SUPPLEMENTAL MATERIAL

MATERIALS AND METHODS

Immunofluorescent Labeling of Human Coronary Arteries

Coronary artery tissue was obtained from autopsy material that was destined to be disposed according to university health system policy. Tissue was taken from autopsy patients where the autopsy consent specifically approved use of tissue for research purposes. Details on each patient are reported in **Supplemental Table I**. Left anterior descending coronary arteries were excised up to 1 cm past bifucation after prolonged fixation (1 week to 2 years). Arteries were fixed in 10% neutral buffered formalin, pH of 6.8 to 7.2. Tissue was then prepared for sectioning by dehydration, and was oriented for transverse sectioning in paraffin. Sections 5μm thick were cut and mounted onto slides. Samples were immunofluorescently labeled, as previously described.[1] Samples were incubated with primary antibody for VCAM-1 (BBA19, R&D Systems, 1:100 and secondary AlexaFluor546 donkey anti-goat IgG (Molecular Probes, 1:300), CXCR2 (ab24963, Abcam, 10μg/mL) and secondary AlexaFluor546 goat anti-mouse IgG (Molecular Probes, 1:300), or SMαA (Clone 1A4-Cy3, Sigma, 1:500). Imaging was performed on a confocal microscope (Nikon Eclipse Microscope TE2000-E2 and Melles Griot Argon Ion Laser System no. 35-IMA-840). Montages of cross-sections were reconstructed using Adobe Photoshop software.

Human Cell Isolation and Culture Plating Conditions

Primary human ECs were isolated from umbilical cord veins, expanded and used as previously described.[2] Human umbilical vein SMCs were purchased from Cell

Applications, Inc. at passage 2 and used until passage 10, as previously described.[2] All tissue procurement was approved by the Human Investigation Committees of the University of Virginia and Martha Jefferson Hospital (#10486).

EC/SMC co-culture plating conditions were used for all flow experiments and performed as previously described.[2] Briefly, porous transwell membranes are treated with 0.1% gelatin and SMCs are seeded onto an inverted well and grown to confluence for 48h in the transwell holding dish in reduced serum growth medium. ECs are then plated on the top surface of the membrane under the same media conditions for an additional 24 hours to ensure confluence. Hemodynamic flow patterns are derived from magnetic resonance imaging (MRI) of human common carotid artery and internal carotid sinus and applied to the EC surface of the dish to simulate atheroprotective and atheroprone shear stress patterns *in vitro*, respectively.[3] The two hemodynamic flow conditions were run in parallel for each EC/SMC subpopulation or identical flow conditions were used in parallel in the presence and absence of a treatment condition.

For monoculture SMC experiments, cells were plated in 12 well or 6 well plates at a density of 10,000 cells/cm^2 in low serum containing media (M199 supplemented with 2% FBS, 2mM L-glutamine, and 100 U/ml penicillin-streptomycin). Twenty-four hours later, cells were washed twice with PBS and then serum starved for 48 hours prior to treatment.

IL-8 Inhibition during Flow

Co-cultured EC/SMCs were incubated with IL-8 antisera (1:300) raised in rabbit, kindly donated by R.M. Strieter, M.D. (University of Virginia) or with normal rabbit

serum 10 minutes prior to the onset of atheroprone or atheroprotective flow. Fresh media containing the IL-8 antisera or vehicle was perfused into the system throughout the flow experiment for 24h, and cells were isolated for mRNA or protein analysis.

Additionally, siRNA for IL-8 (ON-TARGETplus SMARTpool, Thermo Scientific Dharmacon) was optimized in HUVEC (EC-siIL8) using Oligofectamine Reagent (Invitrogen) and Optimem Media. ECs were plated at 60,000 cells/cm^2 in normal media overnight. Then cells were rinsed twice with PBS and treated with ON-TARGET*plus*™ Control Pool (EC-CP, Thermo Scientific Dharmacon) or EC-siIL8 oligonucleotides, Oligofectamine, and Optimem for five hours, after which an equal volume of HUVEC media with 20% FBS was added to the dish. For flow experiments, ECs were plated in two 100mm dishes at 60,000 cells/cm^2 for one day, then transfected with either EC-CP or EC-siIL8 (185 pmol) for one day. Twenty-four hours before the onset of atheroprone flow, transfected ECs were trypsinized and replated onto the inner surface of the transwell membranes. After one day of co-culturing, transfected ECs were exposed to atheroprone flow in parallel. SMC and EC genes were analyzed for modulation of VCAM-1 and IL-8 mRNA.

