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## Cathepsin K Deficiency Reduces Elastase Perfusion-Induced Abdominal Aortic Aneurysms in Mice

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# Arteriosclerosis, Thrombosis, and Vascular Biology



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# Cathepsin K Deficiency Reduces Elastase Perfusion–Induced Abdominal Aortic Aneurysms in Mice

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**Objective**—Cathepsin K (CatK) is one of the most potent mammalian elastases. We have previously shown increased expression of CatK in human abdominal aortic aneurysm (AAA) lesions. Whether this protease participates directly in AAA formation, however, remains unknown.

**Methods and Results**—Mouse experimental AAA was induced with aortic perfusion of a porcine pancreatic elastase. Using this experimental model, we demonstrated that absence of CatK prevented AAA formation in mice 14 days postperfusion. CatK deficiency significantly reduced lesion CD4<sup>+</sup> T-cell content, total lesion and medial cell proliferation and apoptosis, medial smooth muscle cell (SMC) loss, elastolytic CatL and CatS expression, and elastin fragmentation, but it did not affect AAA lesion Mac-3<sup>+</sup> macrophage accumulation or CD31<sup>+</sup> microvessel numbers. In vitro studies revealed that CatK contributed importantly to CD4<sup>+</sup> T-cell proliferation, SMC apoptosis, and other cysteinyl cathepsin and matrix metalloproteinase expression and activities in SMCs and endothelial cells but played negligible roles in microvessel growth and monocyte migration. AAA lesions from CatK-deficient mice showed reduced elastolytic cathepsin activities compared with those from wild-type control mice.

**Conclusion**—This study demonstrates that CatK plays an essential role in AAA formation by promoting T-cell proliferation, vascular SMC apoptosis, and elastin degradation and by affecting vascular cell protease expression and activities. (*Arterioscler Thromb Vasc Biol.* 2012;32:15-23.)

**Key Words:** aneurysms ■ cathepsin K ■ elastase ■ smooth muscle cell ■ T cell

The pathogenesis of human abdominal aortic aneurysm (AAA) involves extensive vascular wall matrix protein remodeling, which requires the participation of proteases.<sup>1–3</sup> Cysteine protease cathepsins K, L, and S are among the most potent mammalian elastases.<sup>4</sup> We showed previously that human atherosclerosis and AAA lesions contained high levels of these proteases.<sup>5</sup> In contrast, their endogenous inhibitor cystatin C was deficient in these lesions.<sup>6</sup> Absence of these proteases protected mice from diet-induced atherosclerosis, whereas cystatin C-deficient mice had enlarged aortic diameters,<sup>7–10</sup> suggesting essential roles of cysteine proteases in aortic wall remodeling. Since we first showed increased expression of cathepsins S, L, and K in human AAA lesions,<sup>6</sup> other studies have found similar expression patterns. Aortic tissue extract cathepsin activities were significantly higher in AAA patients than in those with aortic occlusion diseases, but cystatin C levels were regulated inversely.<sup>11</sup> Aortic tissues from patients with growing AAA and ruptured AAA contained significantly higher cathepsin mRNA and protein levels than did control aortas,<sup>12</sup> suggesting the involvement of

cathepsins in AAA pathogenesis, but a direct role of this class of proteases has never been proven. In this study, we used cathepsin K (CatK)–deficient mice and aorta elastase perfusion–induced experimental AAA to test whether this elastase contributes directly to AAA formation.

