SUPPLEMENT MATERIAL

Supplemental Methods

Mouse Models of AAA

The generation of PKCδ target deletion in mice was described elsewhere[1]. PKCδ knockout mice and their wildtype littermates were generated by mating heterozygous pairs. C57BL/6 mice and apoE$^{-/-}$ mice were purchased from Harlan Laboratories (Madison, WI) and Jackson Laboratory (Bar Harbor, ME), respectively.

Male mice, 12 weeks of age, underwent a $CaCl_2$- or elastase- induced abdominal aortic aneurysm model as described previously[2-5]. Briefly, animals were anesthetized using continuous flow of 1-2% Isofluorane. For the $CaCl_2$, the infrarenal region of the aorta was isolated and treated with 0.5M $CaCl_2$ perivascularly via gauze for 20 minutes. Control mice were similarly treated with 0.5M of sodium chloride (NaCl). For the elastase model, the infrarenal region of the aorta was isolated temporary silk ligatures were placed around proximal and distal portions of the aorta. An aortotomy was created near the bifurcation using a 30-gauge needle and heat-tapered polyethylene tubing (Baxter Healthcare Corp., Illinois, USA) was introduced through the aortotomy and secured with a silk tie. The aorta was filled with saline containing 0.295 U/mL Type I porcine pancreatic elastase (Sigma, St. Louis, MO) at a constant pressure of 100mm Hg. For control, the elastase solution was heat-inactivated (100ºC) before use.

In $CaCl_2$ and Elastase model surgical procedures, Buprenorphine was administered subcutaneously at a dose of 0.05mg/kg immediately after surgery. Subsequently, a 2.5% Xylocaine topical ointment was applied to the suture site. Additional doses of Buprenorphine were given via intraperitoneal injection every 8-12

hours after surgery for the first 48 hours. The maximum external diameter of the infrarenal aorta was measured using a digital caliper (VWR Scientific, Radnor, PA) prior to treatment and at the time of tissue harvest.

For the Angiotensin II model, male, 24-week-old, apoE-deficient mice with a C57BL/6 background were implanted with a Alzet osmotic minipump (model 2004; Alzet, CA) delivering Angiotensin II (1000ng/kg/min) or saline subcutaneously for up to 4 weeks. The external aortic diameter was measured at the region showing maximum dilatation with a digital caliper (VWR, PA).

At selective time points, mice were killed by an overdose of isoflourane and tissues were perfusion-fixed with 4% formaldehyde in phospho-buffered saline (PBS). Tissues meant for immunohistochemical analyses were imbedded in O.C.T. Compound (Sakura Tissue Tek, Netherlands), and tissues meant for morphological analyses were processed for paraffin embedding. All frozen sections were cut to 6μm thick using a Leica CM3050S cryostat and paraffin sections were cut to 8μm thick using a Reichert-Jung 2050 SuperCut Microtome. All experiments were conducted in accordance with experimental protocols that were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin-Madison (Protocol M02284).

	C57B6	PKCd WT	PKCd KO	PKCd HZ	GFP-/-	GFP+/+	ApoE-/-
PKCd and TUNEL timecourse and co-localization	12						
Fresh tissue western blot	8						
PKCd WT vs. KO Aneurysm		18	11				
Fresh tissue RNA extraction		6	6				
Adenovirus PKCd rescue in KO animals			14				
PKCd Dominant Negative	14						
Pluronic Gel MCP-1 Delivery		7	14				
Establish CaCl2 Model	18						
Elastase Model in WT/KO		8	4				
Angiotensin Upregulates PKCd in ApoE-/- mice							9
CBC table		3	3				
Bone Marrow Harvest and Monocyte Count		4	4				
Adenoviral-mediated Gene Delivery Test	4		4		2	2	
Breeding Pairs				40			
TOTALS	**56**	**46**	**60**	**80**	**2**	**2**	**9**

Immunohistochemistry

Van Geison stains were carried out using Chromaview Van Gieson kit (Richard Allan Scientific, Kalamazoo, MI) according to provided protocol. Elastin integrity was evaluated using a semi-quantitative methodology described previously: (1, no elastin degradation or mild elastin degradation; 2, moderate; 3, moderate to severe; and 4, severe elastin degradation)[6, 7]. Representative images for each grading score are provided in Supplemental Figure X. Each section was numbered and photographed at 10x and 20x magnification, maintaining their respective numbers. Then, an objective participant graded the photographs according to the aforementioned scale and recorded the grade with the section number.

Arterial sections were permeabilized with 0.1% TritonX for 10 minutes at room temperature. Non-specific sites were blocked using 5% bovine serum albumin (BSA), 3% normal donkey serum in Tris-buffered Saline and Tween 20 (TBS-T) for 1 hour at room temperature. Primary antibodies to CD3, MCP-1, and CD68 were purchased from Santa Cruz (Santa Cruz, CA), IFN-γ, IL-6, MOMA2, CD45, and anti-smooth muscle specific Myosin heavy chain 11 (MHC) antibodies from Abcam (Cambridge, MA), and Cleaved Caspase 3 from Cell Signaling (Danvers, MA). All antibodies were diluted in previously described blocking solution and incubated overnight at 4°C. Fluorescent stains were completed using secondary antibodies purchased from Invitrogen Molecular Probes (Carlsbad, CA) and 4'6-diamidino-2-phenyl-indole, dihydrochloride (DAPI, Invitrogen, CA) was used to detect nuclei. Control images, included in Supplemental Figure 11A and B, show primary and secondary only antibody stains, respectively. Conventional stains were developed using secondary antibodies purchased from Bio Rad (Hercules, CA) with hematoxylin counter-stain. TUNEL staining kit was purchased from Roche (Madison, WI) and carried out according to the provided protocol. Staining was visualized with a Nikon Eclipse E800 upright microscope or Nikon A1RSi Confocal system, and digital images were acquired using a RetigaEXi CCD digital camera.

Microscope exposure settings were held constant for all images taken amongst experimental group sets. For example, the representative images shown in Figure 5C were all taken with ISO Sensitivity ISO400, with exposure time for MOMA (FITC-HYQ) at 1/7s, TUNEL (G-1A) at 1/3s, and DAPI (UV) at 1/180s. Similarly, representative values for confocal images shown in Figure 1C were Gain 5.95B and Offset 127 for green (PKCδ) and Gain 6.65B and Offset 127 for red (TUNEL).

Quantification of stains was performed in a manner to that previously described [8] using Image J Software as provided by the National Institutes of Health. Data quantification was performed using at least 3 sections per artery.

Fresh Tissue Western Blot

Aorta was harvested from NaCl or $CaCl_2$-treated WT and KO mice and the tissue was flash-frozen in liquid nitrogen to be processed at a later time. Aortic tissue was ground to a fine powder in a small amount of liquid nitrogen, and protein was extracted from the powder using radioimmunoprecipitation assay (RIPA) buffer consisting of 50mM TRIZMA HCl, 150mM NaCl, 1% Nonidet-P40, 0.5% sodium dioxycholate, 0.1% SDS (all reagents from Sigma-Aldrich, St. Louis, MO). Equal amounts of protein extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. The membranes were incubated with rabbit polyclonal antibodies to Protein Kinase Cδ, C-17 fragment from Santa Cruz (Santa Cruz, CA) and mouse monoclonal antibodies to β-actin (Sigma, MO) followed by horseradish peroxidase labeled goat anti-rabbit or anti-mouse IgG (Bio-Rad, Hercules, CA). Labeled proteins were visualized with an enhanced chemiluminescence system (Perkin-Elmer, Boston, MA).