Monocyte Adhesion Assay

Atheroprone and atheroprotective flow patterns were applied to EC/SMC co-culture dishes for 24 hours, after which calcein AM (Molecular Probes) labeled human MM6 monocytes donated by C. L. Hedrick, Ph.D. (University of Virginia) were seeded onto either the EC surface of the transwell dish or the SMC surface at 100,000 cells/cm^2 and allowed to adhere for 25 minutes at 37°C. Cells were then fixed in 1%

gluteraldehyde. For MM6 adhesion to SMCs, a barrier was placed around the exterior of the inverted transwell using parafilm to contain monocytes on the transwell surface. To determine specificity of binding to VCAM-1, a neutralizing antibody for VCAM-1 (R&D, BBA5) was incubated on ECs or SMCs for 45 minutes, prior to seeding labeled monocytes.

For each condition a minimum of three sections approximately 1cm^2 area were cut from the membrane and mounted with DAPI reagent for en face imaging via confocal microscopy. Each section was then imaged at least three times in different locations and an average number of monocytes per field of view (total of nine) were counted using a thresholding function via ImageJ (NIH) software.

IL-1β/IL8 Combinatorial Effects

Growth arrested SMCs were treated with human recombinant IL-1β (5ng/mL, Peprotech, 200-01B), IL-8 (10ng/mL, Peprotech, 200-08M) or the combination of both. A neutralizing antibody for CXCR2 (2ug/mL, Abcam, ab24963) was used to inhibit IL-8 signaling through this receptor. A p38 inhibitor SB202190 (Sigma, 5μM) was used to block p38 activation. To determine NF-κB activity, cells were infected with Ad-NF-κB-luc recporter construct (Vector Biolabs, 7.3 MOI) during serum starvation.

SMC Proliferation and Migration Assays

SMCs were growth arrested and then treated with 10% FBS, IL-1β, IL-8 or IL-1β/IL-8 for 24 hours. SMC proliferation was assayed via Click-iT™ EdU Flow Cytometry Kit (Invitrogen, C35002). Cells were pulsed with EdU (10μM)

simultaneously with treatment. Cells were then trypsinized, fixed and permeabilized, prior to detection of S-phase gated cells using flow cytometry. Alexa Fluor-488 azide was used for S-phase detection in conjunction with Alexa Fluor-633 for cell cycle detection. Negative controls with no EdU treatment plus dye and no EdU treatment without dye were also examined.

Migration of SMCs due to 10% FBS, IL-1β, IL-8 or IL-1β/IL-8 treatment for 24 hours was determined with a Boyden Chamber Assay. SMCs were initially plated on top of a porous polycarbonate transwell membrane (6.5mm diameter, 8.0μm pore diameter, Corning #3422) and allowed to adhere before potential chemo-attractant agents were placed in the bottom of the transwell. The cells were fixed and stained with 0.02% w/v Crystal Violet in 10% EtOH/90% diH2O to quantify cell chemotactic behavior. The bottoms of the transwells were imaged and intensity values per area were measured as an indicator for relative cell chemotaxis.

Real-time reverse transcriptase polymerase chain reaction

Total RNA is extracted using PureLink™ Micro-to-Midi™ Total RNA Purification System (Invitrogen, Cat#12183018) and reverse transcribed using the iScript cDNA Synthesis Kit (BioRad, Cat#170-8897). Previously designed primer sequences are used for the genes SMαA, myocardin, and VCAM-1.[2] The expression of mRNA was analyzed via real-time reverse transcriptase polymerase chain reaction (RT-PCR) using AmpliTaq Gold (Applied Biosystems), SYBR Green (Invitrogen) and an iCycler (BioRad). Real-time RT-PCR results are reported as relative quantity of mRNA and normalized to endogenously expressed gene β-2-microglobulin.

Western Blot

Cells were lysed in MAPK buffer (63.5mM Tris HCL pH 6.8, 2% w/v SDS, 10% glycerol, 50mM DTT, 0.01% bromophenol blue). Total protein was collected and lysates resolved on a 7.5% SDS-PAGE gel and blotted on a polyvinyl derivative (PVD) membrane. Primary antibodies [VCAM-1 (R&D systems, 1:500), SMαA (Sigma, 1:1000), and actin (Sigma, 1:1000)] were incubated with the blot for 1 hour at room temperature or overnight at 4°C. Horseradish peroxidase conjugated secondary antibodies [goat anti-rabbit, goat anti-mouse, donkey anti-goat (Santa Cruz, 1:5000)] were incubated with the blot for 1 hour at room temperature. An AlphaImager 8900 and AlphaEaseFC software were used for acquisition of blot image and densitometry analysis, respectively.

