Supplement Material

DETAILED METHODS

Cell Culture
Mouse aorta vascular smooth muscle cells (MOVAS) were purchased from the ATCC and maintained in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 0.2 mg/mL G418 in 5% CO_2 at 37 °C. *Cercopithecus aethiops* kidney cells (COS7) were maintained in DMEM supplemented with 10% FBS, 1% P/S in 5% CO_2 at 37 °C. Primary SMCs from wild-type, *PAR1*$^{-/-}$, and *PAR2*$^{-/-}$ C57BL/6 mice were isolated from carotid arteries. Arteries were split and scraped to remove the intimal endothelial layer, and then plated on tissue culture plates pre-coated with Type I collagen (Sigma). SMCs were maintained in DMEM supplemented with 15% FBS, 1% P/S in 5% CO_2 at 37 °C.

Flow Cytometry
MOVAS or primary SMC cells were harvested by lifting with 3 mM EDTA/PBS. Cells were treated with a mPAR1 polyclonal Ab, a mPAR2 polyclonal Ab, or a PAR4 polyclonal Ab[1] (GYPGQVSANDSDTLELPC) and subsequently with FITC-labeled secondary Ab. The polyclonal antibody mPAR1-Ab directed against the mouse thrombin receptor (*F2r*) ligand region, residues 42-56, and mPAR2-Ab directed against the mouse *F2rl1* ligand region, residues 39-56 were generated as previously described.[1] Briefly, the epitope peptides mPAR1 S_{42}FFLRNPSENTFELV$_{56}$ and mPAR2 S_{39}LIGRLETQPPITGKGVC$_{56}$ were synthesized, purified by HPLC, conjugated to maleimide-activated keyhole limpet hemocyanin (Thermo), and used to produce antisera from two rabbits each (SDIX, Newark, DE). Polyclonal antibodies were purified from rabbit serum using a SFFLRNPSENTFELVC or SLIGRLETQPPITGKGVC peptide-coupled CNBr-activated Sepharose 4B (GE Healthcare) affinity column. PAR expression was quantified by mean fluorescence intensity relative to isotype control on a BD Canto II flow cytometer and results were analyzed using FlowJo Software (Tree Star).

^{3}H Thymidine Incorporation
MOVAS or primary smooth muscle cells were lifted with 3 mM EDTA, plated at 2000 cells/well in 24-well plates, and allowed to attach overnight in complete media (DMEM, 10% FBS, P/S). Cells were starved overnight in DMEM, 0.4% BSA, 1% pencillin/ steptomcyin (P/S) and treated as indicated once a day under starvation conditions. One day prior to harvesting, 1 mL of methyl ^{3}H-Thymidine (NEN; 1 mCi) was added to each well. Cells were harvested 48-72 h after the first treatment. Media was aspirated, cells were washed with PBS, 2 mL of ice-cold 6% trichloroacetic acid (TCA) was used to fix cells and 1 mL of 0.2 N NaOH for 10 min was used to lift cells. The entire volume of each well was transferred to a scintillation vial, 7.5 mL of scintillation fluid was added, and vials were analyzed on a Tri-Carb 2900 TR Liquid Scintillation Analyzer (Packard).

Calcium Flux Assay
Intracellular Ca^{2+} flux was measured in MOVAS labeled with fura-2AM, by the ratio of fluorescence excitation intensity at 340/380 nm on a LS 50B Luminescence Spectrometer (Perkin Elmer).

MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) Assay
MOVAS were plated at 1000 cells/well in a 96-well plate and allowed to adhere overnight. Cells were incubated in starvation media (DMEM, P/S, 0.4% BSA) and

subjected to specific treatment conditions, as indicated. After daily treatment for 4 days under low serum conditions, MTT reagent (Sigma) was added at a concentration of 0.5 mg/mL and allowed to incubate at 37 °C for 5 h. The resulting formazan crystals were dissolved in 100% DMSO and OD_{570}-OD_{650} was measured using a SPECTRAmax 340 microplate reader (Molecular Devices Corporation).