Cell Culture

The murine macrophage cell line RAW264.7 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). Primary mouse aortic SMCs from the thoracic aorta of both PKCδ KO and WT mice were isolated based on a protocol

described by Clowes et al [9]. Briefly, aortas were perfused with phospho-buffered saline supplemented with 2% penicillin/streptavidin antibiotics. The aorta was isolated from the aortic arch to the iliac bifurcation and incubated 30 minutes in digestion buffer (DMEM, Bovine serum albumin, Collagenase, Soybean trypsin inhibitor, and Elastase Type III) at 37°C. Adventitia was pulled away from the medial layer, tissues were minced, and further digested for 4 hours at 37°C. Tissue was spun to a pellet by centrifugation and washed with 10%FBS DMEM once, before suspension in a small volume of 10%FBS-DMEM and left undisturbed for 48 hours to allow cells to migrate from tissue. All cell types were maintained in DMEM supplemented with 10% fetal calf serum (FCS), 100 units/mL penicillin, and 100μg/mL streptomycin in a 5% CO_2/water-saturated incubator at 37°C.

MCP-1 ELISA

The BD OptEIA ELISA kit was obtained from BD Biosciences (San Jose, CA), and carried out according to the manufacturer's protocol.

Real-Time PCR Analysis

Total RNA was isolated from cultured cells using Trizol reagent (Invitrogen, CA) according to manufacturer's protocol. For fresh tissue, aortic tissue was ground to a fine powder in a small amount of liquid nitrogen, and RNA was isolated from the powder using the RNeasy Plus Mini Kit (Qiagen, CA).

cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA) on a Veriti 96-well Thermal Cycler (Applied Biosystems, CA). Primers

were purchased from Invitrogen and amplification was detected using SYBR Green PCR Master Mix (Applied Biosystems, CA). Real-time PCR was carried out using a 7500 Fast Real-time PCR System Machine (Applied Biosystems, CA). RQ value, where RQ= $(E_{target}^{\Delta CP_{target}(control-sample)})/ (E_{reference}^{\Delta CP_{ref}(control-sample)})$, the reference gene was GAPDH, and CP is defined as a 'crossing point', was used to compare expression of target cytokines.

Adenovirus Infection

The adenoviral vector that expresses wildtype PKCδ was created as described previously [10]. A recombinant adenoviral vector containing the dominant negative PKCδ (AdPKCδDN) [11] was obtained from Dr. T. Biden (Garvan Institute, Australia). *In vitro* adenovirus infection was carried out as described previously [12]. For perivascular adenovirus delivery, a small piece of latex was inserted underneath the aorta to create a 'cup' cradling the infrarenal aorta. After removal of the $CaCl_2$ –containing gauze from the artery, adenovirus suspended in saline ($2.5x10^9$ particle forming units (PFU)/mL) was added to the latex cup to bathe the artery for 20 minutes. Precautions were taken to avoid direct contact of viral solution with surrounding tissues.

Migration Assay

In vitro migration assay was carried out as previously described [13]. RAW 264.7 macrophages, or $CD11b^+$ cells isolated from bone marrow harvested from PKCδ KO or WT mice, were placed in a 5μm pore transwell insert (Corning Inc, Wilkes Barre, PA) to a density of 500,000cells/mL. Media conditioned by TNFα (R&D Systems, Minneapolis,

MN) stimulated aortic SMCs, or recombinant MCP-1 protein (R&D Systems, Minneapolis, MN) were used as chemoattractants. Following 6 hour incubation at 37°C, inserts were removed and washed with PBS, fixed with ice cold 70% Ethanol and stained with hematoxylin for nuclei visualization. The mean value of migrated cells counted in eight high-power fields per membrane was used as a measurement of migration.

Bone Marrow Isolation and Sorting

Bone marrow cells from both PKCδ KO and WT animals were isolated from long bones, washed with PBS, and counted. Monocytes were isolated from bone marrow by magnetic sorting using anti-CD11b microbeads (Miltenyi Biotec, Auburn CA). Purity of the resulting CD11b+ cells was assessed by flow cytometry using antibodies to CD3 (FITC), CD11b (APC), and CD45/B220 (APC-Cy7) (BD Pharmigen, San Diego, CA). Flow cytometric data was collected on a BD FACS Calibur Flow Cytometer equipped with a Cytek 633nm laser (Freemont, CA) and analysis was performed using Flow Jo software (TreeStar, Inc.).

Migration Assay

Chemotaxis assay was performed as described previously (cite). 2x10^5 macrophages (RAW264.7) were placed in the upper chamber of Costar 24-well transwell plates with 5um pore filters (Corning, Inc., Corning, NY). Cultured conditioned medium or control media was placed into the lower chambers or wells. Anti-MCP-1 antibody (Biolegend, CA) was used for neutralization of MCP-1. After incubating plates for 6h at 37C,

migrated cells were collected from the lower chambers and on the bottom of the filters were counted.

Statistical analysis

Values were expressed as mean ± standard error. Experiments were repeated at least three times unless stated otherwise. Differences between 2 groups were analyzed by Student's *t* test. For time course comparison, one-way ANOVA analysis was followed by Bonferroni correction to adjust for multiple comparisons. Values of $p<0.05$ were considered significant.