Interleukin-8 Secretion

Growth arrested SMCs were treated with IL-1β for 4, 24, or 48 hours. Cell culture supernatants were collected and frozen, until assayed as previously described.[2]

Data analysis and statistics

Real-time RT-PCR results for flow experiments were reported as the fold induction of cycle amplification times for paired experiments of treated flow samples compared with control flow samples and normalized to endogenously expressed gene β2-microglobulin. Student's t-test or ANOVA was conducted for all experiments. Data from at least three independent experiments per condition were used for analysis and evaluated at $p < 0.05$.

REFERENCES

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2. Hastings NE, Simmers MB, McDonald OG, Wamhoff BR, Blackman BR. Atherosclerosis-prone hemodynamics differentially regulates endothelial and smooth muscle cell phenotypes and promotes pro-inflammatory priming. *Am J Physiol Cell Physiol.* Dec 2007;293(6):C1824-1833.

3. Gelfand BD, Epstein FH, Blackman BR. Spatial and spectral heterogeneity of time-varying shear stress profiles in the carotid bifurcation by phase-contrast MRI. *J Magn Reson Imaging.* 2006 2006.

SUPPLEMENTAL FIGURES

Supplemental Figure I. VCAM-1 expression is heterogeneous, correlating with SMαA localization. Coronary artery cross-sections show SMαA (**A,B**) where white box highlights detail **(B)**. Images co-stained for VCAM-1 (blue, **C**), SMαA (red, **D**) indicate expression overlap localization. Patient A08.4. (L, lumen; I, intima, M, media). Bars: 50μm.

Supplemental Figure II. VCAM-1 and CXCR2 is present in intimal and medial layers of human coronary arteries. Cross-sections of human patients labeled for VCAM-1 or CXCR-2 in coronary arteries. **(Top, Left)** A montage shows a coronary cross-section stained for VCAM-1. **(A)** VCAM-1 staining was present in ECs and intimal/medial layers. **(B)** Within the same section, a region indicates stronger expression in ECs than SMCs. **(C, D)** Higher magnification of the image in (A) shows

distinct VCAM-1 staining of intimal cells (white arrows) and expression in the media. **(E)** VCAM-1 secondary only control indicates low non-specific staining. **(F)** CXCR-2 was present in ECs and intimal/medial layers. See supplement for data from additional donors. (EC, endothelial cell; I, intima; M, media. n=5). Bars: 50μm.

Supplemental Figure III. Human coronary artery expression of VCAM-1. Images show additional data from coronary arteries of different human patients, labeled for VCAM-1 expression to identify its localization to endothelium, intima, and media. See **Supplemental Table I** for specific patient information. Bars: 50μm (L,lumen; I, intima, M, media)

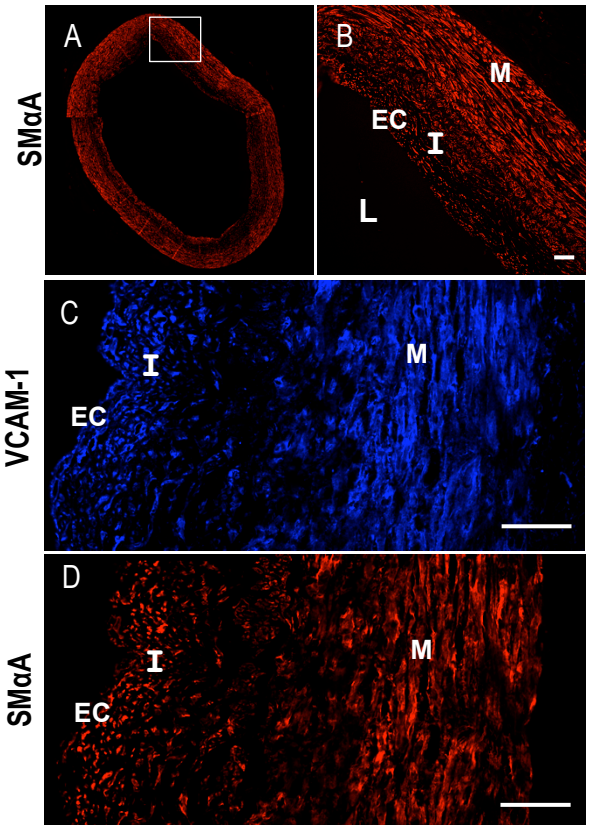
Supplemental Figure IV. Human coronary artery expression of SMαA.
Images show coronary arteries from different human patients, labeled for SMαA expression to highlight SMC presence in the intima and media. Autofluorescence of matrix components (green) show further detail of vessel composition. See **Supplemental Table I** for specific patient information. Bars: 50μm (L,lumen; I, intima, M, media)

