The Anti-Inflammatory Cytokine Interleukin 19 Is Expressed By and Angiogenic for Human Endothelial Cells

Surbhi Jain, Khatuna Gabunia, Sheri E. Kelemen, Tracee S. Panetti, Michael V. Autieri

Objective—To characterize the expression and function of interleukin (IL) 19, a recently described T-helper 2 anti-inflammatory IL, on endothelial cell (EC) pathophysiological features.

Methods and Results—The expression and effects of anti-inflammatory ILs on EC activation and development of angiogenesis are uncharacterized. We demonstrate by immunohistochemistry and immunoblot that IL-19 is expressed in inflamed, but not normal, human coronary endothelium and can be induced in cultured human ECs by serum and basic fibroblast growth factor. IL-19 is mitogenic and chemotactic, and it promotes EC spreading. IL-19 activates the signaling proteins STAT3, p44/42, and Rac1. In functional ex vivo studies, IL-19 promotes cordlike structure formation of cultured ECs and enhances microvessel sprouting in the mouse aortic ring assay. IL-19 induces tube formation in gelatinous protein (Matrigel) plugs in vivo.

Conclusion—To our knowledge, these data are the first to report expression of the anti-inflammatory agent, IL-19, in ECs; and the first to indicate that IL-19 is mitogenic and chemotactic for ECs and can induce the angiogenic potential of ECs. (Arterioscler Thromb Vasc Biol. 2011;31:167-175.)

Key Words: angiogenesis ■ cytokines ■ endothelium ■ vascular biology ■ interleukin 19

Endothelial cell (EC) paracrine and autocrine stimulation can result in migration and proliferation and is an essential component of multiple normal and pathophysiological processes, including atherosclerosis, permeability, wound healing, and angiogenesis.1–3 Angiogenesis is the growth of new blood vessels and normally occurs in the process of healing wounds and restoring blood flow after injury or insult. The expressions of both inflammatory and anti-inflammatory cytokines participate in wound healing and neovascularization, and many proinflammatory cytokines play an established role in angiogenesis. For example, proinflammatory cytokines (ie, interleukin [IL] 1β, IL-8, and IL-18) promote angiogenesis with direct increased effects on EC migration, proliferation, matrix metalloproteinase production, tube formation; and vascularity in vivo.4–6 Although it may be intuitive that the inflammatory state of ischemic tissue may dictate whether that tissue is neovascularized or becomes necrotic, the role and direct effects of anti-inflammatory ILs on ECs in initiation and progression of neovascularization are less clear and are somewhat inconsistent. For example, it is recognized that the M2 anti-inflammatory macrophage phenotype promotes tissue regeneration and angiogenesis by secretion of soluble factors. However, the prototypical anti-inflammatory cytokine, IL-10, has antiangiogenic activity and is associated with vascular endothelial growth factor (VEGF) downregulation and reduction of fibroblast growth factor (FGF)– and VEGF-induced proliferation of microvascular ECs.7 IL-10 also induced tissue inhibitor of metalloproteinase 1 (TIMP1) and reduced matrix metalloproteinase 2 production in ECs.8 IL-4 can inhibit VEGF production and reduce vascularization; it can also induce migration and tubelike structure formation in ECs, activities consistent with angiogenesis.9–11 IL-20 also has reported proangiogenic and antiangiogenic effects.12–14 The identification of cytokines and their receptors (common to leukocytes and ECs) has the potential to link inflammatory and angiogenic processes. Characterization of these molecules and their function in these processes could lead to new therapies for tissue repair and neovascularization; this requires further investigation.

IL-19 is an IL-10 family member expressed in monocytes and T and B lymphocytes and can be upregulated in these cells by lipopolysaccharide and granulocyte colony-stimulating factor.15–18 Like IL-10, IL-19 has been ascribed to be a T-helper 2 (Th2) anti-inflammatory IL.18 Treatment of maturing antigen-presenting cells with IL-19 promotes the Th2 (regulatory), rather than the Th1, response; and induces IL-10, and decreases interferon-γ, expression in T cells.17,18 The expression and function of anti-inflammatory ILs on EC pathophysiological features remain uncharacterized; to our knowledge, the role of IL-19 in particular in wound healing or neovascularization has not been reported. To test the hypoth-
esis that IL-19 has a non-Th2 regulatory function in ECs, we
designed experiments to determine a role for this IL in EC
activation and angiogenesis. This is an inaugural study
describing IL-19 effects in ECs, and there are several unique
findings in this report. We report that IL-19 expression can be
induced in cultured ECs and can activate multiple signal
transduction proteins. The IL-20 receptor, the receptor for
IL-19, is expressed on ECs. IL-19 is mitogenic for ECs; IL-19
also promotes tubulike structure formation on gelatinous
protein (Matrigel) by ECs and microvessel formation in the
mouse aortic ring assay and in vivo in Matrigel plugs.
Because it is expressed by both leukocytes and ECs, we
propose that IL-19 be considered an inducible mediator of
leukocyte/EC bidirectional communication in regulation of
tissue repair and vessel regeneration. This has important
implications for the role of anti-inflammatory factors in
general in the promotion of wound healing and vascular
inflammation.