## Materials and Methods

### Mouse AAA Model and Lesion Characterization

Male 10-week-old CatK-deficient mice (*Ctsk*<sup>−/−</sup>, C57BL/6J129S background)<sup>13</sup> and male wild-type (*Ctsk*<sup>+/+</sup>) littermate controls underwent aortic perfusion with 0.411 U/mL type I porcine pancreatic elastase (E1250, Sigma, St. Louis, MO) to produce experimental AAA, as previously described.<sup>14</sup> We used 9 or 10 mice per experimental group and harvested the aortic tissues at 7 or 14 days postperfusion. Mouse aortic diameters were measured using a surgical microscope (Zeiss Stemi SV11) equipped with a micrometer eyepiece (14 mm/0.1, SG02.T0218c, Motic Instruments, Inc, Vancouver, British Columbia, Canada), which allowed us to read aortic diameters at any time during the surgical procedure or during tissue harvesting.<sup>14,15</sup> The micrometer eyepiece was calibrated under each magnification. Preperfusion, immediate postperfusion (5 minutes after perfusion restoration), 7-day postperfusion, and 14-day postperfusion aortic diameters were measured to a resolution of 0.1 mm

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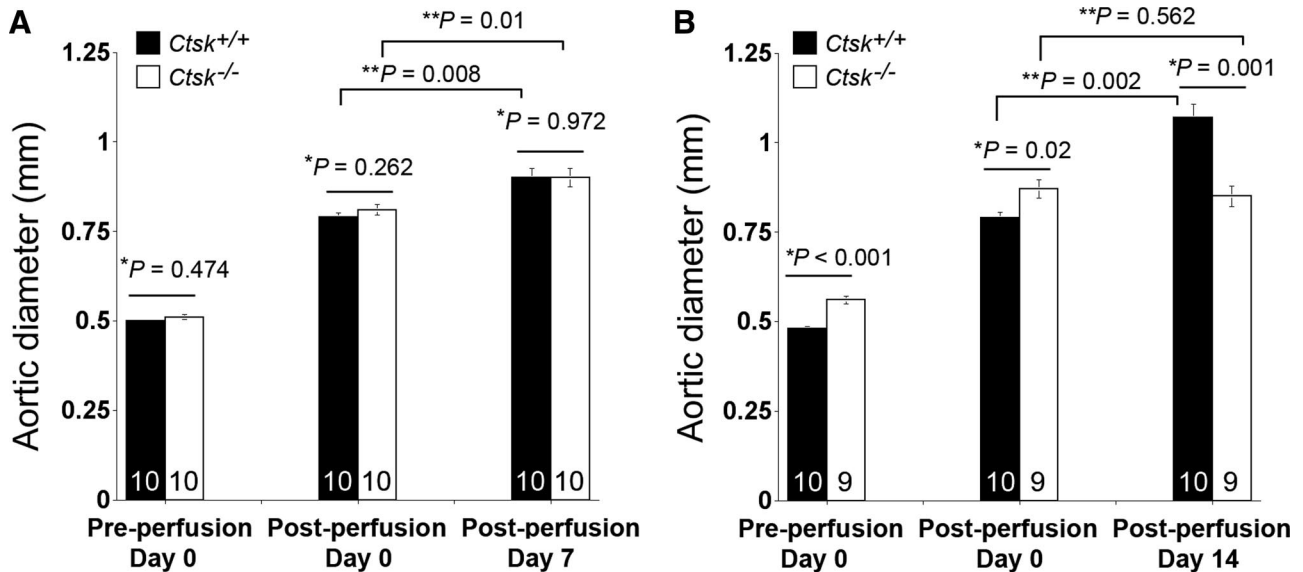
Drs Sun and Sukhova contributed equally to this work.

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**Figure 1.** Reduced abdominal aortic aneurysm (AAA) formation in *Ctsk*<sup>-/-</sup> mice. Aortic diameters in both *Ctsk*<sup>+/+</sup> mice and *Ctsk*<sup>-/-</sup> mice were measured preperfusion, immediately postperfusion, and at 7 and 14 days postperfusion. The number of mice per group is indicated in each bar. Data are mean ± SE. *P* < 0.05 is considered statistically significant; \*nonpaired Mann-Whitney *U* test, \*\*paired Wilcoxon signed-rank test.

