Supplement Material:

Deletion of EP4 on bone marrow–derived cells enhances inflammation and angiotensin II-induced abdominal aortic aneurysm formation

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Mice

Homozygous EP4 deficient ($EP4^{-/-}$) and wild-type ($EP4^{+/+}$) mice on the same genetic background were obtained by crossing mice heterozygous for the *ptger4* gene mutation ($EP4^{+/-}$), and verified as previously described.[1] Because $EP4^{-/-}$ mice only survive on a recombinant inbred strain, all $EP4^{-/-}$ and $EP4^{+/+}$ mice used in this study were on the mixed background, composed of 129/Olac, C57BL/6, and DBA/2. Male and female 8- to 10-week-old low-density lipoprotein receptor knockout ($LDLR^{-/-}$; B6, 129S-*Ldlr*^{tm1Her}) mice on the C57BL/6 background were purchased from the Jackson Laboratory (Bar Harbor, ME). All experiments were performed under protocols approved by the Animal Research Committee of Harvard Medical School and in accordance with institutional guidelines.

Bone marrow transplantation

Bone marrow transplantation was performed as described previously.[2] Bone marrow–derived cells were obtained from the tibias and femurs of donor $EP4^{+/+}$ or $EP4^{-/-}$ mice and were injected ($1x10^7$ cells per mouse) into the tail veins of $LDLR^{-/-}$ recipient mice, which had been irradiated with 1000 rads from a cesium source 1 day before injection. $EP4^{+/+}$ and $EP4^{-/-}$ bone marrow chimera will be referred as $EP4^{+/+}$/$LDLR^{-/-}$ and $EP4^{-/-}$/$LDLR^{-/-}$, respectively, throughout the manuscript.

Angiotensin II–induced abdominal aortic aneurysm (***AAA)***

Five weeks after transplantation, $EP4^{+/+}$/$LDLR^{-/-}$ and $EP4^{-/-}$/$LDLR^{-/-}$ mice were fed *ad libitum* with a high-fat diet (D12108 from Research Diets; 40% Kcal from fat, 1.25%

cholesterol) for 5 weeks. Osmotic mini-pumps (Alzet, Model 2004; Cupertino, CA) containing Ang II (1000 ng/kg/min; Sigma-Aldrich, St. Louis, MO) were implanted subcutaneously 1 week after initiation of fat feeding to induce AAA formation.[3] Mean blood pressure was measured weekly on conscious, restrained mice using the Visitech tail cuff system (Apex, NC).

Preparation of mouse aortas and plasma analysis

Suprarenal abdominal aortas were harvested after 4 weeks of Ang II infusion.[4] On the day of harvesting, mice were anesthetized by intraperitoneal injection with 2,2,2-tribromoethanol (2.5 mg/10 g body weight). Blood was drawn by cardiac puncture using a needle with heparin. Plasma triglycerides and total cholesterol were determined using Infinity triglyceride or cholesterol lipid stable reagent with the appropriate standards (Thermo Scientific, Middletown, VA). Whole aortas were cleaned of adhered fat. Thoracic aortas were stored in 10% formalin until staining with Oil red O for lipid deposits, as described previously.[4] The widest parts of the suprarenal regions of the aortas (where aneurysm occurred) were embedded in OCT for subsequent morphology analysis.

Quantification of aneurysms

A commonly used clinic standard to diagnose AAA is an increase in aortic diameter of ≈50%.[5] The average diameter of the normal suprarenal aorta in control mice is ≈ 0.7 ± 0.02 mm (n=7). We therefore set a threshold of 1.05 mm as evidence of an incidence of aneurysm formation. Aneurysm severity was assessed with a scoring system: Type 0, no aneurysm (the suprarenal region of the aorta was not obviously different from control mice without Ang II treatment); type 1, suprarenal dilation (>50% increase in aortic diameter) without thrombus; type 2, suprarenal dilation (>50% increase in aortic diameter) with thrombus; type 3, multiple aneurysms, including thoracic aneurysms and dissections; type 4, death due to aneurysmal rupture [death due to rupture of aneurysm was qualified by the presence of retroperitoneal hematoma in addition to an AAA].[6] The diameter of the suprarenal aorta (where aneurysm formed) was determined with a micrometer eyepiece.