Methods

Immunohistochemistry

Human coronary arteries were removed from nonfailing hearts from
donor organs that were deemed not appropriate for transplantation
and hearts that needed to be removed from patients with severe
coronary artery transplant vasculopathy (CAV) at retransplantation
and processed as previously described.19

Cell culture, cell proliferation, migration assays, Rac1 activation,
Western blotting, cordlike structure analysis, flow cytometry, aortic
ring assay, RT-PCR, mouse Matrigel plug, and statistical analysis
were all performed as previously described.19 All animal procedures
were approved by the Institutional Animal Care and Use Committee
of Temple University, Philadelphia, Pa.

Cell Spreading

Spreading was performed as described and calculated by tracing the
cell and measuring the area using computer software (Image-Pro
Plus).20

The supplemental material (available online at http://atvb.
ahaajournals.org) provides further details on the methods.

Results

ECs Express IL-19

Immunohistochemical staining of healthy and diseased hu-
man coronary arteries established IL-19 expression in human
endothelium. Figure 1A demonstrates that IL-19 is expressed
in ECs in human coronary arteries with CAV, a disease in
which endothelium is continuously inflamed. Previously, it
was shown that IL-19 is expressed in some smooth muscle
cells and CD45-positive cells in inflamed arteries.21 IL-19
immunoreactivity in endothelium in arteries with CAV was
confirmed by colocalization with the EC marker von Wille-
brand factor (Figure 1B). In contrast, healthy control coro-
nary arteries did not show any expression of IL-19 in
endothelium or other medial cells. Because CAV arteries
exist in a milieu of cytokines and growth factors, we
performed experiments to investigate differential expression
of IL-19 by cytokines in cultured ECs under more defined
conditions. Human vascular ECs (HVECs), coronary artery
ECs (CaECs), and coronary micro-ECs (mECs) were cultured
in serum-reduced media for 24 hours; and then stimulated
with a variety of soluble factors. Figure 1C shows that all
cultured ECs express basal levels of IL-19 protein but can be
induced to express significantly increased IL-19 (×10% FCS
and basic FGF (bFGF); P<0.05; n=3 experiments) (supple-
mental Figure I). Other factors can also increase IL-19
expression, but not to a significant degree. Expression of
IL-19 mRNA mirrors protein expression (data not shown).
These data are the first to indicate that IL-19 expression is
inducible in several EC types. Subsequent experiments were
focused to determine a function and potential mechanism of
IL-19 activity in ECs.

IL-19 Activates EC Signal Transduction and
Enhances Proliferation

IL-19 can significantly induce rapid and transient activation
of STAT3, Rac1, and mitogen-activated protein kinase (MAPK)
p44/42 in all EC types examined (P<0.05 or
P<0.01 compared with unstimulated for all) (Figure 2A-2D
and supplemental Figure II). IL-19 signals through the IL-20
receptor, which is composed of α and β subunits and is
known to activate STAT proteins. Each EC type expressed
detectable amounts of mRNA for each of the IL-20 receptor
subunits (supplemental Figure III).