Carotid Artery Ligation Injury Model

All animal experiments were performed in accordance with the US National Institutes for Health guidelines and approved by Tufts University School of Medicine Institutional Animal Care and Use Committee. Six-month old C57BL/6 were purchased from Jackson Laboratories Strain C57BL/6, Stock number 000664, $PAR1^{-/-}$ C57BL/6 (originally from Jackson Laboratories Strain B6.129S4-$F2r^{tm1Ajc}$/J, Stock number 002862) were bred in-house, $PAR2^{-/-}$ C57BL/6 female mice were bred in-house from breeding pairs kindly provided by Dr. Patricia Andrade-Gordon (Johnson and Johnson Pharmaceutical Research and Development, Spring House, PA) were anesthetized with isoflurane and aseptically surgically prepared. A midline incision was made to expose the trachea and carotid artery. The left carotid artery was isolated and a 6-0 silk suture was placed around the common carotid and ligated to completely restrict blood flow. The wound was closed with 6-0 nylon/monofilament non-absorbable sutures. Post-operative analgesia, buprenorphine at 0.05 mg/kg was administered as needed. P1pal-13 (Pal-AVANRSKKSRALF-NH_2, s.c. 2.5 mg/kg), P1pal-7 (Pal-KKSRALF-NH_2, s.c. 10 mg/kg) pepducins or vehicle (20% DMSO) were administered subcutaneously in 100 µL volumes on a daily basis. After 2 h to 21 days, mice were anesthetized with an intraperitoneal injection of ketamine/xylazine (90-120 mg/kg, 10 mg/kg) and a midline incision was made to expose both carotid arteries. The chest was entered through anterior thoracotomy and a 25-gauge butterfly needle was inserted into the left ventricle of the heart. A pressurized bag at 125 mm Hg with 10% formalin was used to perfuse the animals for 2 min. Left and right common carotid arteries were harvested and further fixed in 10% formalin. Samples were vertically embedded, slides were stained with H&E, elastin, or the proliferation marker Ki67, and cross-sections were analyzed by microscopy.

Carotid Artery Morphometry

Images of H&E carotid artery cross-sections were captured using a Nikon Eclipse 80i microscope and a Spot 7.4 Slider camera (Diagnostic Instruments, Inc) at a magnification of 10x and 40x. Images were analyzed for the intimal, medial, and adventitial layers by measuring area (μm^2) of at least two images from each left carotid artery. Area was measured by weighing the portions of each arterial section of printed images and converted from mg to mm^2 by a standard curve of known sizes by capturing images using an objective micrometer. Areas for each left carotid were averaged and reported for each group.

Coimmuno-precipitation of PAR1 and PAR2

A pcDEF3-PAR1 construct was tagged at the N-terminus with a T7-epitope (MASMTGGQQMGT) as previously described[3]. A pcDEF3-PAR2 construct was tagged at the C-terminus with a myc-epitope tag (EQKLISEEDL) and a CXCR4 construct was tagged at the N-terminus with a HA epitope (MYPYDVPDYA). COS7 fibroblast cells were transiently transfected with pcDEF3 alone (vector), T7-PAR1 alone, PAR2-myc alone, HA-CXCR4 alone, T7-PAR1 and PAR2-myc, T7-PAR1 and HA-CXCR4, or PAR2-myc and HA-CXCR4. Cell lysates were prepared 48 h after transfection in M-PER lysate buffer (Pierce) with Halt-Protease Inhibitor cocktail (Thermo Scientific) and protein was

quantified using a Bradford assay. Cell lysates were pre-cleared with 25 mL of Protein A-Agarose (Calbiochem) for 1 h, then incubated overnight at 4 °C with 25 mL of either a-T7 agarose beads (Novagen) or a-cMyc Monoclonal Ab-agarose beads (Clontech) in a final volume of 500 mL. Agarose beads were collected by centrifugation and washed x3 with 1% of M-PER lysis buffer. Immunoprecipitated protein was eluted in 50 mL of 10 mM citric acid pH 2.2 in SDS loading buffer for 30 min at 37 °C. Samples were centrifuged to precipitate beads and 10 mL of neutralization buffer was added to the protein samples before gel electrophoresis.