Supplemental Figure V. SMCs treated with IL-1β (5ng/mL), IL-8 (10ng/mL) or IL-1β/IL-8 were examined for mRNA changes of SMαA and myocardin via real-time RT-PCR and was normalized to the housekeeping gene β-2-microglobulin. (Mean±SE, n=3, *$p<0.05$).

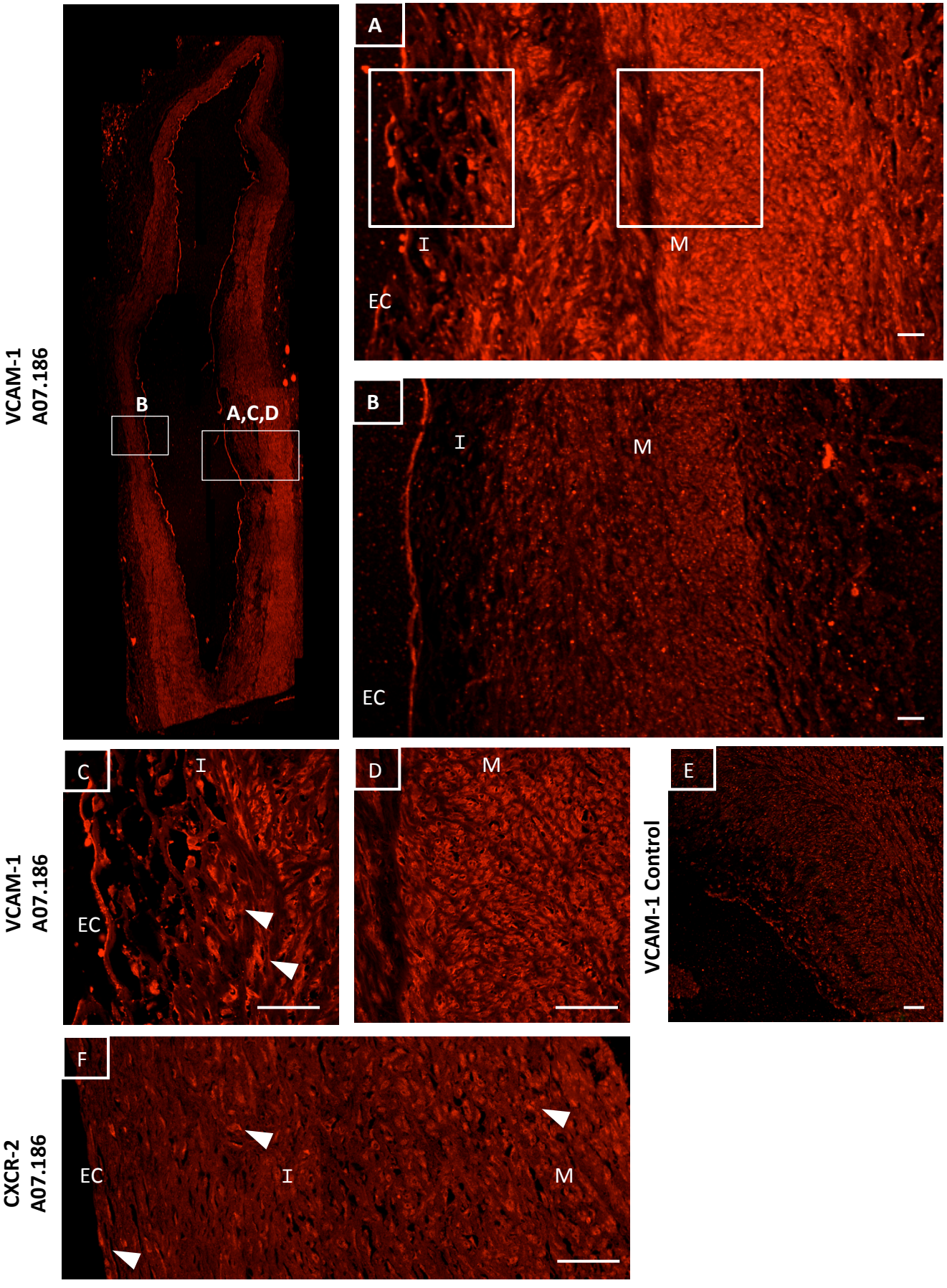
Supplemental Table I. Autopsy number, age, and cause of death of patients whose coronary artery samples were used in immunofluorescent labeling.

