Suppressing STAT3 activity protects the endothelial barrier from VEGF-mediated vascular permeability

SUPPLEMENTAL METHODS:

Cell Culture

Human endothelial cells were cultured in plates that had been pretreated for 30 minutes with Collagen I (Corning, Catalog No. 354231). HUVEC were maintained in EBM Endothelial Cell Growth Basal Medium (Catalog No. CC-3121, Lonza) supplemented with EGM Endothelial Cell Growth Medium SingleQuots (Catalog No. CC-4143, Lonza). HPAEC were cultured in EBM-2 Basal Medium (Catalog No. CC-3156, Lonza) supplemented with EGM SingleQuots (Catalog No. CC-4176, Lonza). HMVEC-L were cultured in EBM-2 Basal Medium (Catalog No. CC-3156, Lonza) supplemented with EGM SingleQuots (Catalog No. CC-4147, Lonza). For in vitro studies, HUVEC, HPAEC or HMVEC-L grown in culture to approximately 80% confluence were serum starved for 16 hours and subsequently stimulated with human recombinant VEGF-165 protein (25 ng/ml; R&D Systems; MNPHARM) for indicated durations. When applicable, cells were treated with inhibitors or control vehicle for various indicated periods of time following serum starvation and preceding VEGF-165 protein stimulation. For an indicated subset of experiments, HUVEC were maintained in a 1:1 mixture of astrocyte conditioned medium (ScienCell Research Laboratories, Catalog No. 1811) and HUVEC standard medium.

Antibodies

Immunoblotting was performed using antibodies were purchased from Cell Signaling Technology (CST) to detect ZO-1 (Catalog No.13663; dilution 1:1000), phosphorylated VEGFR-2 (Tyr1175, Catalog No. 2478; dilution 1:1000), phosphorylated STAT3 (Tyr705, Catalog No. 9145; dilution 1:500), phosphorylated JAK2 (Tyr 1007/1008, Catalog No. 3771; dilution 1:500), phosphorylated JAK1 (Tyr 1034/1035, Catalog No. 3331S; dilution 1:500), phosphorylated TYK2 (Tyr1054/1055, Catalog No. 9321S; dilution 1:500), VEGFR-2 (Catalog No. 2479; dilution 1:1000), STAT3 (Catalog No. 12640; dilution 1:1000), JAK2 (Catalog No. 3230; dilution 1:500), JAK1 (Catalog No. 3332; dilution 1:500) and TYK2 (Catalog No. 14193; dilution 1:500). Additional antibodies were obtained from Santa Cruz Biotechnology to detect phosphorylated STAT3 (Tyr705, Catalog No. sc-8059; dilution 1:500), ICAM1 (Catalog No. sc-18853; dilution 1:500), and tubulin (Catalog No. sc-5286; dilution 1:500). The monoclonal antibody for detecting GST (Catalog No. MA4-004; dilution 1:1000) was bought from Thermo Fisher Scientific. The monoclonal antibody against phospho-STAT3 (Tyr708, Catalog No. D128-3) in zebrafish was obtained from MBL International Corporation. Horseradish peroxidase-conjugated anti-rabbit (Catalog No. 7074; dilution 1:5000) and anti-mouse (Catalog No. 7076; dilution 1:5000) secondary antibodies (1 µg/µl) were purchased from Cell Signaling Technology.

Immunofluorescence was performed using antibodies against ZO-1 (CST, Catalog No.13663; dilution 1:200), p-STAT3 (Santa Cruz Biotechnology; Tyr705, Catalog No. sc-8059; dilution 1:50) or STAT3 (CST, Catalog No. 12640; dilution 1:100). The cells were then washed with PBS and incubated with CF™ 488A goat anti-rabbit secondary antibody (Sigma-Aldrich; Catalog No. SAB4600389; dilution 1:1000) or CF™ 594 goat anti-mouse secondary antibody (VWR; Catalog No. 20110; dilution 1:1000).

Immunoprecipitation

HUVEC were lysed in RIPA buffer (Millipore) supplemented with protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail set V (Sigma). After quantifying protein using the Quick Start Bradford protein assay (Bio-Rad), 500 µg protein lysate was loaded to the supplied spin column and immunoprecipitation was achieved following the manufacturer's protocol (Catch and Release Immunoprecipitation Kit; Catalog No.: 17-500; Millipore).