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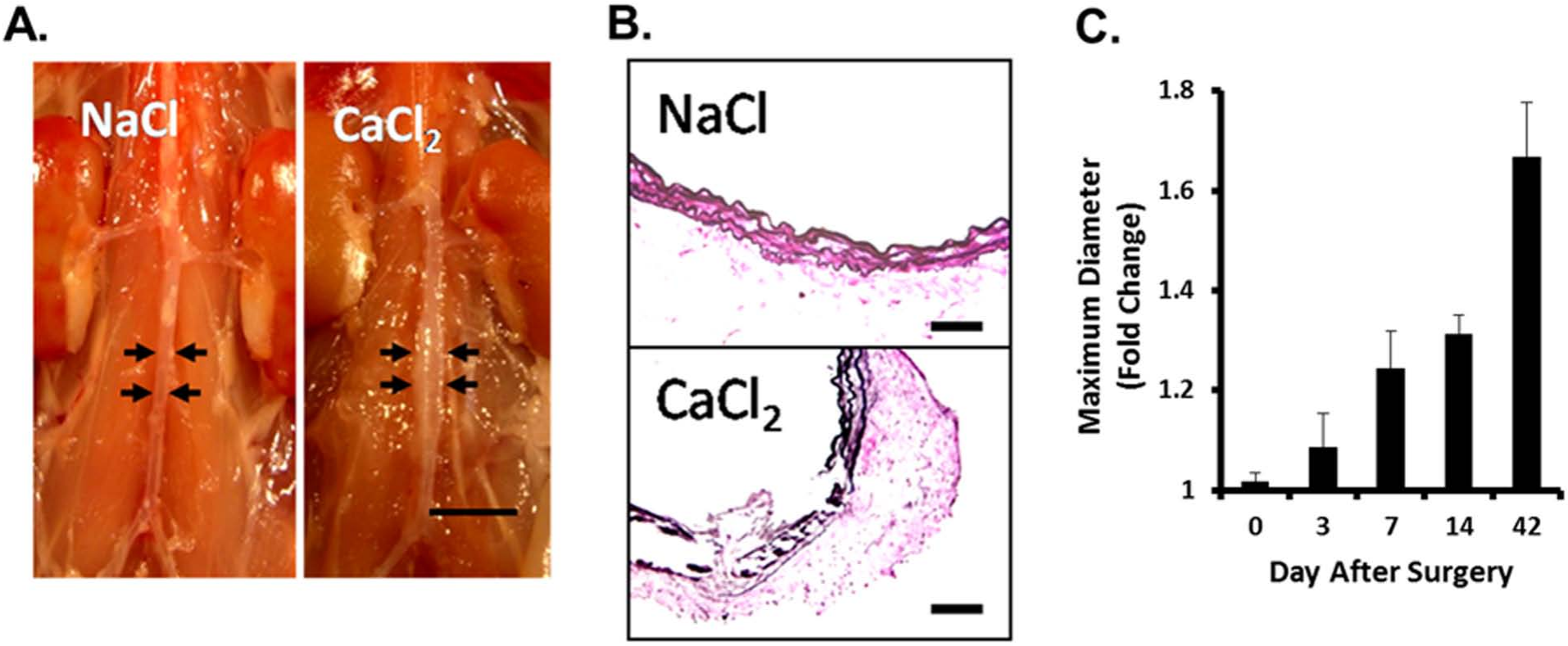
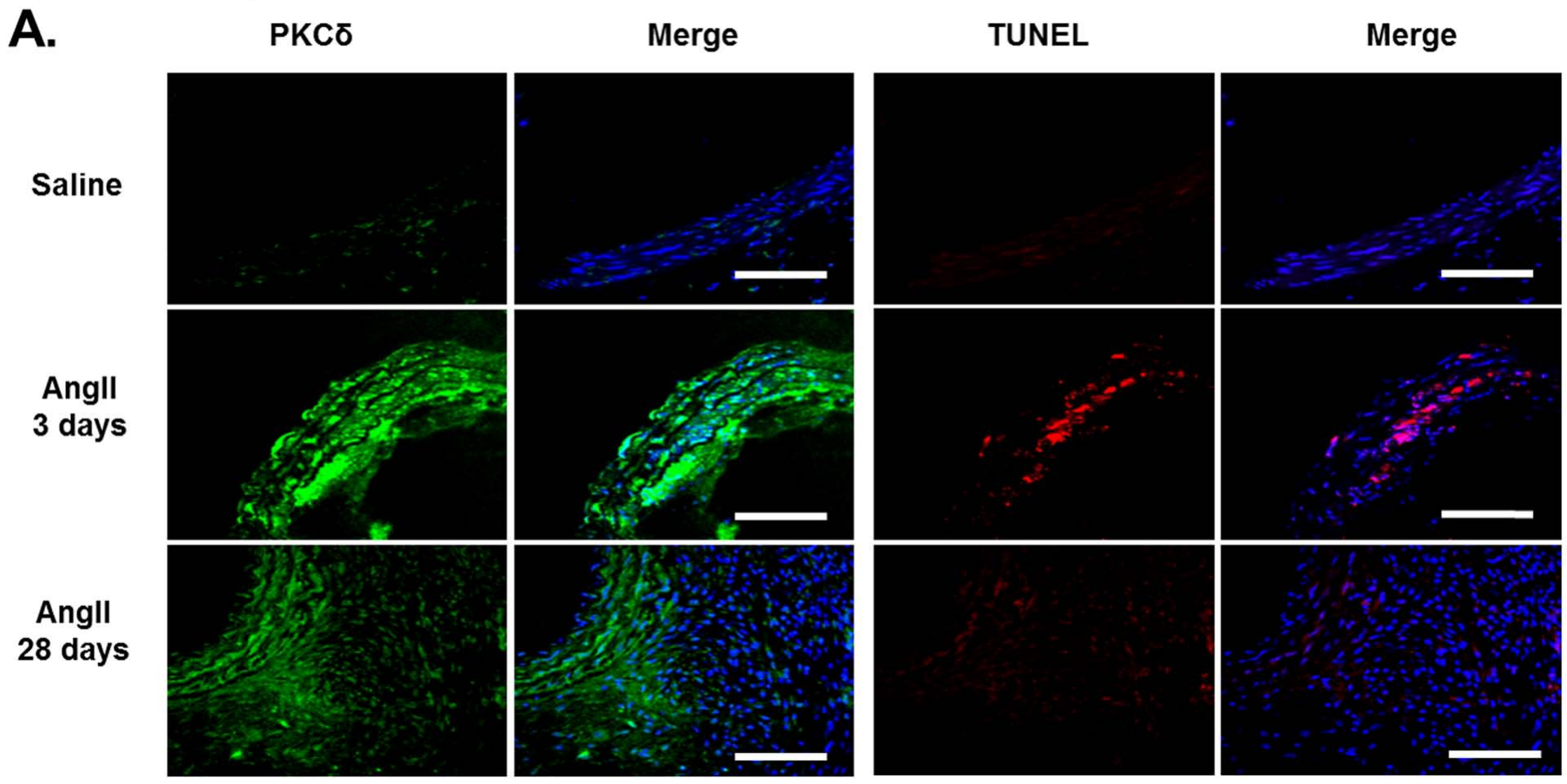
Supplemental Table I

	WT	KO
RBC (x10^6/µl)	7.78 ±0.56	8.49 ±0.34