Autopsy No.		Age	Cause of Death
A04	151	55 yr	indeterminate
A05	106	11 mo	heterotaxy
A07	112	15 yr	enchephalocele, meningitis
A07	186	6 m	complex congential heart disease
A07	17.1H	73 yr	necrotizing pancreatitis
A07	25.2D	79 yr	pulmonary fibrosis
A07	28.4D	49 yr	ischemic heart disease
A07	37	29 yr	toxic shock syndrome
A08	4	41 yr	arrhythmia
A08	48	75 yr	arrhythmia

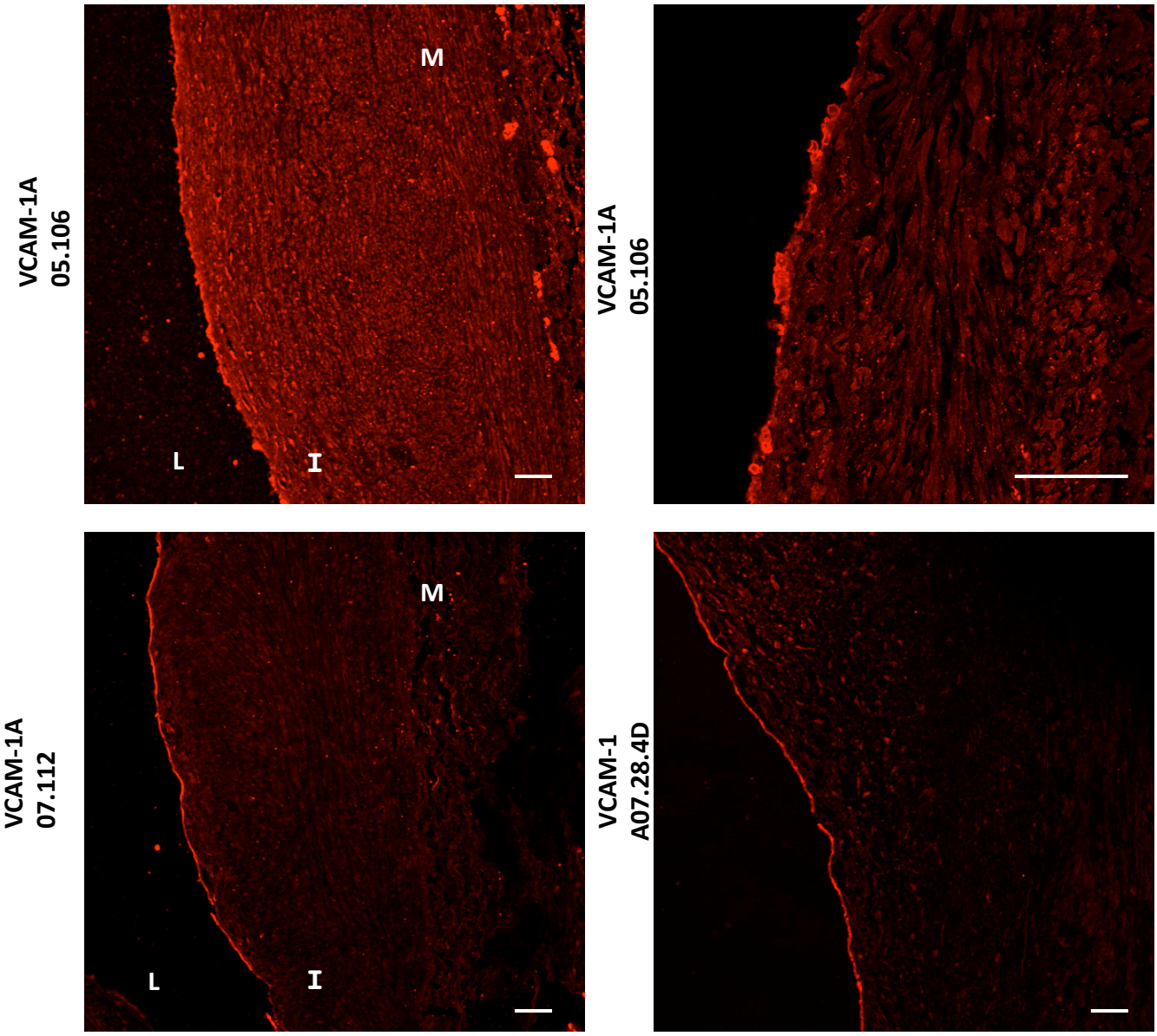
Supplemental Figure I.