Genotyping

Mice: Genotyping primer pairs for genotyping the Stat3^{tm1Xyfu}/J mice are forward primer 5'-TTGACCTGTGCTCCTACAAAAA-3' and reverse primer 5'-CCCTAGATTAGGCCAGCACA-3'. The genotyping primer pairs for Tg(Tek-cre)1Ywa/J mice are forward primer 5'-CGCATAACCAGTGAAACAGCATTGC-3' and reverse primer 5'-CCCTGTGCTCAGACAGAAATGAGA-3'.

Zebrafish: Following imaging of vascular permeability, each individual zebrafish was euthanized and genomic DNA was extracted using standard techniques for subsequent STAT3 genotyping using the following pair of primers: 5'-GGTCTTCCACAACCTGCTG-3' and 5'- TAGACGCTGCTCTTCCCAC-3'.

Compounds

Pyrimethamine (PYR; 75 mg/kg; Sigma Aldrich; Catalog No. 46706) or control vehicle was delivered to mice by intraperitoneal injection daily for 15 days. PYR was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 167 mg/ml and then diluted in PBS before injection into mice. For zebrafish studies, 10μM PYR or vehicle (DMSO) was added to zebrafish embryo water for 3 days prior to assessment of vascular permeability and immunoblotting studies. Atovaquone (AQ; Sigma Aldrich; Catalog No. A7986), C188-9 (Sigma Aldrich; Catalog No. 573128), and PYR were each dissolved in DMSO and used for in vitro studies at the indicated concentrations. For in vivo studies, C188-9 was dissolved in DMSO at 208mg/ml and diluted in 5% dextrose in water (D5W) before injection. Mice received C188-9 (100 mg/kg) or vehicle (DMSO in D5W) by intraperitoneal injection daily for 1 week. For in vivo studies, Tyrphostin AG 490 (40 mg/kg, Sigma Aldrich; Catalog No.T3434) was dissolved in DMSO at the concentration of 250 mg/ml and diluted in PBS before injection**.** Mice received AG490 or vehicle (DMSO in PBS) by intraperitoneal injection daily for 1 week.

Glutathione S-transferase (GST) pull-down assays

The cDNA fragment encoding full-length human STAT3 was subcloned from pLEGFP-WT-STAT3[1] into the mammalian expression vector pEBG[2] using KpnI and NotI restriction enzyme sites and the following subcloning primer pairs: forward: 5'-GGGGTACCTCGAGCTCAAGCTTCAGGATGG-3' and reverse: 5'-ATAAGAATGCGGCCGCTCACTTGTAGTCCATGGGGGAGGTA-3'. pLEGFP-WT-STAT3 was a gift from Dr. George Stark (Addgene plasmid # 71450; http://n2t.net/addgene:71450; RRID:Addgene_71450)[1]. pEBG was generously shared by Dr. David Baltimore (Addgene plasmid # 22227; http://n2t.net/addgene:22227; RRID:Addgene_22227)[2]. Freestyle 293F cells (Thermo Fisher Scientific) grown in FreeStyle™ 293 Expression Medium (Thermo Fisher Scientific; 12338018) in an incubator at 37°C with 8% CO_2 were transfected with pEBG-STAT3 plasmid according to the instructions of the Freestyle 293 Expression System (Thermo Fisher Scientific) and then harvested by centrifugation at 1500 x g for 10 minutes. Cell pellets were suspended in PBS containing protease inhibitor cocktail (Roche) and lysed on ice using 15 s pulses of sonication repeated 7 times with a Sonic Dismembrator, Model 100 (Fisher Scientific). Lysates in 1% Triton, 5 mM DTT were centrifuged at 12000g for 10 minutes at 4°C, and Glutathione Sepharose 4B (GE Healthcare) was used to bind the GST fusion proteins from the supernatant. GST protein-coated beads were incubated with pre-cleared HUVEC cell lysates at 4°C overnight. The bead-protein complexes were then washed 5 times with pre-chilled PBS, and the proteins were eluted using 10mM Glutathione elution buffer at room temperature. The proteins were boiled for 5 minutes in the Laemmli sample buffer and then analyzed by immunoblotting.