WBC (x10^3/µl)	7.01 ±2.75	6.98 ±3.21

Supplemental Figure I



Supplemental Figure II



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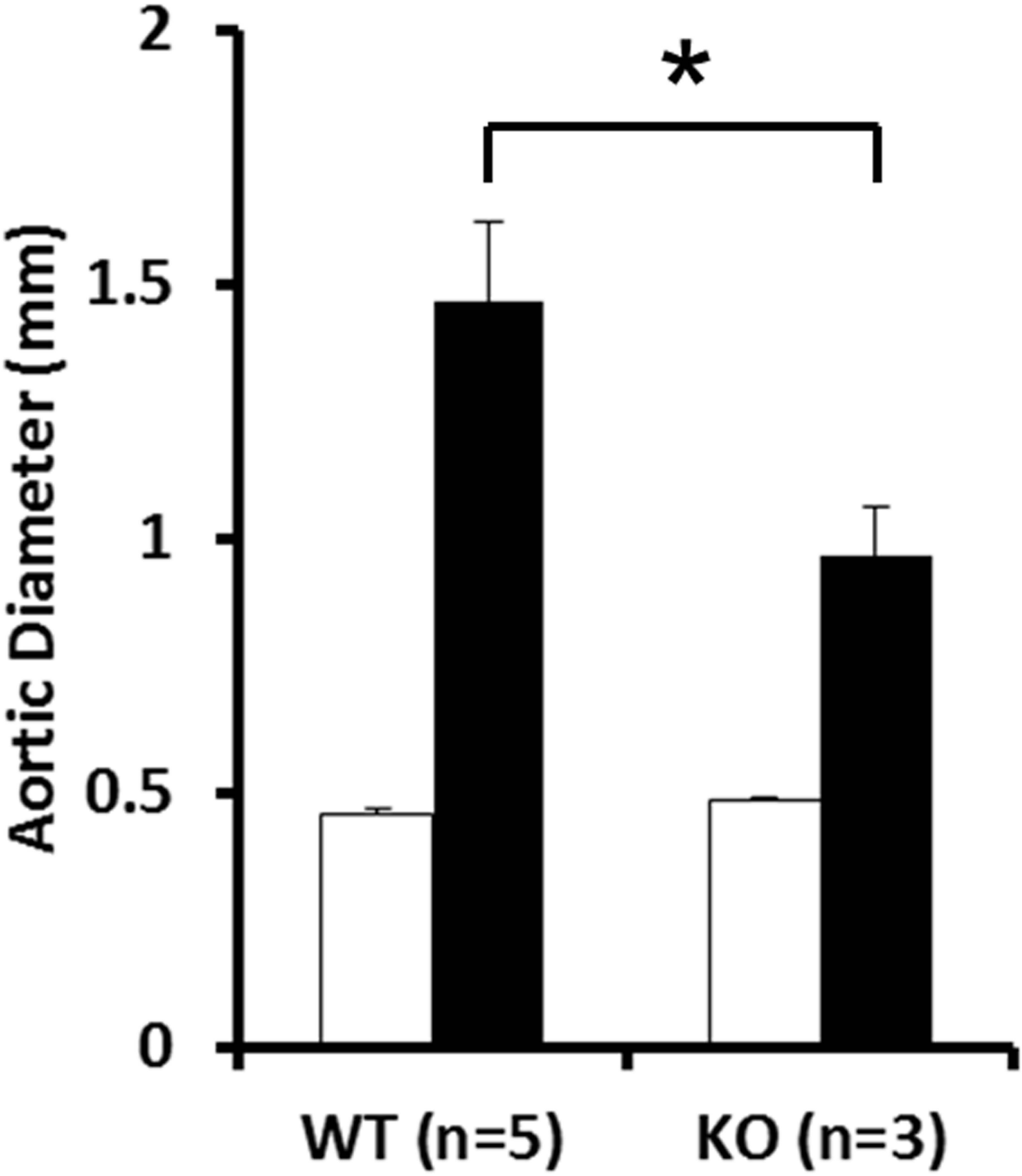
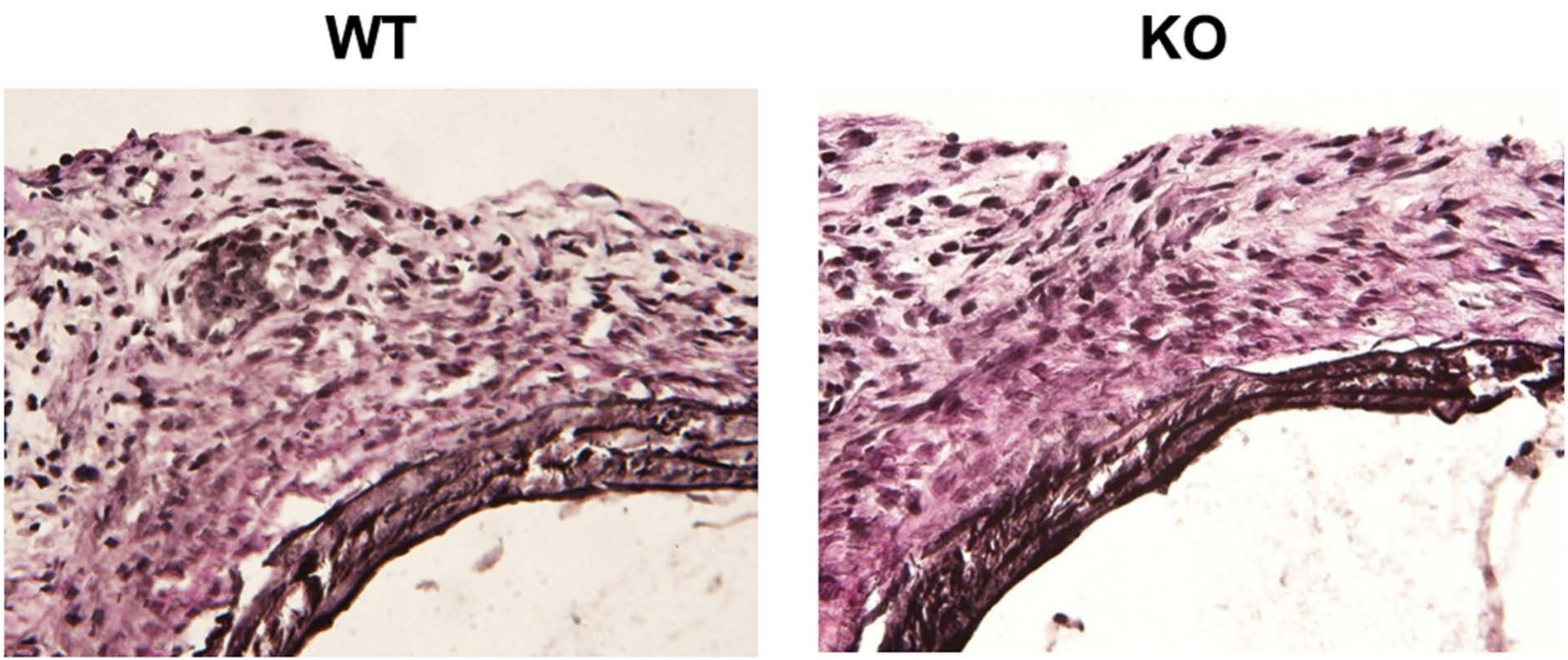