Because activation of p44/42 MAPK participates in EC
mitogenesis, we tested the hypothesis that IL-19 could
promote proliferation of ECs by itself and/or enhance VEGF-
or bFGF-mediated EC proliferation. HVECs, CaECs, and
mECs cultured in growth media were treated with IL-19 (100
ng/mL); at 1, 3, and 5 days, they were trypsinized and
counted. In each EC type, IL-19 significantly increased EC
proliferation at 5 days: 43.7±1.6 versus 27.8±1.4, 38.4±1.3
versus 26.3±1.5, and 23.9±1.8 versus 15.0±1.3 ECs/mL for
HVECs, CaECs, and mECs, respectively (P<0.001 for all
(Figure 2E). This is not because of decreased apoptosis of
these cells, as ascertained by annexin V staining (supple-
mental Figure IV). IL-19 did not induce VEGF or bFGF protein
expression (supplemental Figure V) nor enhance either
VEGF- or bFGF-mediated HVEC proliferation (supplemental
Figure VI). To determine whether IL-19 proliferative effects
are contingent on p44/42 MAPK activity, each EC type was
cultured in the presence of 2 different MAPK inhibitors
(PD98059 or U0126). Each of these inhibitors, at published
median effective concentrations (5 μmol/L each), signifi-
cantly reduced IL-19–driven EC proliferation in the range of
23.3% to 66.0% (P<0.01 for each EC type) (Figure 2F). This
indicates that IL-19 is mitogenic for multiple EC types and
uses the p44/42 MAPK pathway in this process.

IL-19 Activates EC Spreading and Migration

p44/42 MAPK, Rac1, and STAT3 regulate cytoskeletal orga-
nization and actin dynamics; we hypothesized that IL-19
would influence EC spreading and/or migration. In all 3 EC
types, compared with unstimulated cells, IL-19 significantly
enhanced cell spreading: 3060±110 versus 4650±110, 2620±
170 versus 4087±190, and 205±120 versus 4260±290 μm²
for HVECs, CaECs, and mECs, respectively (P<0.001)
(Figure 3A and 3B).

To determine whether IL-19 induced EC migration,
HVECs were seeded onto the top chamber in medium
containing 0.2% BSA, with or without 100-ng/mL IL-19, or
VEGF as a positive control, in the lower chamber. Some ECs were pretreated with IL-19 for 16 hours before trypsinization and seeding in migration chambers. Figure 3C shows that the addition of IL-19 significantly increases HVEC migration (107.5 ± 19.3 compared with 357.6 ± 29.9 ECs/high-power field [HPF] for unstimulated versus IL-19 treated; \( P < 0.01, n = 3 \)), which is comparable to values obtained for VEGF (317.0 ± 36.9 ECs/HPF); also, there was no significant difference between IL-19– and VEGF-treated HVECs. Interestingly, coinoculation of VEGF and IL-19 resulted in significantly more migration than VEGF alone (503.1 ± 26.4 versus 317.0 ± 36.9 ECs/HPF for combined versus VEGF; \( P < 0.05 \)). Also, ECs preincubated with IL-19 before seeding in the chamber, which did not have IL-19 in the lower chamber, also migrated significantly more rapidly than VEGF alone (447.3 ± 24.3 versus 317.0 ± 36.9 ECs/HPF for pretreated versus unstimulated ECs; \( P < 0.5; n = 3 \)) (Figure 3D). There was no statistical difference in migration between HVECs that had VEGF and IL-19 in the lower chamber and those that were pretreated with IL-19 but only had VEGF in the lower chamber. Experiments were also performed in which IL-19 neutralizing antibody was added to the lower chamber. Figure 3B shows that IL-19 neutralizing antibody significantly reduced IL-19–induced HVEC migration at all concentrations of neutralizing antibody used. VEGF does not induce IL-19 expression (supplemental Figure VII) nor does IL-19 neutralizing antibody reduce VEGF-induced migration (supplemental Figure VIII). Together, these data are the first to show that IL-19 stimulates EC signaling, spreading, and migration.

**IL-19 Promotes EC Cordlike Structure Formation**

We hypothesized that IL-19 might play a role in endothelial migration and organization, leading to tubelike or cordlike structure formation. HVECs were seeded onto growth factor–reduced Matrigel, in the presence of 10% FCS. Some HVECs
were treated with IL-19, some were cotreated with IL-19 and VEGF, and some were pretreated with IL-19 and then treated with VEGF or with VEGF alone as a positive control. The number of cordlike structures was counted manually per multiple representative images, and an average was calculated for each condition. Data presented in Figure 4 show that IL-19 can induce significantly more cordlike structures than control samples (31.3 ± 3.9 compared with 20.0 ± 1.2 cords; \( P<0.05 \); \( n=3 \)). These results were similar to migration experiments in that ECs pretreated with IL-19, but not incubated with IL-19 in the assay, continued to form cordlike structures to a similar degree compared with IL-19, VEGF, or cotreated HVECs (40.6 ± 2.9 versus 31.3 ± 3.9, 35.6 ± 3.6, and 43.0 ± 2.1). Together, this suggests that IL-19 plays a role in directed EC migration and organization.