Histological examination of lesion morphology

Frozen 6-μm sections were prepared and stained for macrophages (Mac-3, 1:900; Pharmingen, San Diego, CA), T cells (CD4, 1:100; Pharmingen), smooth-muscle cells (SMC; α-actin, 1:75; Santa Cruz Biotech Inc., Santa Cruz, CA), MCP-1 (1:50, Pharmingen), and elastin (Verhoeff-Van Gieson; Accustain elastin stain kit, Sigma-Aldrich), as described previously.[4,7] Aneurysm lesional apoptotic cells were

determined with the *in situ* apoptosis detection kit according to the manufacturer's instructions (Chemicon International, Temecula, CA). Elastin fragmentation was graded as previously described using a 1-to-4 scoring system: 1 = intact internal elastin lamina; 2 = mild elastin fragmentation; 3 = severe elastin digestion; 4 = severe elastin digestion with visible ruptured sites.[7] A pictorial elastin fragmentation scale is illustrated in Figure 2G. All images were analyzed using the Image-Pro Plus program (Media Cybernetics, Silver Spring**,** MD) to obtain numerical values for perimeter length, maximal intimal-medial thickness, aneurysmal lesion areas, and positive stained areas. Grading for all specimens (for a particular measurement) was done on the same day, and all analyses were performed on coded specimens without knowledge of genotype. Only one person was assigned as the grader throughout the study.

In situ zymography

AAA lesion elastinolytic activity was determined on 6-µM frozen sections using elastin conjugated with quenched fluorescein (DQ elastin; Invitrogen, Carlsbad, CA) as a substrate, which requires cleavage by elastinolytic enzymes to become fluorescent. DQ elastin (1 mg/ml in H_2O) was mixed 1:10 with 1% low-melting agarose (Sigma-Aldrich). This mixture (20 µl) was added on top of each section, coverslipped, and gelled at 4˚C. Following incubation at 37˚C for 48 hours, fluorescence was examined using a fluorescent microscope. Cysteine protease activity was determined using a pH 5.5 buffer containing EDTA (a chelator of calcium to inhibit MMP activity; 10 mM). MMP activity was determined using a pH 7.4 buffer containing E64 (a non-selective inhibitor of cysteine proteases; 20 µM).[7] Negative controls (EDTA and E64 were added to MMP and cathepsin measurements, respectively) and no-substrate controls (data not shown; fluorescence emission was negligible) were performed in parallel sections on the same slides for all experiments. Fluorescence intensity, as measured using computer-assisted image quantification, is expressed in percentage of fluorescence area over aneurysm lesion area of each cross-section, excluding the media area due to medial elastin filament autofluoresence.

RNA isolation and quantitative PCR

Total RNA was isolated from suprarenal aortas of mice by mechanical homogenization and RNeasy columns (Qiagen, Valencia, CA). Equivalent amounts of RNA were reverse-transcribed by Superscript II (Invitrogen, Caslsbad, CA), according to the manufacturer's instructions. Quantitative PCR was performed in a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The sequences of mouse primers were: CD68, 5'-CTCTCTAAGGCTACAGGCTGCT-3'

and 5'-TCACGGTTGCAAGAGAAACA-3'; MCP-1, 5'-GGCTGGAGAGCTACAAGAGG-3' and 5'-TCTTGAGCTTGGTGACAAAAAC-3'; CD3, 5'-TCCCAACCCAGACTATGAGC-3' and 5'-GCGATGTCTCTCCTATCTGTCA-3'; GAPDH, 5'-TGGGTGTGAACCATGAGAAG-3' and 5'-GCTAAGCAGTTGGTGGTGC-3'. The mRNA levels of the various genes tested were normalized to glyceraldehyde 3-phosophate dehydrogenase (GAPDH), used as an internal control in all experiments.

Statistical analysis

Data are expressed as mean $\pm$ standard deviation (SD). All statistical analysis was performed using the GraphPad Prism software 5.0 (San Diego, CA). Two-tailed nonparametric Mann-Whitney U test was used for comparisons between $EP4^{+/+}/LDLR^{-/-}$ mice and $EP4^{-/-}/LDLR^{-/-}$ mice (of the same sex). Mean blood pressure before and after 4 weeks of Ang II infusion was compared using the paired t-test. Total blood pressure changes between $EP4^{+/+}/LDLR^{-/-}$ mice and $EP4^{-/-}/LDLR^{-/-}$ mice were compared using the Mann-Whitney U test of its area under curve. Blood pressure changes between $EP4^{+/+}/LDLR^{-/-}$ mice and $EP4^{-/-}/LDLR^{-/-}$ mice at particular time points were also compared using the Mann-Whitney U test. Elastin fragmentation scores between groups were compared using the chi-square test. Differences were considered statistically significant at $P<0.05$.

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