Supplemental Figure IV



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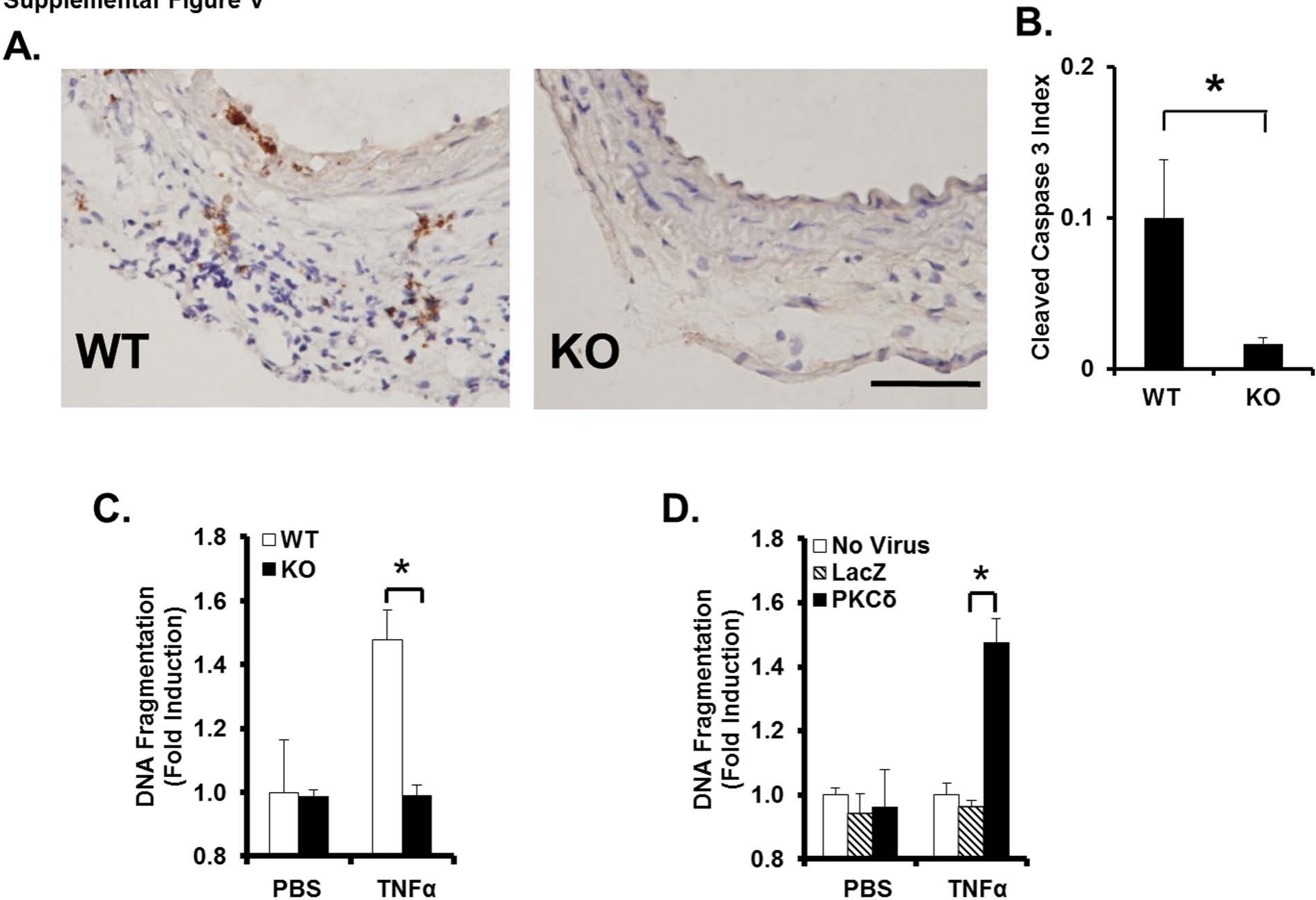
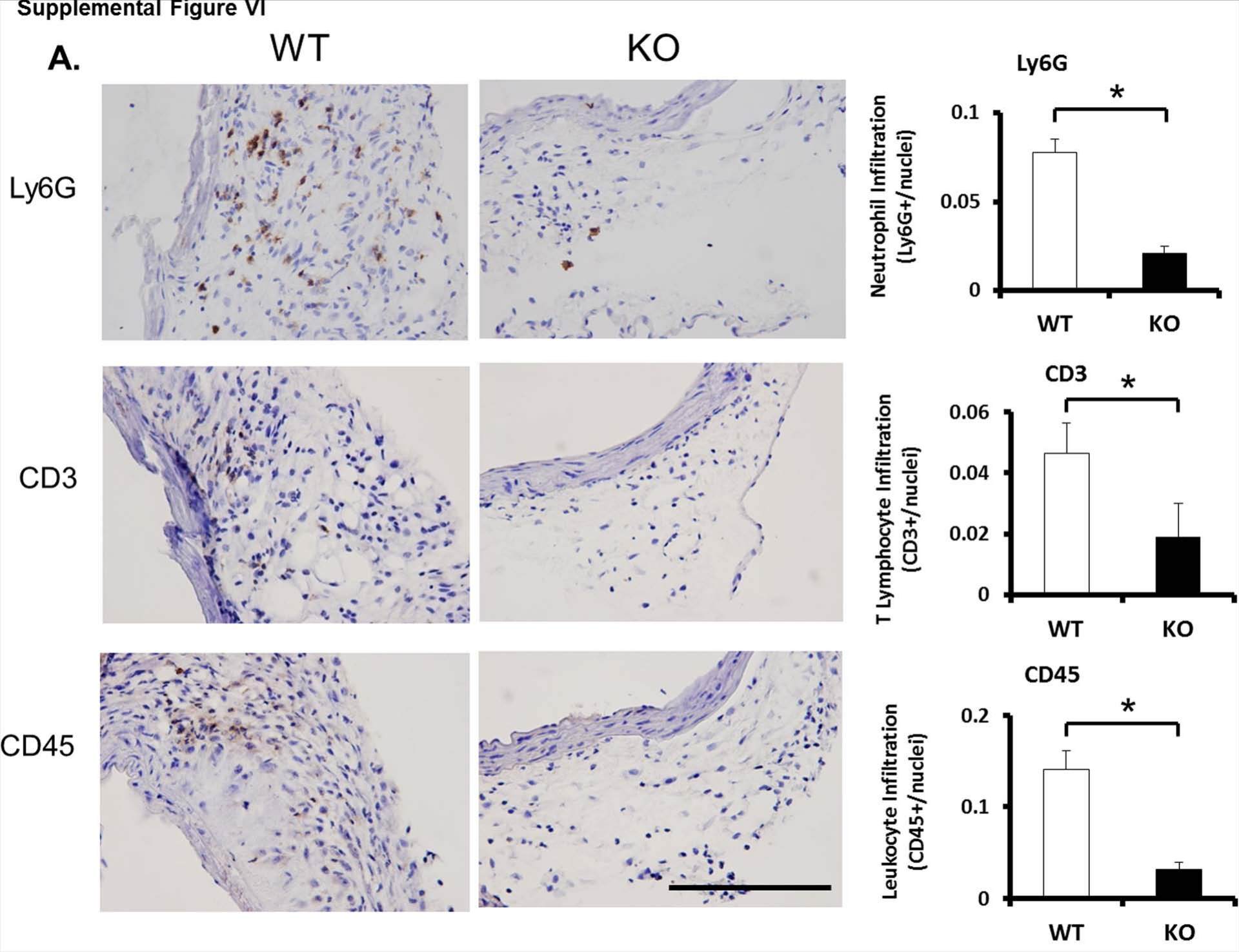
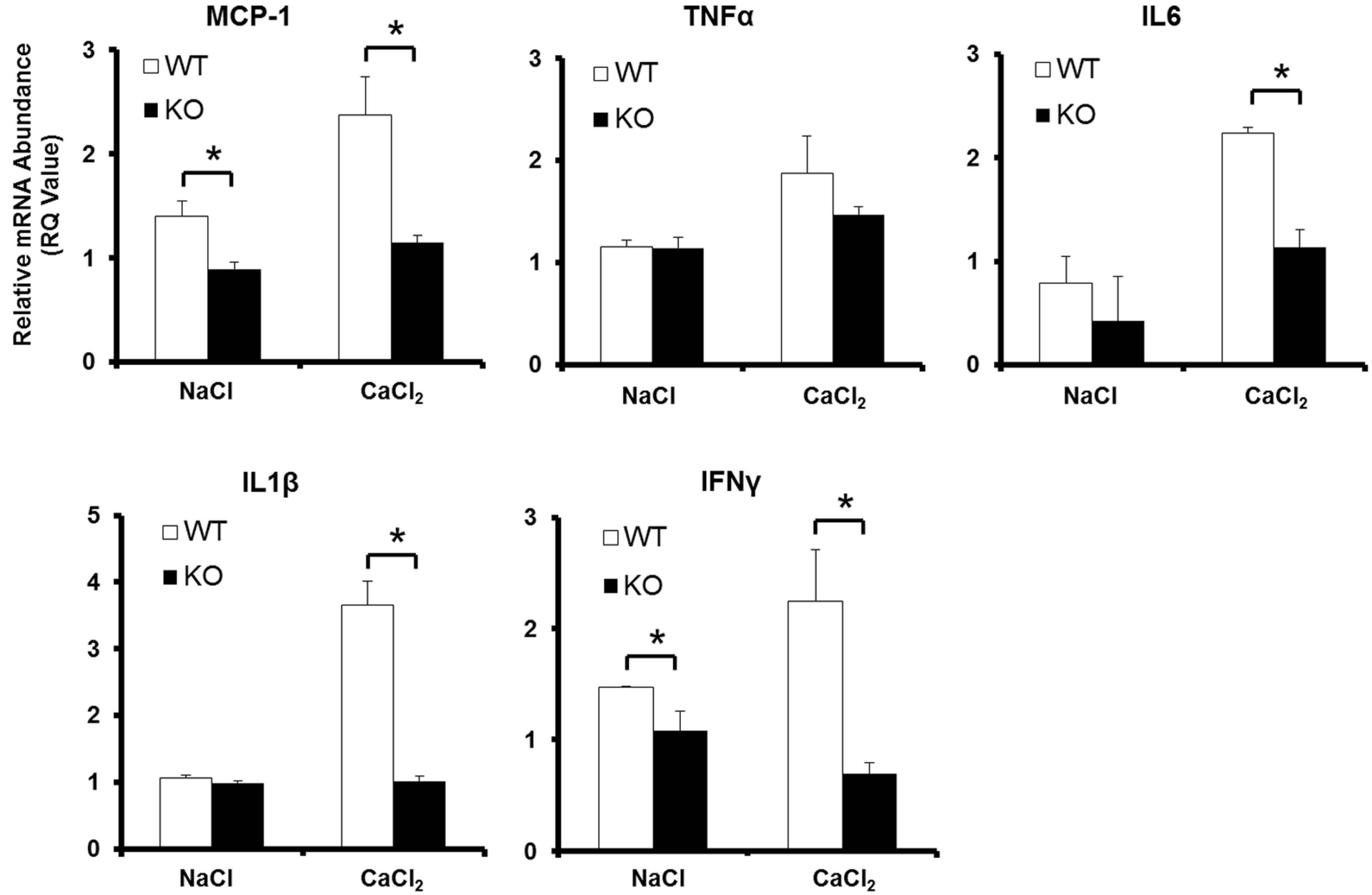