while mice were under anesthesia and physiological blood pressure. Two independent investigators measured aortic diameters, with no significant interobserver or intraobserver variability. Aortic diameter expansion ≥100% of that before perfusion defined AAA.<sup>14</sup> Each mouse aorta was isolated for frozen section preparation (a total of 20 sections per aorta were prepared for immunohistology analysis) and tissue protein extraction in a pH 5.5 buffer containing 1% Triton X-100, 40 mmol/L sodium acetate, and 1 mmol/L EDTA.<sup>5</sup> Frozen sections were used for immunostaining for macrophages (Mac-3), smooth muscle cells (SMCs) ( $\alpha$ -actin), T cells (CD4), endothelial cells (ECs) (CD31), elastin (Verhoeff-van Gieson), apoptotic cells (terminal deoxynucleotidyl transferase dUTP nick-end labeling) and proliferating cells (Ki67), as described previously.<sup>7,15,16</sup> Medial elastin fragmentation and SMC contents were graded as described previously, except that SMC content was presented as grades for medial SMC loss.<sup>15</sup> T cells, apoptotic cells, proliferating cells, and microvessels in the adventitia were counted and compared blindly by 2 independent observers to ensure reproducibility. Adventitial macrophage-positive areas were measured with the use of computer-assisted image analysis software (Image-Pro Plus; Media Cybernetics, Bethesda, MD).

### Cysteine Protease Active Site Labeling and Gelatin Gel Zymogram

SMCs and ECs were lysed into a pH 5.5 buffer. Five micrograms of protein from each sample were incubated with 12 mmol/L dithiothreitol and 1  $\mu$ L of biotin-conjugated JPM in 30  $\mu$ L of a pH 5.5 buffer for 1 hour at 37°C. Protein samples were then separated on a 12% SDS-PAGE gel, followed by immunoblot detection with horseradish peroxidase-conjugated avidin (1:10 000, Sigma).<sup>3</sup> Matrix metalloproteinase (MMP) activity was detected with a gelatin gel zymogram, essentially the same as reported previously.<sup>17</sup> Equal protein loading (20  $\mu$ g protein/lane) was confirmed with immunoblot analysis with goat anti-mouse  $\beta$ -actin polyclonal antibodies (1:2000, Santa Cruz Biotechnologies, Santa Cruz, CA).

### Real-Time Polymerase Chain Reaction

Real-time polymerase chain reaction determined protease mRNA levels in SMCs and ECs. Mouse aortic SMCs and ECs were prepared from *Ctsk*<sup>+/+</sup> mice and *Ctsk*<sup>-/-</sup> mice, as reported previously.<sup>7,18</sup> Aortic SMC purity was examined with the expression of  $\alpha$ -smooth muscle actin and calponin.<sup>7</sup> Total cellular RNA was extracted from SMCs and ECs using TRIzol reagent (Invitrogen, Carlsbad, CA) and treated with RNase-free DNase (Ambion, Austin, TX) to remove

genomic DNA contaminants. Equal amounts of RNA were reverse transcribed, and quantitative polymerase chain reaction was assessed in a single-color reverse transcription-polymerase chain reaction detection system (Stratagene, La Jolla, CA). The level of each protease transcript was normalized to that of the  $\beta$ -actin transcript.