Immunofluorescence

Primary SMCs were plated at 1000-3000 cells/well on BD chamber Culture-Slides and allowed to adhere for 24-48 h. Media was aspirated and cells were washed with PBS. Cells were fixed with 4% formaldehyde in PBS for 20 min at 37 ºC. Cells were blocked with 1% BSA at 25 ºC for 30 min and 10% goat serum for 20 min, and then incubated with 1:50 or 1:100 dilutions of the indicated antibodies for 1 h at 25 ºC. Cells were then incubated with a 1:500 dilution of the appropriate secondary antibody (Alexa 488-rabbit or Alexa 546-mouse, Invitrogen) in the dark for 1 h at 25 ºC. Cells were washed, ProLong antifade solution with DAPI (Invitrogen) was added, and coverslips were mounted on glass slides. Fluorescence was observed on a Nikon Eclipse 80i microscope and images were captured by a Spot 7.4 Slider camera (Diagnostic Instruments, Inc).

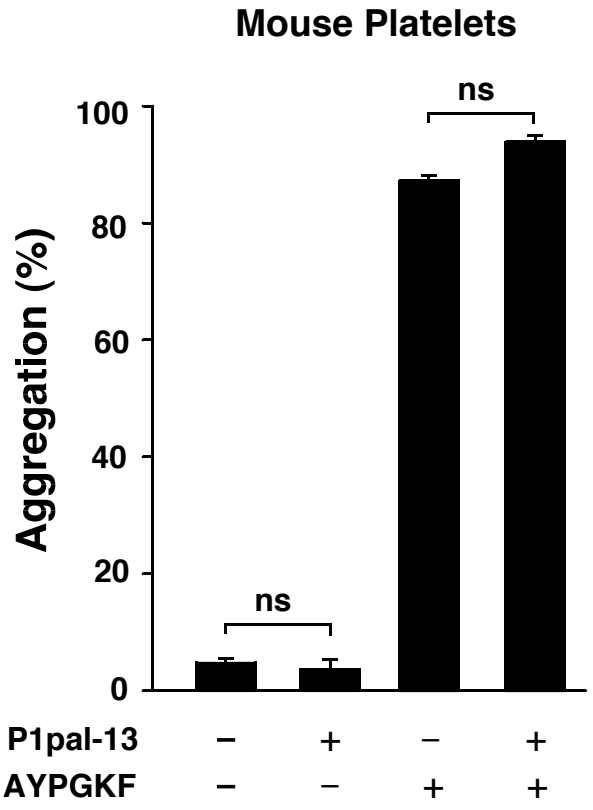
RT-PCR Analysis

Total RNA was extracted using the RNeasy mini kit (Qiagen) and 1 mg of RNA was reversed transcribed using Moloney murine leukemia virus reverse transcriptase and dNTPs (Invitrogen). Real-time PCR was conducted in 25 mL volumes with 12.5 mL of SYBR Green, 1.25 mL of each primer and 8 mL of RNA-free water. All reactions were performed in triplicate in a DNA Engine Opticon 2, Continuous Fluorescence Detector (MJ Research). After 15 min of denaturation at 95 °C, 30 s at 94 °C, 1 h at 55 °C, 30 s at 72 °C, PCR was performed at 95 °C for 15 s, 55 °C for 20 s, and 72 °C for 15 s for 40 cycles. The primers used are listed in Online Table I. Relative expression was normalized to GAPDH expression (rER or relative expression ratios) were further compared to uninjured vehicle control for fold change.