Purification of human STAT3 protein

STAT3 cDNA (human α isoform, residues 1-770) was subcloned from pLEGFP-WT-STAT3[1] into the RGS-6xHis-pcDNA3.1 plasmid[3], which was a gift from Dr. Adam Antebi (Addgene plasmid # 52534; http://n2t.net/addgene:52534; RRID: Addgene_52534)[3]. The 6xHis-STAT3α fragment was cloned into the pFastBac™Dial vector, generously provided by Dr. George Aslanidi at the University of Minnesota, The Hormel Institute. The plasmid was first transformed into *E. coli* DH10 MultiBac. Single colonies were inoculated into 2 ml antibiotic LB broth containing 50 μg/ml Kanamycin, 7 μg/ml Gentamycin, and 10 μg/ml Tetracycline and grown at 37°C overnight. After isolating recombinant Bacmid DNA, we transfected Sf9 insect cells, grew cells in Gibco™ Sf-900™ II SFM medium (Gibco, Catalog NO.10902088) in a 27°C incubator, harvested P0 baculovirus stock, and amplified P1 and P2 baculovirus in a 27°C, 90 rpm shaker. Sf9 cells were infected with P2 baculovirus (MOI=10) and cells were harvested by centrifugation at 1500 rpm for 10 minutes after incubation for 3 days. The pellet was resuspended in binding buffer [50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 2 mM $MgCl_2$, 10% glycerol, 1mM Tris (2-Carboxyethyl) Phosphine (T-CEP), 10 mM Imidazole] supplemented with complete protease inhibitors (Roche) and then lysed by 7 cycles of sonication (Fisher Scientific; Sonic Dismembrator, Model 100) each consisting of constant pulse for 15s on ice. The lysate was cleared by centrifugation at 30,000 x g for 30 minutes at 4°C. The supernatant was bound to high performance HisTrap column (GE Healthcare) using binding buffer [Sodium phosphate 20 mM, NaCl 500 mM, Imidazole 20 mM and T-CEP 0.5 mM] and eluted with an imidazole gradient (0 to 500 mM). The protein was then concentrated and loaded onto a Superdex 200 size exclusion column equilibrated in 50 mM sodium phosphate, 150 mM NaCl and 0.5 mM T-CEP. Peak fractions were analyzed by SDS-PAGE.

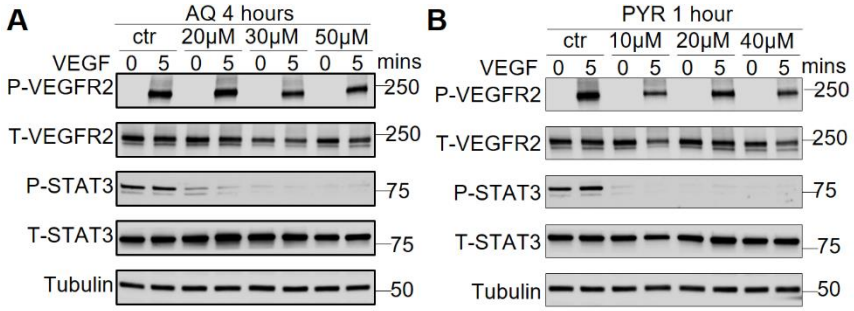
Quantitation of zebrafish vascular permeability

ImageJ software was used to quantitate the extent of Texas Red-dextran in the extravascular space. Mean gray value was measured after converting the image type to 8-bit gray, setting scale to pixels, inverting the image and identifying 4 areas of extravascular space in the middle of the zebrafish trunk region using drawing/selection tools to avoid intersegmental vessels evident by green fluorescent signal originating from FITC-dextran. The background gray value minus the average gray value of the four regions is used for statistical analysis.

