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 ($0.32mm^2$) 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 (10^5 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 (25nM 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^{o}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/mm^2 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 μg 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: AGCCTCGCCTTTGCCGA, reverse: GCGCGGCGATATCATCATC. MMP2: forward: TCCAGGGCACATCCTATGACAG, reverse: TCTCAGGGCAGAAGCCATACTTC. 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'-ATGATCCTTGAACACCCTGTC-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 H_2O_2 for 18 hours and harvested. After washing in PBS, pellets were resuspended in 100μl Annexin Binding Buffer at concentration $2X10^6$ cell/ml. 2μl of Annexin V-FITC (BD Pharmingen) was added to each pellet and incubated for 15min at room temperature in the dark. 200μl of binding buffer and 5μl PI (BD Pharmingen) was added to each tube before collection of cells. Fluorescence was monitored using FACSCalibur (BD Biosciences) and analysed using FLowJo softwere.

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

1. Tian Y, Jain S, Kelemen SE, Autieri MV. AIF-1 expression regulates endothelial cell activation, signal transduction, and vasculogenesis. Am J Physiol Cell Physiol. 2009. 296:C256-266.

2. Tian Y, Sommerville LJ, Cuneo A, Kelemen SE, Autieri MV. Expression and suppressive effects of interleukin-19 on vascular smooth muscle cell pathophysiology and development of intimal hyperplasia. Am J Pathol. 2008:173:901-909.

3. Panetti TS, Nowlen J, Mosher DF. Sphingosine-1-phosphate and lysophosphatidic acid stimulate endothelial cell migration. Arterioscler Thromb Vasc Biol. 2000. 20:1013-1019.

4. Wu Y, Rizzo V, Liu Y, Sainz IM, Schmuckler NG, Colman RW. Kininostatin Associates With Membrane Rafts and Inhibits vß3 Integrin Activation in Human Umbilical Vein Endothelial Cells. Arterioscler. Thromb. Vasc. Biol. 2007 27:1968-1975

5. Autieri, MV., Kelemen, SE, Wendt, KW. AIF-1 is an Actin-polymerizing and Rac1-activating protein that Promotes Vascular Smooth Muscle Cell Migration. Circulation Research, 2003. 92:1107-1114.

6. Zhu WH, Iurlaro M, MacIntyre A, Fogel E, Nicosia RF. The mouse aorta model: influence of genetic background and aging on bFGF- and VEGF-induced angiogenic sprouting. Angiogenesis. 2003;6:193-199.

7. Li Q, Olsen BR. Increased angiogenic response in aortic explants of collagen XVIII/endostatin-null mice. Am J Pathol. 2004;165:415-24.

8. Kundumani-Sridharan V, Niu J, Wang D, Van Quyen D, Zhang Q, Singh NK, Subramani J, Karri S, Rao GN. 15(S)-hydroxyeicosatetraenoic acid-induced angiogenesis requires Src-mediated Egr-1-dependent rapid induction of FGF-2 expression. Blood. 2010;115:2105-16.

9. O'Connor RA, Wittmer S, Dalton DK. 2005. Infection-induced apoptosis deletes bystander CD4+ T cells: a mechanism for suppression of autoimmunity during BCG infection. J Autoimmun. 24(2):93-100.

10. Hu L, Sun Y, Hu J. 2010. Catalpol inhibits apoptosis in hydrogen peroxide-induced endothelium by activating the PI3K/Akt signaling pathway and modulating expression of Bcl-2 and Bax.Eur J Pharmacol. 628(1-3):155-63.