**SUPPLEMENT**

## Expanded Material and Methods

### Hindlimb Ischemia Model

Male C57Bl/6J mice were purchased from the Jackson Laboratory and used at 6-8 weeks of age. All protocols were approved by the Institutional Animal Care and Use Committee and done in accordance with the federal guidelines on the principles for the care and use of animals in research. Animals were anesthetized by intraperitoneal injection of Xylazine (10 mg/kg) and Ketamine (80 mg/kg). The left superficial femoral artery and vein were isolated from the femoral nerve and ligated proximal to the caudally branching deep femoral artery. A second ligation was then made proximal to the branching of the tibial arteries and the artery and vein were removed between the two ligation points, leaving the femoral nerve intact. The skin was closed by interrupted sutures and the mouse was allowed to recover and ambulate freely[1]. Where indicated, L1-10 (4mg/kg), an FC-fusion protein Ang2 specific inhibitor (Amgen), vehicle (PBS), or $IgG_1$-FC control (R&D systems) was injected subcutaneously every other day starting with 1 day before surgery.

### Whole Mount Immunohistochemistry

On day 2, 5, 7 or 10 after surgery as indicated, the animal was sacrificed by $CO_2$ inhalation and pressure perfused with 0.9% NaCl solution then pressure fixed with 10% formalin. The adductor muscle was isolated, removed and fixed for 1 hour with 1% paraformaldehyde. The tissue was blocked for 1 hour with 5% goat or donkey serum in

Tris buffered saline containing 0.3% Triton-X 100 (TBST) and incubated overnight at 4°C with primary antibody diluted 1:1000 in TBST. After several washes with TBST, the tissue was then incubated with fluorescently tagged secondary antibody diluted in TBST for 4 hours. The tissue was washed and post-fixed with 4% paraformaldehyde for 10 minutes. The whole tissue was placed on a coverslip and imaged using a LSM 510 Meta confocal microscope (Zeiss). Confocal Images were taken from the center, proximal, and distal regions of the adductor muscle. Images were constructed into 3D projections of Z-stacks. Quantification of images was performed using NIH ImageJ for each individual channel and averaged for all anatomical regions of the adductor muscle. Antibodies used were: hamster anti-mouse PECAM-1 (Chemicon), goat anti-mouse Ang2 (Santa Cruz), anti-mouse smooth muscle α-actin-FITC (Sigma) and rat anti-mouse CD11b (BD Biosciences).

**Cell Culture and Shear Studies**

Human umbilical vein endothelial cells (HUVEC) were obtained from the Department of Dermatology, Emory University, cultured in M199 media (Cellgro) with 20% heat inactivated fetal bovine serum (FBS, Atlanta Biologicals), and used between passage 4 and 6. HUVEC one day post-confluence were exposed to laminar shear stress (15 dyn/cm$^2$), oscillatory shear stress (OS) (± 5 dyn/cm$^2$ at 1 Hz frequency), or static control (ST) for 24 hours using a cone-and-plate device as described by us[2].

**Matrigel Tubule Formation Assay**

Following shear, conditioned media (CM) were collected from HUVEC sheared in reduced serum media (M199-2% FBS). Non-sheared HUVEC were then resuspended in the CM and 20,000 cells/well were added to a growth factor reduced Matrigel coated 96-well plate. The Ang2 specific inhibitor, L1-10, was then added at 10 μg/mL and the cells were incubated at 37°C for 6 hours. Tubule formation was observed using a phase contrast microscope at 5x and 10x magnification. Tubule length was quantified using NIH ImageJ and the total length of tubules was summed over 4 high powered fields (5x magnification).

**L1-10 Characterization**

HUVEC were cultured as described above. Rat aortic smooth muscle cells (RASM) were isolated from the aorta of Sprague-Dawley rats, as previously described[3] and cultured in DMEM containing 10% FBS. Confluent HUVEC were serum starved in 0% M199 for 1 hour and confluent RASM were serum starved in 0.01% FBS DMEM for 24 hours. Cells were then treated with 50 ng/mL VEGF-A (HUVEC) or 10 ng/mL PDGF (RASM) for 30 minutes and 10 minutes, respectively, with or without 10-fold molar excess of L1-10.