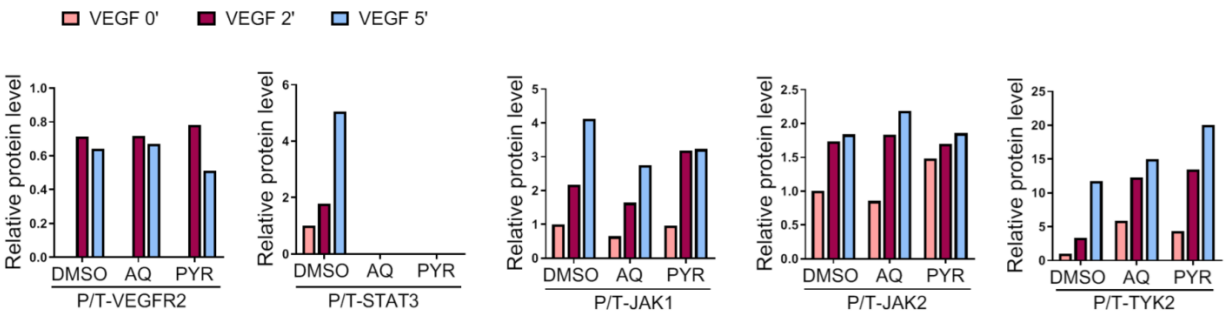
SUPPLEMENTAL REFERENCES:

1. Dasgupta M, Unal H, Willard B, Yang J, Karnik SS, Stark GR. Critical role for lysine 685 in gene expression mediated by transcription factor unphosphorylated STAT3. *J Biol Chem*. 2014;289(44):30763-30771.
2. Tanaka M, Gupta R, Mayer BJ. Differential inhibition of signaling pathways by dominant-negative SH2/SH3 adapter proteins. *Mol Cell Biol*. 1995;15(12):6829-6837.
3. Horn M, Geisen C, Cermak L, et al. DRE-1/FBXO11-dependent degradation of BLMP-1/BLIMP-1 governs C. elegans developmental timing and maturation. *Dev Cell*. 2014;28(6):697-710.

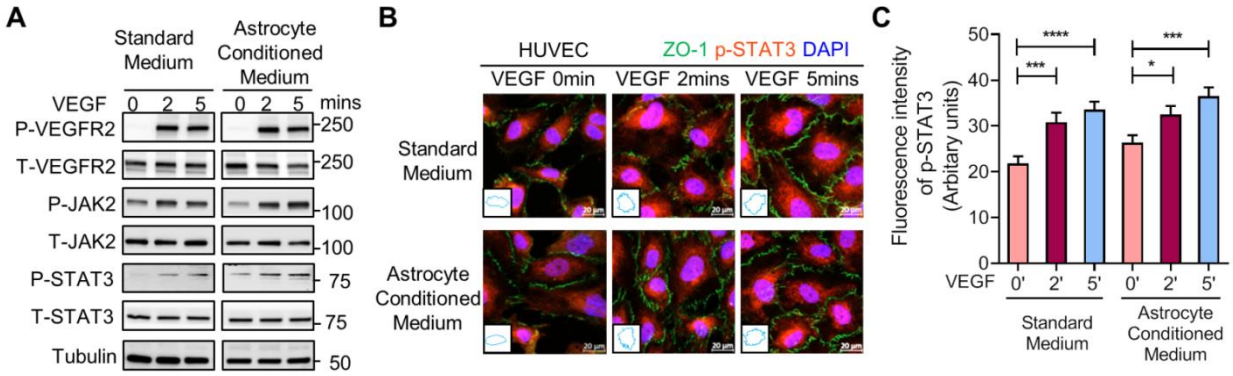
SUPPLEMENTAL FIGURES:



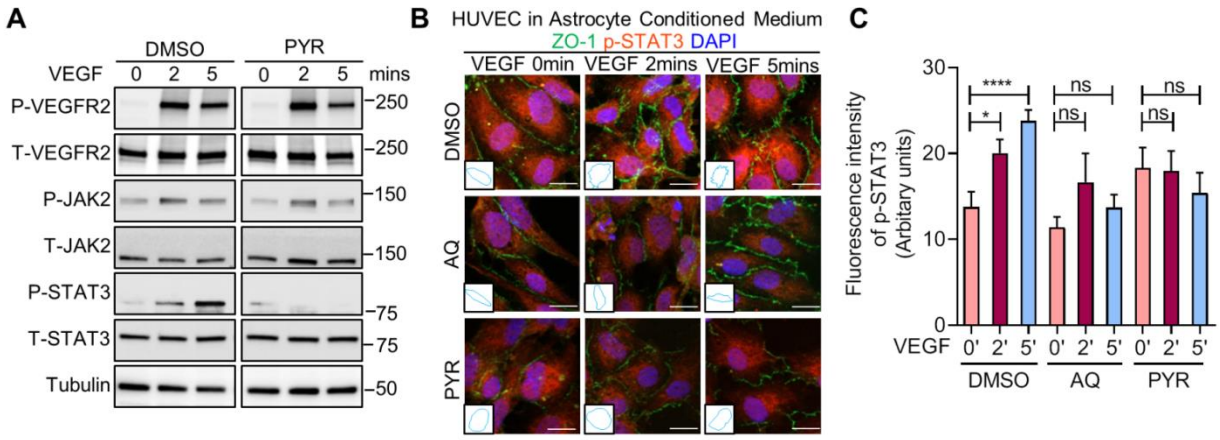
Supplemental Figure 1: Assessment of STAT3 inhibition by atovaquone (AQ) and pyrimethamine (PYR) in VEGF-stimulated HUVEC. Serum-starved HUVEC were pretreated with 0, 20, 30, or 50 μM AQ for 4 hours (left) or 0, 10, 20, or 40 μM PYR for 1 hour (right) prior to human VEGF-165 protein (25 ng/ml) stimulation for 0 or 5 minutes. Cells were lysed and then immunoblotted with the indicated primary antibodies.



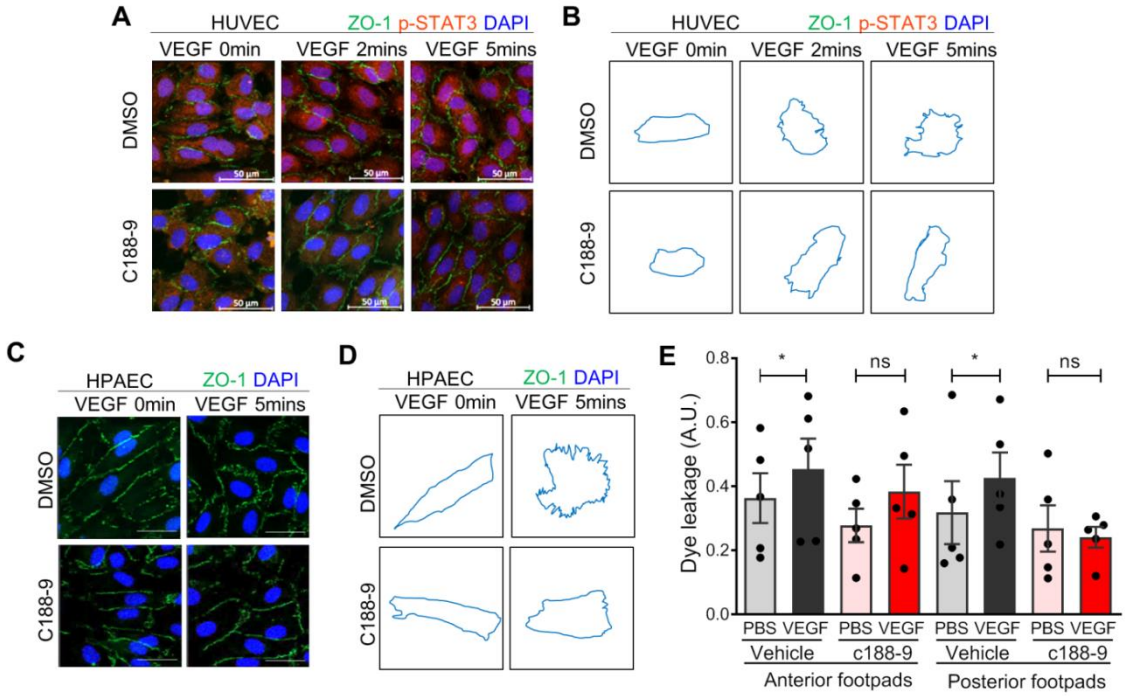
Supplemental Figure 2: Densitometry analysis for immunoblotting of VEGF-stimulated HUVEC upon pretreatment with STAT3 inhibitors, atovaquone (AQ) and pyrimethamine (PYR). Densitometry was performed on the immunoblotting image depicted in Figure 4B by quantitating phosphorylated protein relative to total protein levels as indicated.