**IL-19 Promotes Microvessel Formation**

Because IL-19 effected cordlike structure formation, we hypothesized that IL-19 could also regulate EC sprouting using 2 independent assays: ex vivo from aortic rings and in vivo using the Matrigel plug angiogenesis assay. Mouse thoracic aorta was sectioned, placed onto growth factor–reduced Matrigel, and incubated in media containing IL-19, PBS, or VEGF as a positive control. Sprouting from rings was assessed by counting the number of vessels and comparing them to control samples. Data presented in Figure 4 show that IL-19 significantly increased vessel formation compared with control samples (31.3 ± 3.9 versus 20.0 ± 1.2 cords; \( P<0.05 \); \( n=3 \)). These results were similar to migration experiments in that ECs pretreated with IL-19, but not incubated with IL-19 in the assay, continued to form cordlike structures to a similar degree compared with IL-19, VEGF, or cotreated HVECs (40.6 ± 2.9 versus 31.3 ± 3.9, 35.6 ± 3.6, and 43.0 ± 2.1). Together, this suggests that IL-19 plays a role in directed EC migration and organization.
was analyzed daily; on the sixth day, it was photographed and the outgrowth area was quantified by image analysis. Figure 5 shows that aortic rings incubated with IL-19 showed significantly greater sprouting area than Matrigel alone (770.0±85.1 versus 1877.3±48.1 \( \mu \)m\(^2\)) for unstimulated versus IL-19 treated; \( P<0.01; n=6\). This was similar to values for the VEGF-positive control and rings incubated with both IL-19 and VEGF (1784.8±28.8 and 1730.0±47.8 \( \mu \)m\(^2\)) for VEGF and IL-19 and VEGF cotreated, respectively. Immunostaining was performed on whole mounts of aortic rings. Sprouting microvessels were immunoreactive with plateletendothelial cell adhesion molecule 1 (PECAM1) antibody, demonstrating the presence of ECs in these structures.

To determine whether IL-19 could stimulate microvessel formation in vivo, 0.5 mL of growth factor–free Matrigel was mixed with 200 ng of IL-19, 200 ng of VEGF, or PBS control; and delivered as a single plug subcutaneously. After 10 days, the plug was recovered and microvessels containing lumen were determined by immunohistochemistry using PECAM1 antibody. Figure 6 shows that Matrigel containing IL-19 had significantly more PECAM1-positive microvessels per unit area than did PBS control plugs (2.5±0.08, 6.9±1.0, and 12.8±2.1 microvessels per unit area for PBS, IL-19, and VEGF, respectively; \( P<0.05\)). These PECAM1-positive structures surrounded a lumen, indicating that IL-19 can induce microvessel formation in vivo.

**Discussion**

The major finding of this study is that IL-19 is a novel proangiogenic factor expressed in ECs. IL-19 is a Th2 anti-inflammatory IL, and nothing has been reported on the expression or mechanism(s) of IL-19 effects on ECs. Basal levels of IL-19 can be detected in human monocytes and B and T lymphocytes, and IL-19 expression can be upregulated in these cells by stimulation. To our knowledge, this is the first report regarding IL-19 expression in ECs, and IL-19 expression in inflamed ECs suggests a role distinct from Th1/Th2 phenotype modulation in lymphocytes. The observation that low basal levels of IL-19 expression can be increased by FCS is consistent with the finding that IL-19 is undetectable in uninjured human arteries but is increased in arteries from patients diagnosed as having CAV, a chronic inflammatory disease in allografted hearts. Expression in injured arteries is also consistent with an in vivo murine tissue survey indicating that IL-19 is not present in unstimulated tissue but can be induced in immune and local tissue cells in inflamed tissue and cultured keratinocytes when stimulated with IL-1\( \beta \).\(^{24,25}\) The expression of certain anti-inflammatory cytokines (ie, IL-9, IL-10, or IL-11) in ECs has not been reported. However, IL-4 is detected in ECs from atherosclerotic plaques and can increase vascular cell adhesion molecule-1, monocyte chemoattractant protein-1, and IL-6 levels, consistent with a proinflammatory effect on endothelium.\(^{1,26}\)

IL-19 shares 20% amino acid identity with IL-10 but does not engage the IL-10 receptor; rather, it engages the IL-20 receptor, which is known to activate STAT proteins in several cell types.\(^{17,27}\) In our study in HVECs, IL-19 did activate both STAT3 and p44/42 MAPK activity, but not STAT1. There is a diversity in use of various members of the STAT family by ILs, much of which is regulated by tissue-specific expression of certain anti-inflammatory cytokines (ie, IL-9, IL-10, or IL-11) in ECs has not been reported. However, IL-4 is detected in ECs from atherosclerotic plaques and can increase vascular cell adhesion molecule-1, monocyte chemoattractant protein-1, and IL-6 levels, consistent with a proinflammatory effect on endothelium.\(^{11,26}\)

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it activated STAT1 rather than STAT3. Interestingly, IL-20 stimulated STAT3 in keratinocytes. Some of these differences in signaling and gene expression may be explained by IL-20 receptor complexity and tissue distribution.