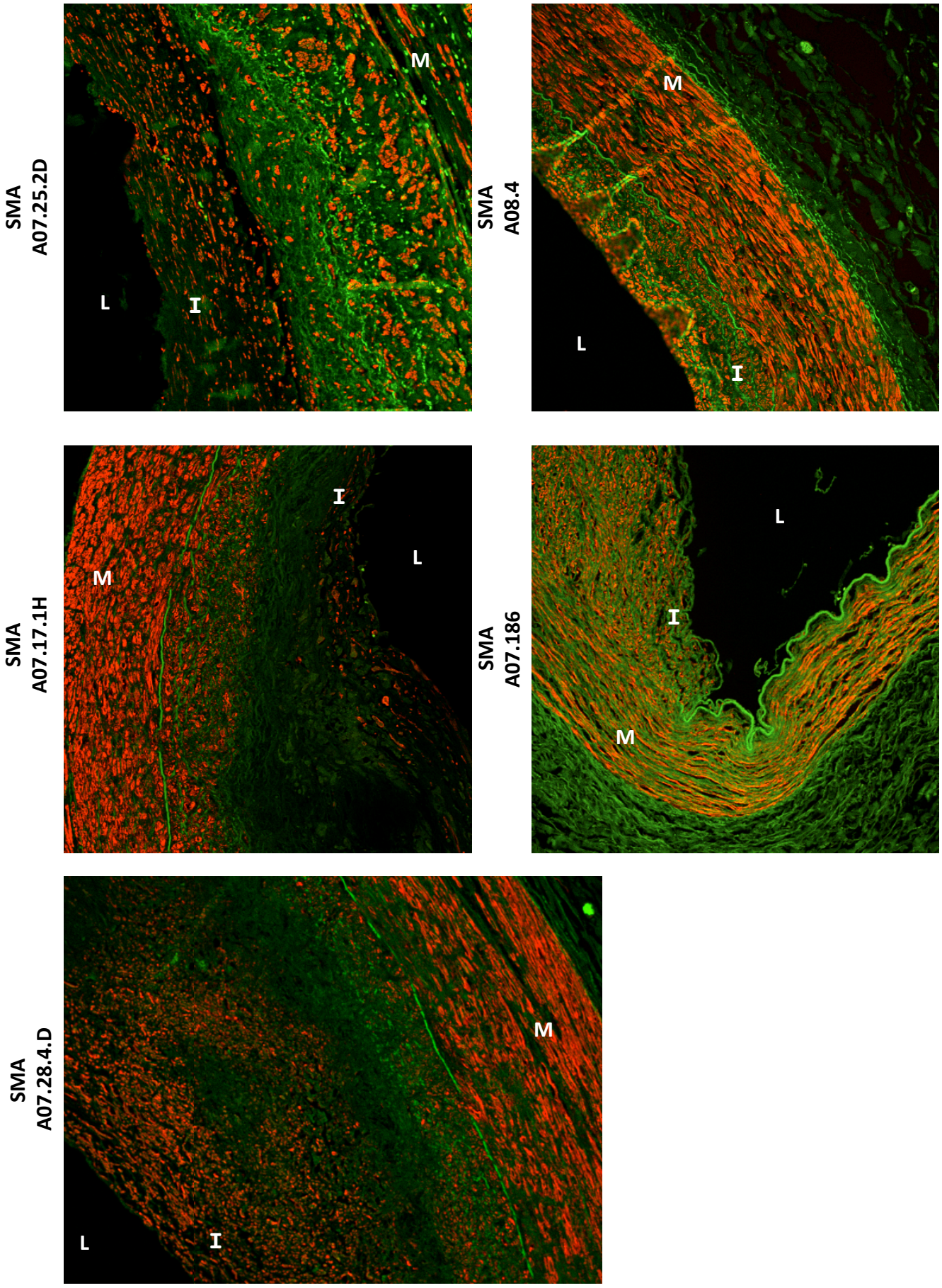
Supplemental Figure II.



Supplemental Figure III.



Supplemental Figure IV.



Supplemental Figure V.