### SMC Apoptosis

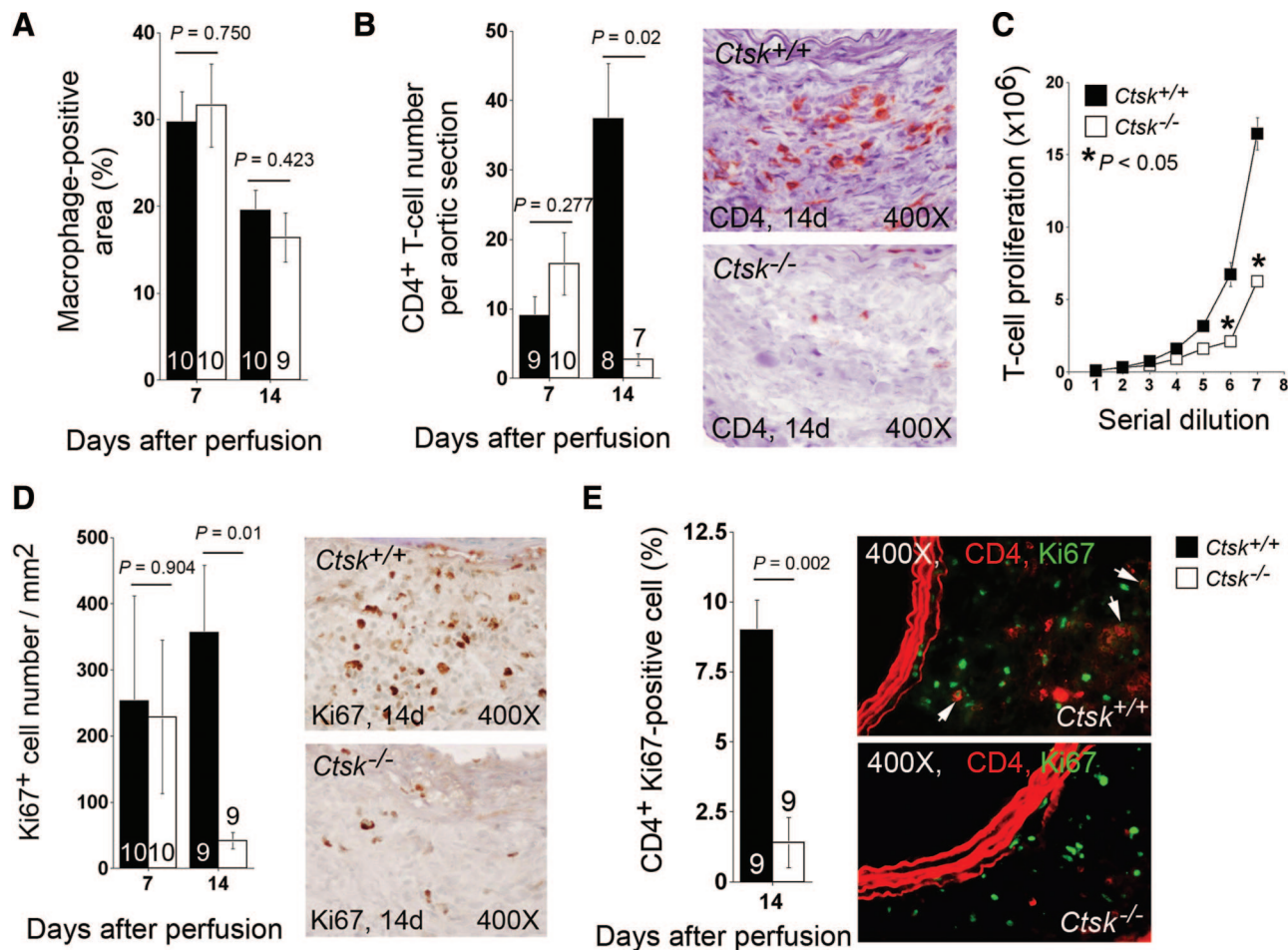
SMC apoptosis was performed using primary cultured *Ctsk*<sup>+/+</sup> and *Ctsk*<sup>-/-</sup> mouse aortic SMCs on an 8-well chamber slide. Confluent SMCs were stimulated to apoptosis overnight with 60  $\mu$ mol/L pyrrolidine dithiocarbamate.<sup>19</sup> Apoptotic cells were detected with *In Situ* Cell Death Detection Kit according to the manufacturer's instructions (Roche Diagnostics Co., Indianapolis, IN).