The present study demonstrated that IL-19 was capable of mediating EC spreading and migration and proliferation. STAT3, p44/42, and the small GTPase Rac1 are involved in EC proliferation, migration, and angiogenesis. STAT3 in particular participates in angiogenesis by multiple mechanisms, including potentiation of microtubule polymerization, leading to increased migration and induction of VEGF expression. p44/42 regulates membrane protrusions and focal adhesion dynamics, and specific p44/42 inhibitors decrease migration of multiple cell types, including ECs. Rac1 also has a key role in cell migration by influencing actin cytoskeleton and membrane ruffling, and IL-19 activation of any 1 of these 3 enzymes may be responsible for EC spreading and migration. Interestingly, ECs pretreated with IL-19 for 24 hours migrated more rapidly than those singly incubated with either IL-19 or VEGF and migrated to the same degree as those coincubated with both VEGF and IL-19 as chemotactic agents in the lower chamber, suggesting that IL-19 could induce expression of a factor(s) that played a part in migration.

Migration and proliferation of ECs in response to peptide factors are key events in angiogenesis. IL-19 promoted cordlike structure formation on Matrigel by human umbilical vein ECs, enhanced microvessel formation in isolated mouse aortic rings, and enhanced microvessel formation in Matrigel plugs in vivo. PECAM1-positive structures in Matrigel plugs surrounded a lumen. These assays assess EC migration, proliferation, differentiation, and polarization of primary quiescent ECs into organized sprouts. Microvessels sprouting from aortic rings were immunoreactive with PECAM1 antibody, confirming the effects of IL-19 on ECs in these structures.

Various proinflammatory ILs (eg, IL-1, IL-8, IL-17, and IL-20) were identified as angiogenic; they increase EC migration, cordlike structure organization, and matrix metalloproteinase production. However, the effects of anti-
inflammatory ILs on angiogenesis are not well characterized.
The archetypal anti-inflammatory cytokine, IL-10, is antiangiogenic because it reduces EC migration and downregulates VEGF production. Although IL-4 is antiangiogenic in that it inhibits VEGF production and reduces vascularization, it can also induce migration and cordlike structure formation in ECs, consistent with angiogenesis. Similarly, IL-13 attenuates EC tube formation by induction of the JAK2-STAT6 pathway. IL-20 is expressed by ECs and has both proangiogenic and antiangiogenic effects on ECs. IL-20 angiogenic effects are indirect because it also induces FGF2 and VEGF expression. In contrast to IL-20, IL-19 does not induce VEGF, or its own expression, and VEGF does not induce IL-19 expression, implying that IL-19 effects on EC proliferation are direct. It also explains why IL-19 neutralizing antibody had no effect on VEGF-induced migration. Neither VEGF nor bFGF could significantly increase the proliferative effects of IL-19 on ECs, implying independent pathways of activity. IL-19 effects on cellular proliferation also appear to be cell type specific because IL-19 has

Figure 5. IL-19 promotes EC microvessel formation in mouse aortic rings. A, Sectioned mouse thoracic aorta was cultured in triplicate in growth factor–reduced Matrigel in the presence or absence of IL-19, VEGF, or both. B, Densitometric measurement of the area of microvessel sprouting. *P<0.05 and **P<0.01 vs unstimulated control. Data are from 3 independent experiments. C, Rings that were cultured with media only (control), IL-19, or VEGF for 6 days were immunostained with PECAM1 antibody or secondary antibody only (neg Ab).
antiproliferative effects in both a breast cancer cell line and human vascular smooth muscle cells. This may also be a function of tissue-specific expression of receptor subunits, and distribution of receptor chain expression on ECs and vascular smooth muscle cells requires further investigation.