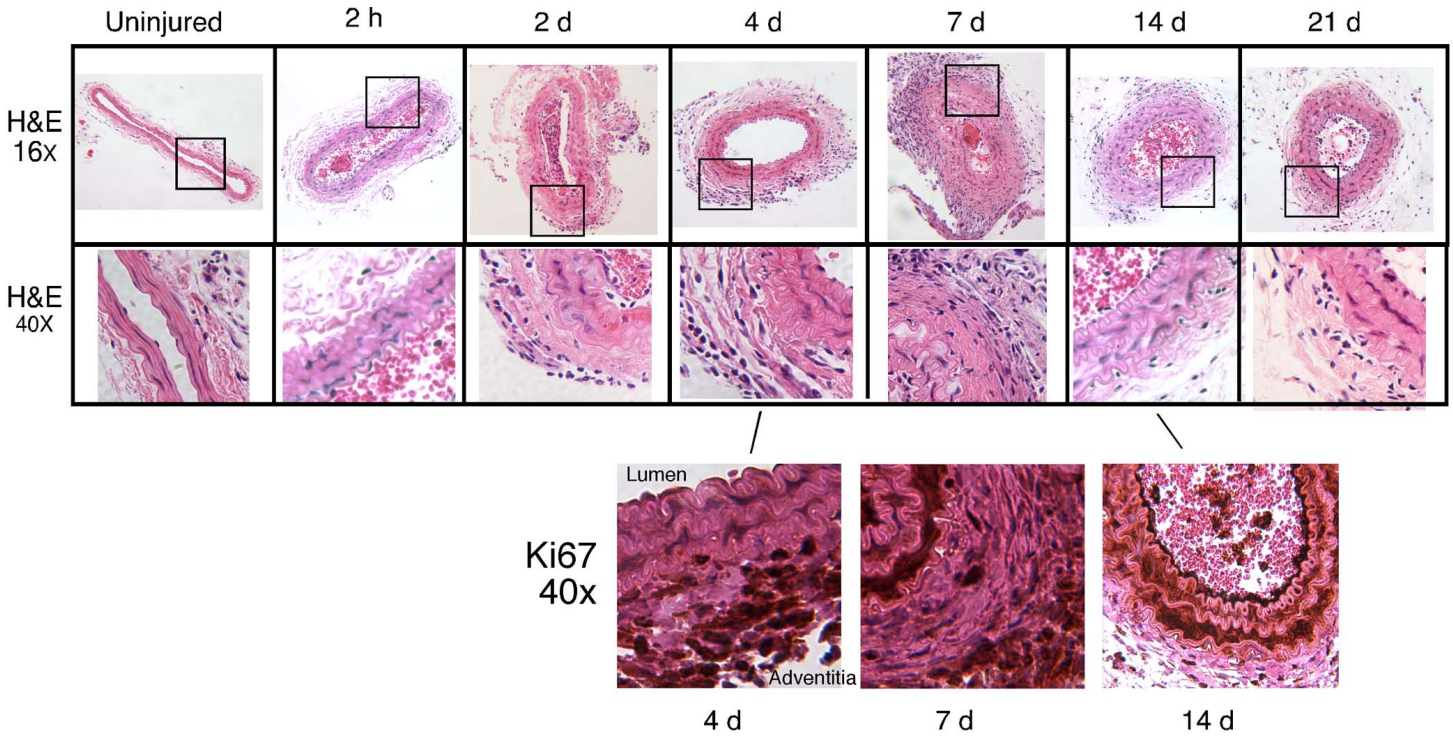
1. Kamath L, Meydani A, Foss F, Kuliopulos A. Signaling from protease-activated receptor-1 inhibits migration and invasion of breast cancer cells. *Cancer Res.* 2001;61(15):5933-5940.
2. Kaneider NC, Leger AJ, Agarwal A, Nguyen N, Perides G, Derian C, Covic L, Kuliopulos A. 'Role reversal' for the receptor PAR1 in sepsis-induced vascular damage. *Nat Immunol.* 2007;8(12):1303-1312.
3. Kuliopulos A, Covic L, Seeley SK, Sheridan PJ, Helin J, Costello CE. Plasmin desensitization of the PAR1 thrombin receptor: kinetics, sites of truncation, and implications for thrombolytic therapy. *Biochemistry.* 1999;38(14):4572-4585.

Supplemental Table I: Real-time PCR primers (for mouse SMCs).			
Gene targeted	Official gene name	Direction	Primer Sequence
α-SMA	*Acta2*	forward	AGCCAGTCGCTGTCAGGAACCCT
		reverse	CACCAGCGAAGCCGGCCTTAC
SM-22	*Tagln*	forward	GGCGGCCTTTAAACCCCTCACC
		reverse	GTTGAGGCAGAGAAGGCTTGGTCG
SM-MHC	*Myh10*	forward	TCTCAAGAACCGGCTCAGGCGGG
		reverse	GCGATGCCCCCTCAATGTGCAG
PDGF-B	*Pdgfb*	forward	CAGCGAGCCAAGACGCCTCAA
		reverse	ACACTCTTGCCGACGCCCCT
Type III collagen	*Col3a1*	forward	AGAGGGGCTCCTGGTGAGCG
		reverse	GGGCCAGGGGGACCAGGTT
GAPDH	*Gapdh*	forward	AGAACATCATCCCTGCATCC
		reverse	CACATTGGGGGTAGGAACAC