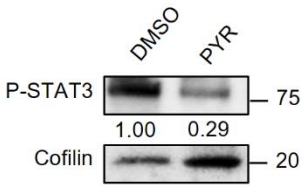
Supplemental Figure 3: VEGF-induced STAT3 activation disrupts ZO-1 junctional stability in HUVEC. **A-B)** Serum-starved HUVEC were cultured in standard medium or astrocyte conditioned medium (mixed 1:1 with standard medium) and stimulated with VEGF (25 ng/ml). **A)** Cells were lysed and immunoblotted using indicated antibodies. **B)** IF was performed using p-STAT3 (Y705; red) and ZO-1 (green) antibodies. VEGF stimulation promotes disorganization of ZO-1 at endothelial cell junctions (i.e. jagged appearance). Nuclei stained with DAPI (blue). Insets: trace of ZO-1 staining. **C)** Quantification of the p-STAT3 staining intensity. *P<0.05, ***P<0.001, ****P<0.0001, one-way ANOVA.



Supplemental Figure 4: STAT3 inhibitors, atovaquone (AQ) and pyrimethamine (PYR), stabilize endothelial barrier integrity following VEGF stimulation in HUVEC cultured in astrocyte conditioned medium. A) Serum-starved HUVEC cultured in astrocyte conditioned medium (mixed 1:1 with standard medium) were pretreated with 20 µM PYR for 1 hour prior to VEGF (25 ng/ml) stimulation for 0, 2, or 5 minutes. **B)** Serum-starved HUVEC cultured in astrocyte conditioned medium (mixed 1:1 with standard medium) were pretreated with 30 µm AQ for 4 hours or 10 µm PYR for 1 hour prior to VEGF (25ng/ml) stimulation. VEGF stimulation promotes disorganization of ZO-1 (green) at endothelial cell junctions (i.e. jagged appearance). ZO-1 organization is maintained when HUVEC are pretreated with AQ or PYR (i.e. smooth appearance). Nuclei were stained with DAPI (blue). Insets: trace of ZO-1 staining on 1 cell. **C)** Quantification of the intensity of phosphorylated STAT3 protein at Y705. *P<0.05, ****P<0.0001, one-way ANOVA.



Supplemental Figure 5: Pharmacological inhibition of STAT3 stabilizes endothelial barrier integrity following VEGF stimulation in HUVEC and mice. A) Serum-starved HUVECs were pretreated with 10 µM C188-9 for 5 minutes prior to human VEGF-165 protein (25ng/ml) stimulation for 0, 2 and 5 minutes. VEGF stimulation promotes disorganization of ZO-1 (green) at endothelial cell junctions. ZO-1 organization is maintained when HUVEC were pretreated with C188-9. p-STAT3 (Y705; red) was reduced upon treatment with C188-9. Nuclei were stained with DAPI (blue). **B)** Depiction of selected ZO-1 staining to help visualize its organization or disorganization upon VEGF treatment in the absence of STAT3 inhibitor, C188-9. **C)** Serum-starved HPAEC were pretreated with 10 µM C188-9 for 5 minutes prior to human VEGF-165 protein (25ng/ml) stimulation for 0 and 5 minutes. After stimulation with VEGF protein for 5 minutes, the structure of tight junction marked with ZO-1 was disrupted (i.e. jagged-like ZO-1 green staining). ZO-1 organization is maintained when HPAEC were pretreated with C188-9. Nuclei were stained with DAPI (blue). **D)** Depiction of selected ZO-1 staining. (E) Mice were administered C188-9 or vehicle prior to tail vein injection with 1% Evans blue and VEGF (2.5 µg/ml) or PBS vehicle being injected into the root of the footpad. Quantitation of Evans blue leakage in C57BL/6 wildtype mice. n=5 mice per group. Each mouse was injected with PBS on right anterior and posterior footpads and VEGF on left anterior and posterior footpads. Multiple biological replicates were performed and depicted findings are representative. *P<0.05, paired t-test.



Supplemental Figure 6: Pyrimethamine (PYR) inhibits STAT3 activity in zebrafish. Zebrafish were exposed to embryo water containing DMSO or 25 μM PYR for 3 days at starting 6 hours post-fertilization. Protein lysates were harvested from 3 days post-fertilization embryos by sonication in RIPA buffer after removing yolk sac and immunoblotting was performed using antibodies against zebrafish p-STAT3 (Y708) and cofilin.