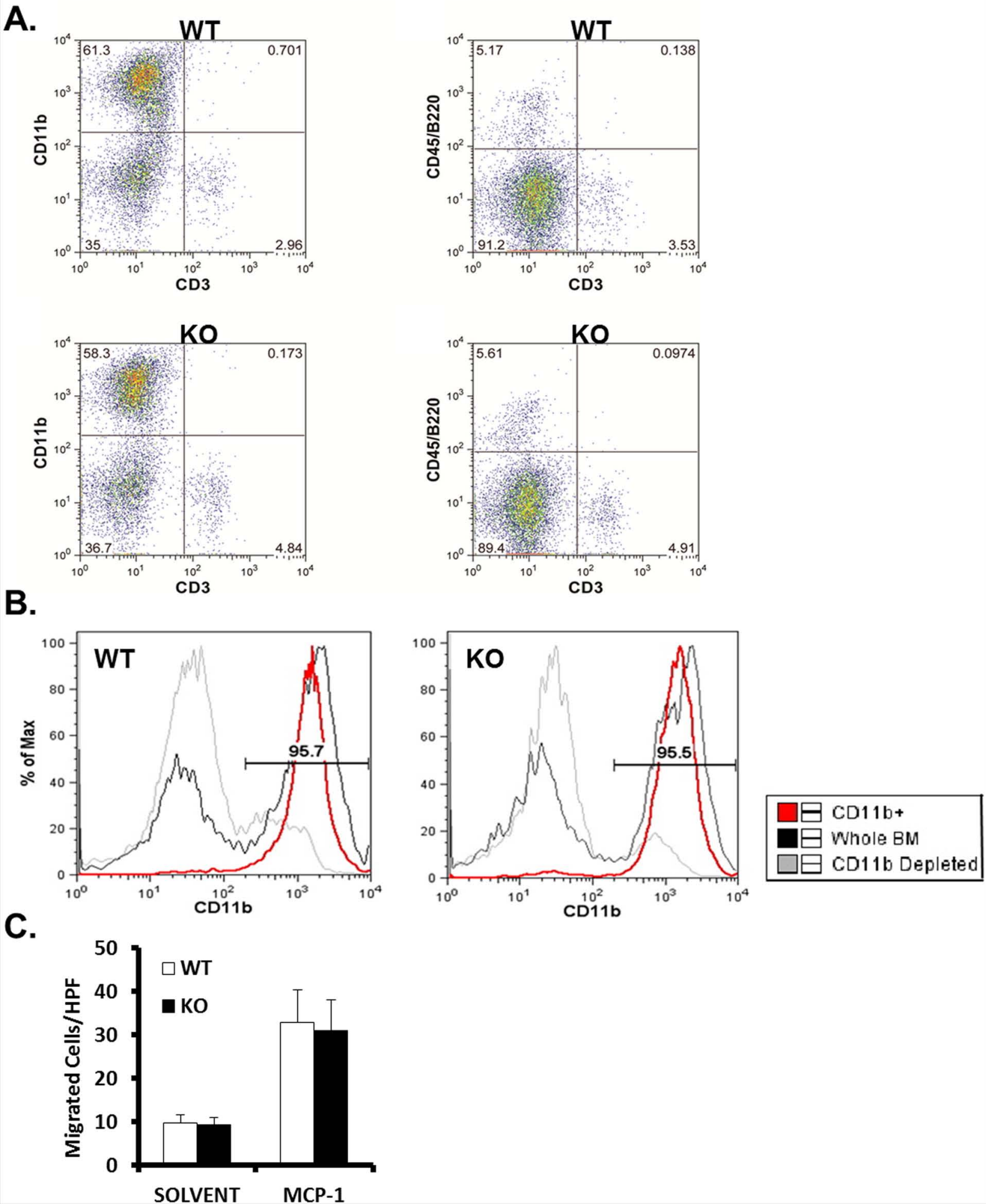
Supplemental Figure VI



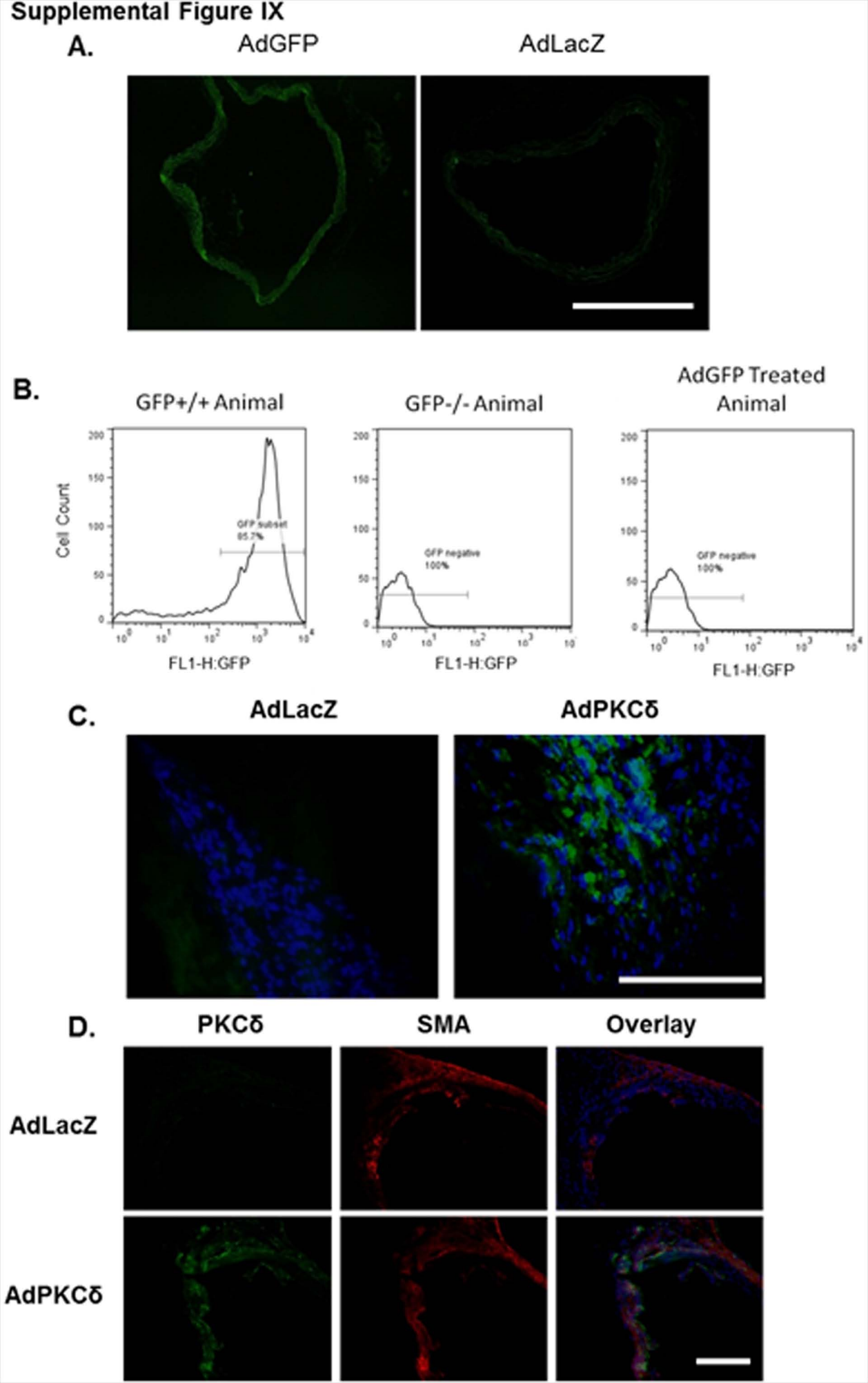
Supplemental Figure VII



Supplemental Figure VIII



Supplemental Figure IX



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