Supplemental Figures



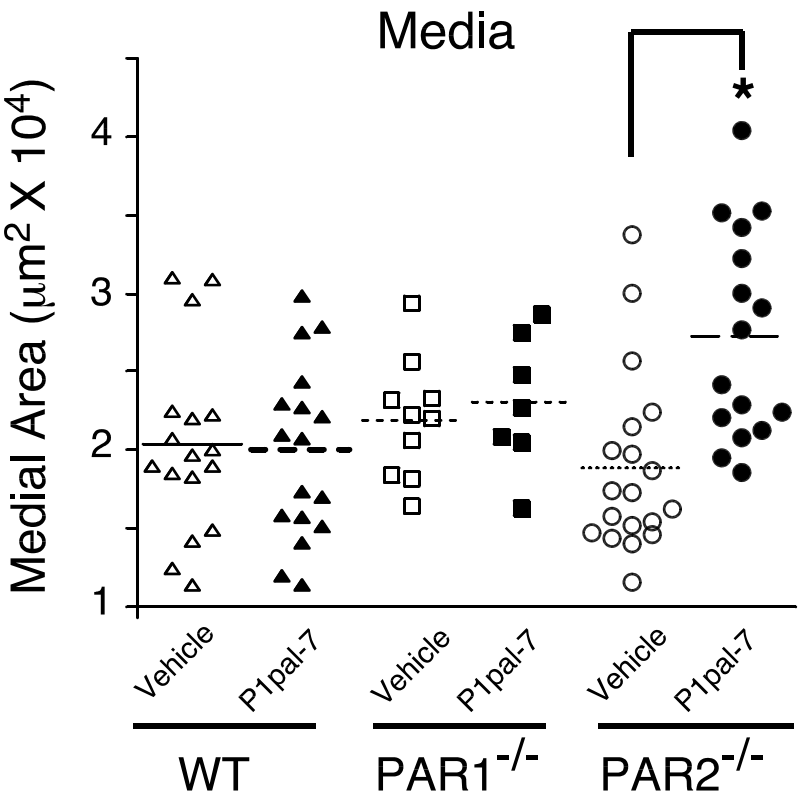
Supplemental Figure I. P1pal-13 is not an agonist of PAR4-dependent aggregation of mouse platelets. Mouse platelets in PRP were prepared from whole blood (0.5-1 ml per mouse) collected from the inferior vena cava into heparinized syringes and pooled from 5 WT C57BL6 mice in 0.1 U/ml apyrase and 10 U heparin. Platelet aggregation was measured by light scattering using a Chronolog 560VS/490-2D aggregometer. Platelets were pre-incubated for 5 min with 3 μM P1pal-13 or 0.2% DMSO vehicle (-) and then challenged with 160 μM AYPGKF to induce PAR4-dependent platelet aggregation. Error bars represent mean ± SD of 4 experiments. Essentially identical results were also obtained with 1 μM P1pal-13 and 10 μM P1pal-13 (data not shown).