The notion that a Th2 anti-inflammatory IL can directly regulate EC pathophysiological features is novel and has important implications for the role of anti-inflammatory mediators in vasculogenesis. This is an inaugural study describing IL-19 effects in ECs, and there are several unique findings in this report. The expression of IL-19, a Th2 IL, can be induced in cultured ECs and in vivo by local inflammation. Treatment of cultured human ECs with IL-19 results in activation of STAT3, p44/42 MAPK, and Rac1. IL-19 treatment of ECs results in increased cell spreading and enhanced migration. IL-19 is mitogenic for ECs and enhances cordlike structure formation and microvessel sprouting from aortic rings. IL-19 can induce microvessel formation in Matrigel plugs in vivo. Angiogenesis is a multistep process requiring EC activation, basement membrane degradation, proliferation, migration, and cord formation. This study suggests that IL-19 can participate in each of these processes and has important implications for wound healing, vasculogenesis, and vascular biology.

**Acknowledgments**

We thank Victor Rizzo, PhD, (Temple University School of Medicine) for critical reading of the manuscript.

**Sources of Funding**

This study was supported by grants HL063810 and HL090885 from the National Heart, Lung, and Blood Institute; and grant 0455562U from the American Heart Association (Dr Autieri).

**Disclosures**

None.

**References**


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Arterioscler Thromb Vasc Biol. 2011;31:167-175; originally published online October 21, 2010;
doi: 10.1161/ATVBAHA.110.214916
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272
Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
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Data Supplement (unedited) at:
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MATERIALS AND METHODS

**Immunohistochemistry.** Arteries and matrigel plugs were obtained and processed as described (1). Briefly, human coronary arteries were removed from non-failing hearts from organ donors which were deemed not appropriate for transplantation, and hearts which needed to be removed from patients with severe CAV at the time of re-transplantation. The length of time for heart removal due to CAV ranged from 3 to 7 years. Representative sections from five different normal and seven different CAV arteries, from males and females, were tested for endothelial IL-19 expression with identical results. Tissue used in this study is from a bank of sections obtained from standard Pathology tissue collection. Use of these tissue blocks were approved by the Institutional Review Board of Temple University Hospital. Von Willebrand and PECAM1 antibody (Neo Markers, Inc, San Diego, CA) was used at a concentration of 2µg/mL. IL-19 antibody (R&D Inc. Minneapolis, MN), which has been previously described, was used at 1.0 µg/mL (2). Sections were then incubated with biotinylated secondary antibody (1:200) followed by avidin-biotin-peroxidase complex in a Vectastain Elite kit (both from Vector Labs, Burlingame, CA). The reaction product was visualized with DAB (Vector Labs) used as the chromogenic substrate, which produces a reddish-brown stain. The sections were counterstained with hematoxylin. For immunofluorescence, primary antibody incubation was followed by a 30-minute incubation with secondary antibody conjugated to AlexaFluor 568 (red) and AlexaFluor 488 (green) (Molecular Probes, Inc., Eugene, OR).

**Cells and Culture.** Primary human vascular endothelial cells (HVECs) were obtained as cryopreserved secondary culture from Cascade Corporation (Portland, OR) and subcultured in MCDB medium 131 with Low Serum Growth Supplement as described (1). Primary human coronary artery microvascular cells (mEC), and coronary artery EC (CaEC), were purchased from Lonza, Inc. Cells were used from passage 3-5. Pre-confluent ECs were serum-starved in 0.3% FBS for 24 hours, and then exposed to 10% FBS, (Fisher Biotech), 10µg/ml oxidized LDL (Intracel, Fredrick MD), 20ng/ml VEGF, 10ng/ml bFGF for another 48 hours, at which times samples were processed for protein isolation. Some samples remained untreated and were used as controls. All cytokines were purchased from Sigma (St. Louis, MO), except recombinant IL-19 purchased from R&D, Inc. (Concord, MA).
Migration Assay. Migration was performed using a 48 well modified Boyden chemotaxis chamber (Neuroprobe, Gaithersburg, MD) using 100μg/ml IL-19, or 40ng/ml VEGF as a positive control in MCDB medium 131 with 0.2% fatty acid free BSA were added to bottom chamber as described (3). Briefly, 50μl of HVEC (50,000 /ml) were suspended in the same medium were added to top chamber. The two chambers were separated by a Polyvinylpyrrolidone-free polycarbonate membrane with 8μ pores (Corning/Costar) coated overnight with collagen 20 ng /ml. EC on the top of the filter were removed by scraping, and EC on the underside of the filter were fixed, stained with Hemacolor (EM Science) and air-dried on a slide. For some experiments, anti-IL-19 antibody was added to the top and bottom chambers. Each condition was performed in triplicate and three fields (0.32mm²) from each well were counted using Image-Pro Plus software.