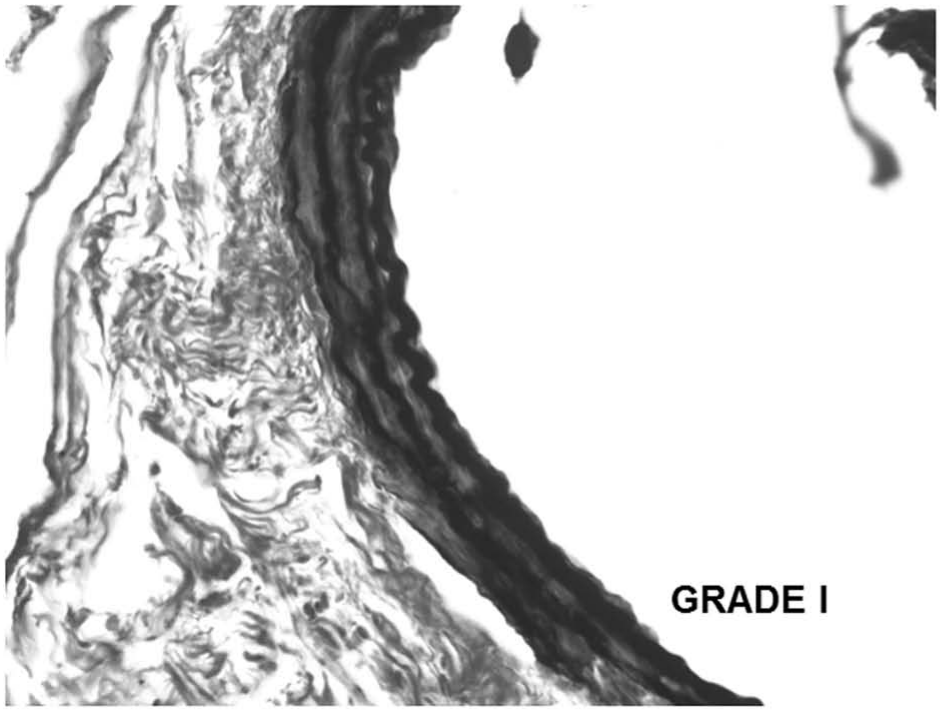
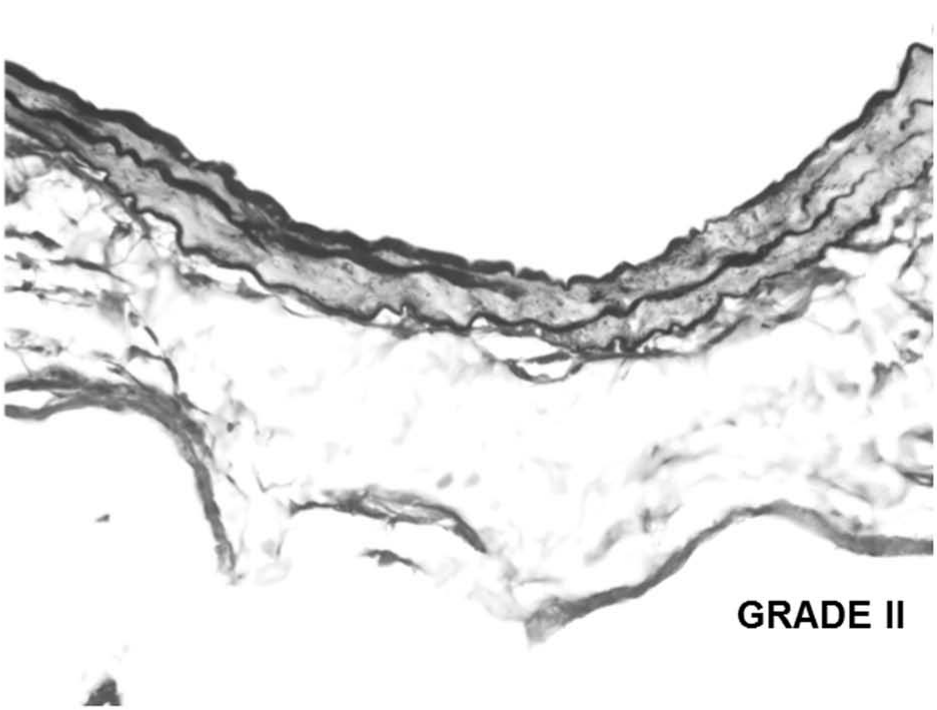
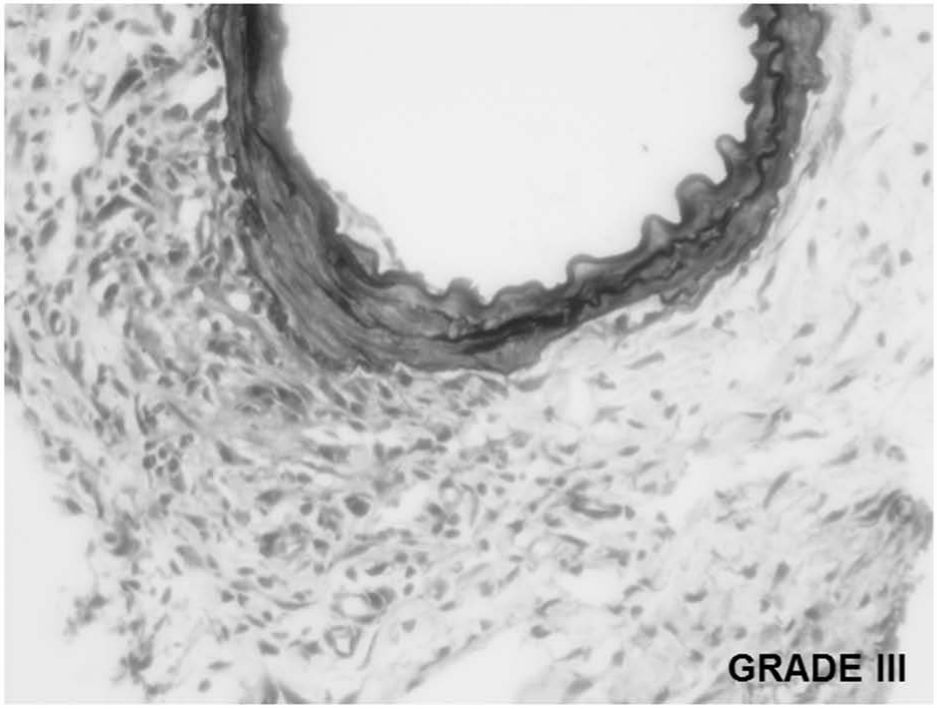
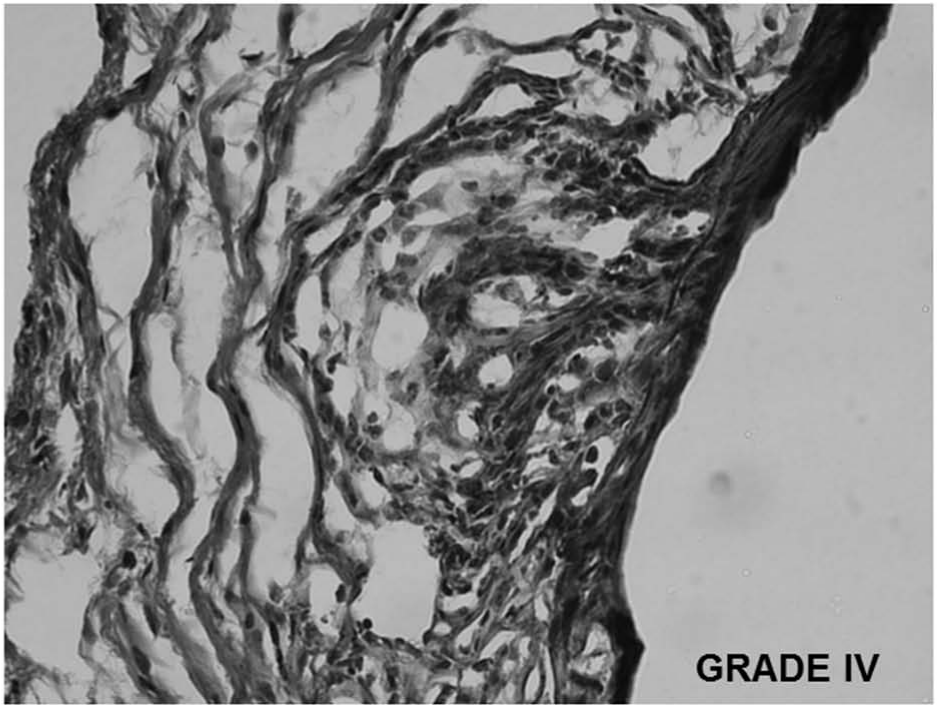
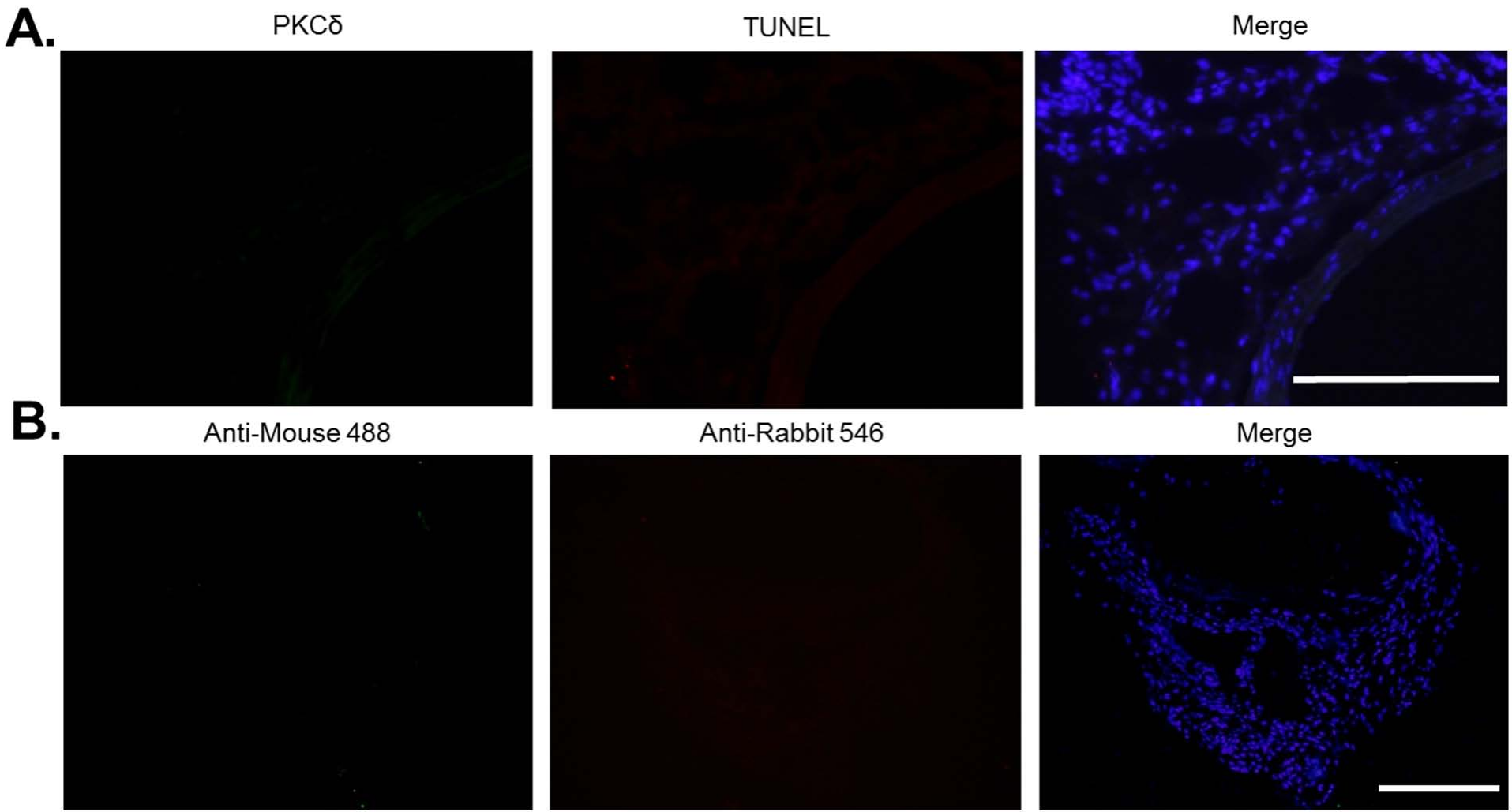