**Preparation of Tissue/Cell lysates and Immunoblotting**

Human umbilical vein endothelial cells (HUVEC) were serum starved for 1 hour and then treated with Ang1 (800ng/mL, R&D systems), Ang2 (800 ng/mL)[4], with or without L1-10 (6.5μg/mL which is equivalent to ~10-fold molar excess). To test the role of L1-10 *in vivo*, C57Bl/6J mice were injected via tail vein with 20μg Ang2 with or without 10-

fold molar excess L1-10. After 30 minutes, the adductor muscle was collected, frozen in liquid nitrogen, and homogenized using a mortar and pestle. Protein content of each sample was measured using a Bio-Rad DC assay. Protein (~50 μg) was resolved on 8% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane (Millipore). The membranes were probed with the following primary antibodies: rabbit anti-pTie2 (Tyr992) (Cell Signaling), rabbit anti-Tie2 (Santa Cruz), and goat-anti-actin (Santa Cruz) and then with secondary antibody conjugated to alkaline phosphatase, which was then detected using chemiluminescence. The intensities of immunoreactive bands were analyzed using Scion Image and NIH ImageJ and normalized to β-actin (Santa Cruz).

**Real time quantitative polymerase chain reaction (PCR)**

Animals were sacrificed by $CO_2$ inhalation and pressure perfused with 0.9% NaCl solution. The adductor muscle was isolated and immediately flash frozen in liquid nitrogen. The frozen tissue was disrupted using a mortar and pestle and homogenized using QIAshredders (Qiagen). Total RNA was isolated and purified using RNeasy Mini Kit (Qiagen). Four μg of total RNA was reverse transcribed by using random primers and a Superscript-II kit (Life Technology) to synthesize first-strand cDNA. The cDNA was assayed by real time PCR using a LightCycler (Roche), recombinant Taq polymerase (Life Technology), Taq start antibody (Clontech) and SYBR green detection of products. Copy numbers were determined based on standard curves generated with mouse standards for Ang2, Ang1, c-fms, and 18S templates. The following primers were used to amplify mouse Ang2 (5' to 3')- forward: AGATCCAACAGAATGTGGTGC and reverse: TGTTGACGGTCTCCATTAGG[5], for mouse Ang1- forward:

CACGAAGGATGCTGATAACG and reverse: AAGTGGCGATTCTGTTGACC[5], for mouse c-fms- forward: CACAGGAGGTGACAGTGGTT and reverse: TGGTCTTGCACACGTAGGTA[6], for mouse ICAM-1- forward: CACCCCAAGGACCCCAAGGAGAT and reverse: CGACGCCGCTCAGAAGAACCAC, for mouse VCAM-1- forward: GGAAGCTGGAACGAAGTATC and reverse: GCTTTGTCTCTCCCAATCAG. Real-time PCR for Ang2, Ang1, c-fms, ICAM-1, and VCAM-1 was carried out with the annealing temperature at 60ºC and 40 cycles. DNA gels were run to confirm specificity of PCR products.

**Laser Doppler Perfusion Imaging (LDPI)**

LDPI was performed 1 day before hindlimb ischemia surgery (Pre-Op) and on days 2, 5, 7, and 10 after surgery. Mice were anesthetized with Xylazine (10mg/kg) and Ketamine (80 mg/kg) and allowed to warm on a 37°C heating pad for 5 minutes. The footpad was then scanned with a PIM II Laser Doppler Perfusion Imager. Average perfusion in the ischemic foot was then normalized to the contralateral control for each animal.

