (1). Transforming growth factor-β2 can induce expression of VEGF and its receptor, as well as promote EC proliferation and angiogenesis (33). Much less is known about IL-5, but it has been reported to induce VEGF-A expression in aortic smooth muscle cells (45). Together, these studies suggest that, in addition to direct effects on aortic rings, IL-19 can indirectly promote angiogenesis by inducing macrophages to synthesize soluble factors that can promote angiogenesis. Thus, in vivo, it is likely that both EC and M2 macrophages respond to IL-19, which mediates angiogenesis.

In summary, this work shows that IL-19, in the absence of hypoxia and inflammation, can induce angiogenesis. Two major pathways may be responsible: 1) direct effects on EC and VSMC and 2) indirect effects by inducing angiogenic gene expression in macrophages. Future studies are necessary to define the specific role of each of these pathways in IL-19-driven angiogenesis in other in vivo models of angiogenesis.

GRANTS
This work was supported by National Heart, Lung, and Blood Institute Grants HL-115575 and HL-117724 and American Heart Association Grant 13GRNT1685003 to M. V. Autieri. K. Gabunia was supported by an American Heart Association postdoctoral fellowship (11POST7530001).

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
F.K., K.G., M.R., S.E.K., R.N.E., B.K., and M.V.A. performed the experiments; S.E.K., R.N.E., R.G.S., and M.V.A. analyzed the data; S.E.K. and M.V.A. edited and revised the manuscript; R.G.S. and M.V.A. interpreted the results of the experiments; M.V.A. developed the concept and designed the research; M.V.A. prepared the figures; M.V.A. drafted the manuscript; M.V.A. approved the final version of the manuscript.

REFERENCES
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