Supplemental Figure XI



Supplementary Table I. Complete Blood Count (CBC) results of PKCδ WT and KO mice. Red blood cells (RBC) and white blood cells (WBC) are shown; n=4.

Supplementary Figure I. Perivascular application of $CaCl_2$ induces progressive dilatation of abdominal aorta. (A) Representative photos of abdominal aortas of C57B/6 mice treated with 0.5M $CaCl_2$ or equal concentration of NaCl, taken 42 days after surgery. (B) Van Gieson stain to identify elastin continuity in aneurysmal ($CaCl_2$) and control (NaCl) aortas. Scale Bar=50μm. (C) Aortic expansion over the time course of 42 days measured by fold change of maximum diameters as calculated by dividing aortic diameter at time of sacrifice by aortic diameter prior to treatment.

Supplementary Figure II. PKCδ expression correlates with apoptosis in an experimental aneurysm model. (A) Infusion of AngII in $ApoE^{-/-}$ animals leads to induction of both PKCδ (green) and TUNEL (red) in the aortic wall, overlay with DAPI (blue).

Supplemental Figure III. PKCδ expression moderates aneurysmal expansion in an Elastase perfusion model of murine AAA. Aortic diameter measured prior to (Pre, white bars), and 14 days after (Post, black bars), Elastase perfusion treatment.

Supplemental Figure IV. $CaCl_2$ treatment causes similar early elastin damage in both PKCδ WT and KO aortas. Representative Van Gieson stains of arterial sections harvested 7 days after surgery.

Supplemental Figure V. The lack of PKCδ causes an apoptosis-resistant phenotype in vivo and in vitro. (A) Representative photos of immunohistochemistry for cleaved caspase-3 at 7 days, scale bar=100μm. (B) Cleaved caspase 3 index. *p<0.05, n=6. (C) PKCδ KO SMCs were resistant to apoptosis induced by TNFα (50ng/mL, for 6h). (D) Adenovirus-mediated expression

of exogenous PKCδ in PKCδ KO SMCs rescued the apoptotic phenotype. *p<0.05 compared to AdLacZ, n=3.

Supplemental Figure VI. PKCδ gene deficiency has a broad inhibitory effect on inflammatory infiltration. (A) IHC for neutrophils (Ly6G), T lymphocytes (CD3), and leukocytes (CD45) at 7 days; Scale Bar=200μm. Quantification of immunohistochemistry stains expressed as ratio of positive cells divided by nuclei. *p<0.05, n=6.

Supplementary Figure VII. PKCδ mediates expression of cytokines and chemokines in aortas. Total RNA was isolated from aortas of PKCδ WT or KO mice 7 days after surgery. mRNA levels of selected cytokines and chemokines were determined by RT-PCR. , *p<0.05, n=4.

Supplementary Figure VIII. PKCδ gene deficiency does not significantly affect the number or function of monocytes. (A) Bone marrow cells isolated from PKCδ WT or KO mice were stained with CD11b (monocytes), B220 APC-CY7 (B lymphocytes), and CD3 (T lymphocytes) for analysis by flow cytometry. (B) Purified monocyte population derived from whole bone marrow using $CD11b^+$ magnetic bead sorting technique compared to whole bone marrow and CD11b-depleted bone marrow. (C) $CD11b^+$ monocytes were isolated from bone marrow of PKCδ WT and KO mice. Migration of monocytes toward recombinant MCP-1 was analyzed by the trans-well assay, n=4.

Supplementary Figure IX. Perivascular application of adenovirus leads to aorta-specific gene transfer. (A&B) AdGFP or AdLacZ were administered perivascularly following $CaCl_2$ treatment. Green fluorescence was evaluated by fluorescent microscopy for GFP expression in

the aortic wall, Scale bar=500μm, (A) or by flow cytometry for GFP expression in peripheral blood, $GFP^{+/+}$ and $GFP^{-/-}$ mice were used as positive and negative controls, respectively (B). (C&D) PKCδ KO mice were treated with Ad PKCδ or AdLacZ following the $CaCl_2$ procedure. Aortic sections were co-stained with antibodies specific to PKCδ (green) and smooth muscle cell-specific α-actin (SMA, red), overlay with DAPI (blue). Scale bar=200μm.

Supplemental Figure X. Elastin degradation scaling. Representative images depicting the grading scale used to evaluate elastin degradation in mouse aneurysmal tissue. Detailed description in Supplemental Methods.

Supplemental Figure XI. Representative control images for immunoflourescent staining. (A) Primary antibody alone for PKCδ and TUNEL (diluent only, no enzyme), overlay with DAPI. Scale bar = 200μm. (B) Secondary antibody only for green fluorescent anti-mouse (Anti-Mouse 488) and red fluorescent anti-rabbit (Anti-Rabbit 546), overlay with DAPI. Scale bar = 200μm.