**References**


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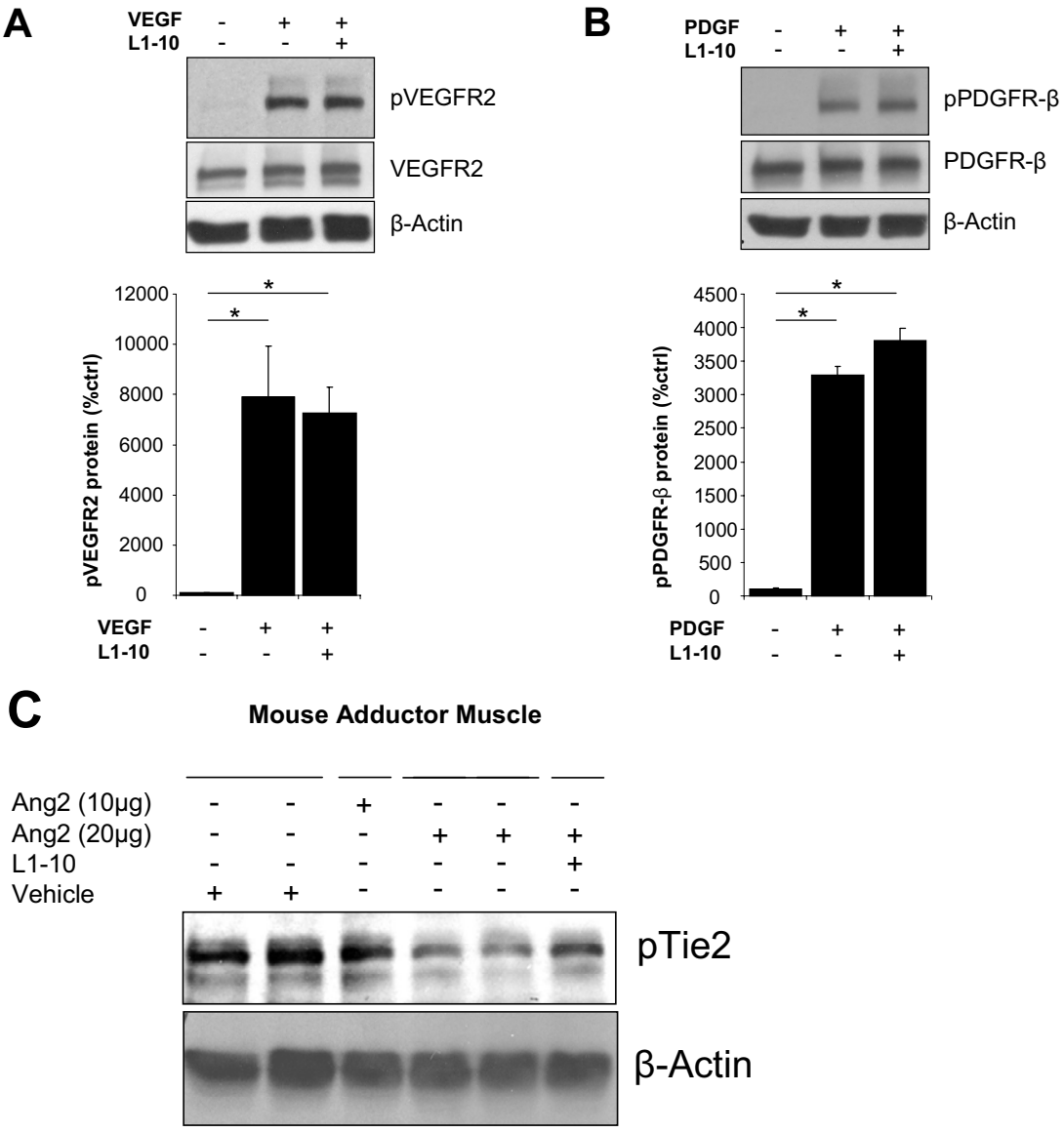
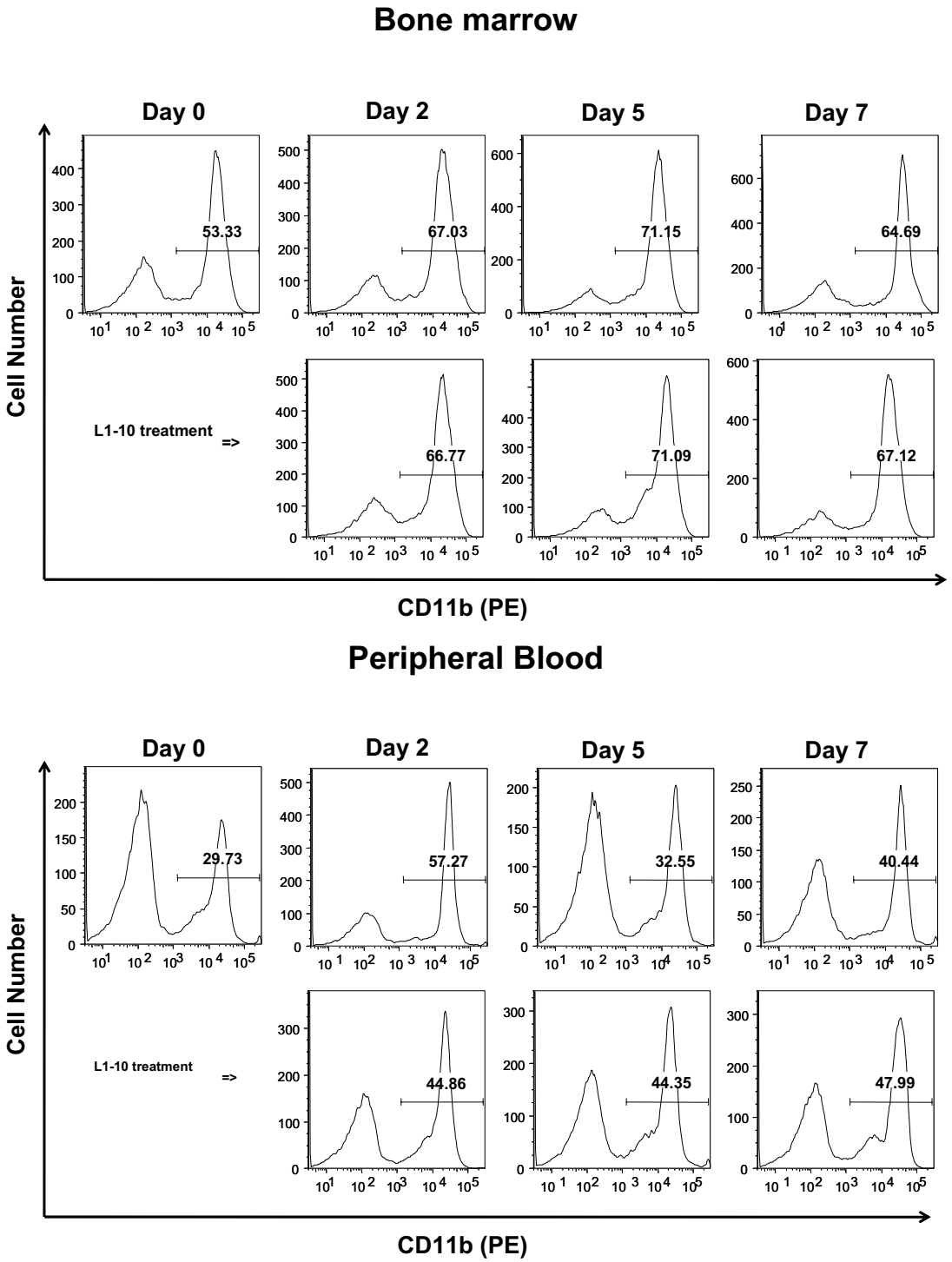



**Supplemental Figure 1.** Specificity and *in vivo* action of L1-10. A, HUVEC were treated with VEGF (50 ng/mL) ± L1-10 for 30 minutes. Cell lysates were used for Western analysis for antibodies against phosphor-VEGFR-2 (pVEGFR-2). B, RASM were treated with PDGF (10 ng/mL) ± L1-10 for 10 minutes. Cell lysates were used for Western analysis with antibodies against phospho-PDGFR-β (pPDGFR- β). C, C57Bl/6J mice were injected via tail vein with vehicle, 10 or 20 μg Ang2 ± L1-10. After 30 minutes, the adductor muscle was collected. Cell and Tissue lysates were used for Western blots with antibodies to phospho-Tie2 and β-actin. Blot densitometry was quantified using Scion Image or ImageJ and normalized to β-actin as a loading control. (mean ±SEM, n=3-6; * $P<0.05$).




**Supplemental Figure 2:** Representative histograms of flow cytometric analysis. Mice underwent hindlimb ischemia surgery and were treated with vehicle or L1-10. Cells from bone marrow and peripheral blood were collected before surgery (day 0) and on day 2, 5, and 7 and analyzed by FACS with antibodies to CD11b.