Cell Proliferation. Eight thousand HVEC, CaEC, or mEC were seeded into 24-well plates. After adhesion, media was replaced with serum reduced (2% FCS) media, in the presence and absence of IL-19, and were counted in the presence of trypan blue on the first, third, and fifth day post seeding, using a standard hemocytometer as described (1). MAPK inhibitors PD98059 and U0126 (5μM each) were purchased from Sigma, Inc., added on the first day of seeding, and replaced along with new medium and IL-19 on the third day. Flow cytometry using Annexin-V kit from BD Biosciences verified that apoptosis was not reduced by IL-19.

Cell Spreading. Spreading was performed as described (4). Briefly, EC (10⁵ cells/ml) were plated on glass coverslips pre-coated with collagen (10ug/ml) in 0.5% charcoal stripped serum in growth media, or in media with IL-19 for three hours. EC were fixed with 3.7% paraformaldehyde and permeabilized with 0.2% Triton-X 100. Rhodamine phalloidin was added to stain filamentous actin. Images were captured with an inverted microscope using epifluorescence. Cell spreading was calculated by tracing the cell and measuring the area using Image-Pro Plus software.

Western blotting. For detection of IL-19, cell culture and extracts were made as we described (1). Briefly, EC grown to confluence in a 6 well tray were rinsed with PBS, starved in 1% 200 medium for 24 hours, then stimulated with soluble factors for 48 hours. To detect activation of intracellular signaling proteins, cells were rinsed with PBS, starved in 0.5% base
media for 24 hours, and stimulated with IL-19 or fetal calf serum for the indicated times. Extract proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and blocked. IL-19, PECAM, (1:3000 dilution), (Santa Cruz Inc, Santa Cruz, CA), anti-phospho p44/42, and anti-phospho STAT3, and antibody to the total protein of each of these kinases were from Cell Signaling Technology (Beverly, MA), and a 1:2000 dilution of secondary antibody were used. Equal loading of protein extracts on gels was verified by Ponceu S staining of the membrane and normalization to either a housekeeping gene, or in the case of phosphoprotein, total protein. Blots were then stripped and re-probed with the housekeeping proteins anti-actin or anti-GAPDH (Biolegend, Inc., San Diego, CA). Reactive proteins were visualized using enhanced chemiluminescence (GE Healthcare, Piscataway, NJ.). Quantitation of protein expression was performed by densiometry using the Image J program, with protein expression normalized to GAPDH abundance as we described (5).

Rac1 activation. The PAK pull-down assay was performed as we previously described (5). Briefly, EC were grown to confluence, serum starved for 24 hours and then stimulated with 100ng/ml IL-19 for 0, 5, 10 and 20 minutes, and lysed in sample buffer (25mM Hepes, 150 mM NaCl, 5 mM MgCl, 0.5mM EGTA, 20mM β-glycerophosphate, 0.5% Triton-X100, 5% glycerol, 10mM NaF, 2mM NaVanadate, plus protease inhibitors). The volume of lysate was adjusted to normalize for equal concentrations of proteins. Cell suspensions were incubated with GST-PAK Sepharose (Cytoskeleton, Inc) for 1 h at 4°C. Only the activated forms of Rac1 and Cdc42 bind the PAK protein. Beads were washed three times, bound proteins detected by Western blotting with Rac1 antibodies (Santa Cruz, Inc) and quantitated by densiometry of corresponding bands.

Cord-like structure and aortic ring assay. Was performed as we previously described (1). 200μl of low-growth matrigel (BD Biosciences) containing either 100ng/ml IL-19 or 40ng/ml VEGF was added to each well of a 24 well tray, and allowed to polymerize at 37°C for 30 minutes. 200 μl of ECs (2x10^5 cells/ml) were added on the top of matrigel suspended in MCDB-131 medium with 10% FCS, and incubated at 37°C for 16 hours. Images were taken on an inverted microscope using a 10X objective. Three images were taken per well from random fields. Each condition was performed in triplicate. The number of cord-like structures were counted manually per image and an average was calculated for each condition. In
some experiments, EC were incubated with for IL-19 24 hours prior to trypsinization and seeding on matrigel. The Aortic Ring Assay was carried out as we described (1) using C57BL/6 mice (3 - 4 months old), according to standard protocols (6). Briefly, thoracic aortas were excised from mice, and peri-adventitial tissues removed. Aortas were then cut into 1-mm rings, rinsed 5 times with DMEM, placed into MCDB medium 131 in 48-well tissue culture plates coated with Matrigel (BD biosciences), and overlaid with an additional 100 µL of Matrigel and allowed to gel for 30 minutes at room temperature. The plates were incubated at 37°C with MCDB medium 131 medium containing 2% autologous mouse serum and either 100ng/ml IL-19 or 40ng/ml VEGF (R&D, Inc, Minneapolis, MN). Aortic rings were examined daily and digital images were taken at day 6 for quantitative analysis of the area of vessel outgrowth by the SPOT Advanced program (Media Cybernetics, Sterling Heights, MI). Microvessel outgrowth was calculated by circling the extent of microvessel outgrowth at 6 days, and subtracting the area of the aortic ring. All animal procedures were approved by the Institutional Animal Care and Use Committee of Temple University.