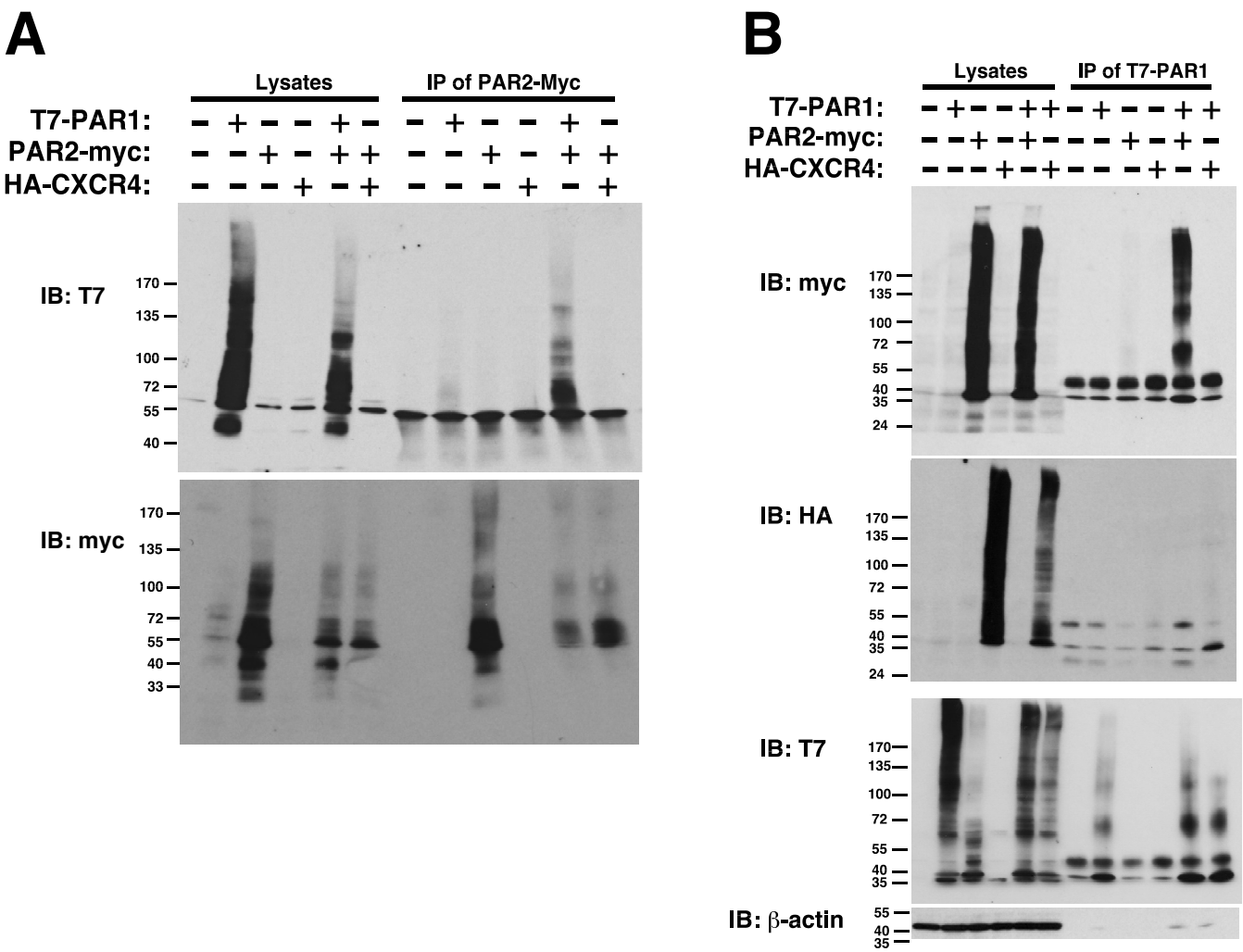
Supplemental Figure II. Time-course and histopathology of the carotid artery ligation injury and repair process.

Top rows-Ligated carotid arteries of wild-type C57BL/6 mice were removed and sectioned for histopathology (Hemotoxylin and Eosin stain, H&E) at the indicated time points. In the beginning stages of vascular injury, cytoplasmic swelling of SMCs (2 h-2 d) and increased infiltration of neutrophils into the intimal area is observed, with neutrophils, lymphocytes and macrophages appearing in the adventitial area (2-4 d). By 7 d, the lymphocytes and macrophages have increased dramatically, especially in the adventitia and occasionally in the medial layer. By 14-21 d, fewer inflammatory cells are observed with resolution of injury nearly complete.

Bottom row-Ki67 staining (brown) indicates proliferation of adventitial cells at day 4, medial cells at days 7-14 and intimal cells at day 14.



Supplemental Figure III. Effect of PAR1 antagonist pepducin, P1pal-7, on medial area after carotid artery ligation injury in mice. C57BL/6 wild-type, PAR1-/- and PAR2-/- mice underwent ligation injury of the left common carotid artery and were treated daily for 21 d with P1pal-7 (10 mg/kg) or vehicle (20% DMSO). The mean medial areas (horizontal lines) for each treatment cohort were calculated from cross sections of the arteries as described above in the Supplemental Material. (n=7-19 with each symbol representing an animal) *, P <0.05.



Supplemental Figure IV. PAR2-Myc Co-immunoprecipitates with T7-PAR1 but not HA-CXCR4. T7-Tagged PAR1, PAR2-Myc and HA-CXCR4 receptors were transiently expressed in COS7 cells as detailed above in the Supplemental Methods. A, T7-PAR1 immunoprecipitates with myc-Ab agarose beads when co-expressed with PAR2-Myc. B, Unlike PAR2-myc, HA-CXCR4 does not immunoprecipitate with co-expressed T7-PAR1 using T7-Ab agarose beads.