Whole mount IHC of aortic rings were performed using the Thin-gel method as described (7). Briefly, aortic rings were explanted and cultured as described above on Nunc chamber slides. After 6 days, gels containing explants were fixed in 10% neutral buffered formalin, washed in PBS, then with 0.25% TritonX-100, and blocked with 5% rabbit serum. Tissue was incubated with anti-PECAM-1 antibody at 3ug/ml for 2 hours, followed by Alexa Fluor 568-conjugated secondary antibody (Molecular Probes, Eugene, OR) for 1 hr. Wells were removed from chamber slides and stained explants were cover slipped with Fluoro-Gel mounting media. Images were captured with an Olympus THX-100 microscope and photographed at 20X magnification.

*Mouse in vivo matrigel plug assay.* Was performed as described (8). Briefly, 0.5 ml growth-factor free matrigel was mixed with 200ng/ml IL-19, VEGF, or PBS, and delivered as a single plug subcutaneously in C57B/6 wild-type mice. After 10 days, the plug was recovered, processed for immunohistochemistry, and microvessels with lumen identified by immunohistochemistry using PECAM antibody. Vessel density was expressed as number of vessels/mm² in >6 independent fields from >4 plugs. Four different mice were used per experimental condition, with the experiment performed three times. All animal procedures were approved by the Institutional Temple University Animal Care and Use Committee.
Quantitative RT-PCR: One \( \mu \)g of total RNA was reverse transcribed by standard methods as we described (1). MMP2, IL-19, and beta actin mRNA was targeted using primer pairs from Integrated DNA Technologies, (Coralville, IA), SYBR green used for detection and amplified using an Eppendorf MCEP RealPlex 4X thermocycler. Product was quantitated by Eppendorf software. Beta actin: forward: AGCCTCGCTTGTGCGGA, reverse: GCGCGGCGATATCATC. MMP2: forward: TCCAGGCGACATCCTATGACAG, reverse: TCTCAGGGGAGGCAGCCACTTTC. Interleukin-20 receptor chain primer sequences were: IL-20R2 forward: 5’-GCCGAGGAACATGTCAAAAT–3’, reverse: 5’-TTCAAGGTGTCTGGGAGGAC-3’ IL-20R1 forward: 5’ - GTGCAG ATG GAAAACTGATGC-3’, reverse: 5’- TTATGGCTGGGATCAAAGGG - 3’. IL-19 forward: 5’-TGT TTCCCTTTGGCTCCT G-3’, Reverse: 5’-ATGATCCTTGAACACCCTGTGC-3’.

Apoptosis assay. Apoptosis in CAEC were determined by flow cytometric analysis of Annexin V and PI stained cells according to standard protocols (9,10). EC were treated with 100ng/ml IL-19, untreated, or 0.5mM H2O2 for 18 hours and harvested. After washing in PBS, pellets were resuspended in 100\( \mu \)l Annexin Binding Buffer at concentration 2\( \times 10^6 \) cell/ml. 2\( \mu \)l of Annexin V-FITC (BD Pharmingen) was added to each pellet and incubated for 15min at room temperature in the dark. 200\( \mu \)l of binding buffer and 5\( \mu \)l PI (BD Pharmingen) was added to each tube before collection of cells. Fluorescence was monitored using FACSCalibur (BD Biosciences) and analysed using FLowJo software.

Statistical analysis. Results are expressed as mean ± SE. Differences between groups were evaluated with the use of ANOVA, with the Newman-Keuls method applied to evaluate differences between individual mean values or by paired \( t \) tests where appropriate. Differences were considered significant at a level of \( P<0.05 \).

REFERENCES