### Aortic Ring Assay

An aortic ring assay was used to test the role of CatK in angiogenesis. In brief, a 96-well plate was coated with 50  $\mu$ L of Matrigel (BD Biosciences, San Diego, CA). Mouse aortic rings of 1 mm in length from *Ctsk*<sup>+/+</sup> and *Ctsk*<sup>-/-</sup> mice were laid on top of the Matrigel and covered with 100  $\mu$ L of Matrigel. After solidification, 150  $\mu$ L of RPMI (10% FBS) was added to each well. After 7 to 10 days of culture, the aortas were photographed, and the endothelial outgrowth was analyzed using Image-Pro Plus software and presented as square millimeters. Basic fibroblast growth factor (10 ng/mL, PeproTech, Inc., Rocky Hill, NJ) was used as a positive control.

### In Situ Elastin Zymography

AAA lesion elastolytic activity was determined in 8- $\mu$ m frozen sections using elastin conjugated with quenched fluorescein (DQ elastin; Invitrogen) as a substrate, which requires cleavage by elastolytic enzymes to become fluorescent. In brief, DQ elastin (1 mg/mL in H<sub>2</sub>O) was mixed 1:10 with 1% low-melting agarose (Sigma). This mixture (20  $\mu$ L) was added on top of each section, coverslipped, and gelled at 4°C. Following incubation at 37°C (48 hours), fluorescence was examined under fluorescent microscopy. Cysteine protease activity was determined using an EDTA-containing pH 5.5 buffer<sup>5</sup> with or without a generic cathepsin inhibitor E64d (20  $\mu$ mol/L). Zymographic images were acquired using identical shutter conditions. The percentage of fluorescence intensity of each cross section, excluding the media area because of medial elastin filament autofluorescence, was measured using computer-assisted image quantification (Image-Pro Plus software). Medial cathepsin elastase activity was calculated by subtracting the



**Figure 2.** Cathepsin K (CatK) activities on T-cell proliferation. Absence of CatK did not affect abdominal aortic aneurysm (AAA) lesion Mac-3<sup>+</sup> macrophage-positive area (A) but reduced CD4<sup>+</sup> T-cell numbers (B) in the adventitia at 14 days postperfusion. In vitro cell proliferation assay showed that CatK deficiency impaired CD4<sup>+</sup> T-cell proliferation (C). In AAA lesion adventitia, numbers of Ki67-positive cells (D) and percentage of CD4<sup>+</sup> T-cells among Ki67-positive cells (E; arrows indicate Ki67<sup>+</sup>CD4<sup>+</sup> T cells) were also reduced in *Ctsk*<sup>-/-</sup> mice at 14 days postperfusion. The number of mice per group is indicated in each bar. All data are mean ± SE.  $P < 0.05$  is considered statistically significant; Mann-Whitney *U* test. Representative images are shown to the right in B, D, and E.

percentages of fluorescence intensity in media regions of E64d-treated sections from those of untreated sections.

### Cell Proliferation Assay

CD4<sup>+</sup> T cells were purified from splenocytes by depleting major histocompatibility complex class II-positive cells and CD8<sup>+</sup> T cells using anti-mouse I-A<sup>b</sup> and CD8 monoclonal antibodies (BD Biosciences), followed by complement depletion, as described previously.<sup>20</sup> Monocytes were isolated from peripheral blood by Percoll (Sigma) gradient centrifugation. T-cell and monocyte proliferation were assessed with the Cell Titer 96AQ Assay kit, according to the manufacturer's instructions (Promega, Madison, WI). Equal numbers of T cells and monocytes from *Ctsk*<sup>+/+</sup> mice and *Ctsk*<sup>-/-</sup> mice were plated on 96-well plates at serial dilutions in 100  $\mu$ L of 10% FBS RPMI 1640 per well and cultured for 2 days at 37°C. Then, 20  $\mu$ L of a mixture of tetrazolium compound and phenazine methosulfate was added, and the absorbance was determined at 492 nm. Monocyte proliferation was determined under spontaneous conditions, whereas CD4<sup>+</sup> T-cell proliferation was measured in the presence of anti-CD3 monoclonal antibodies (1  $\mu$ g/mL, Pharmingen, San Diego, CA). Serial dilutions of known numbers of T cells and monocytes were used to generate standard curves.

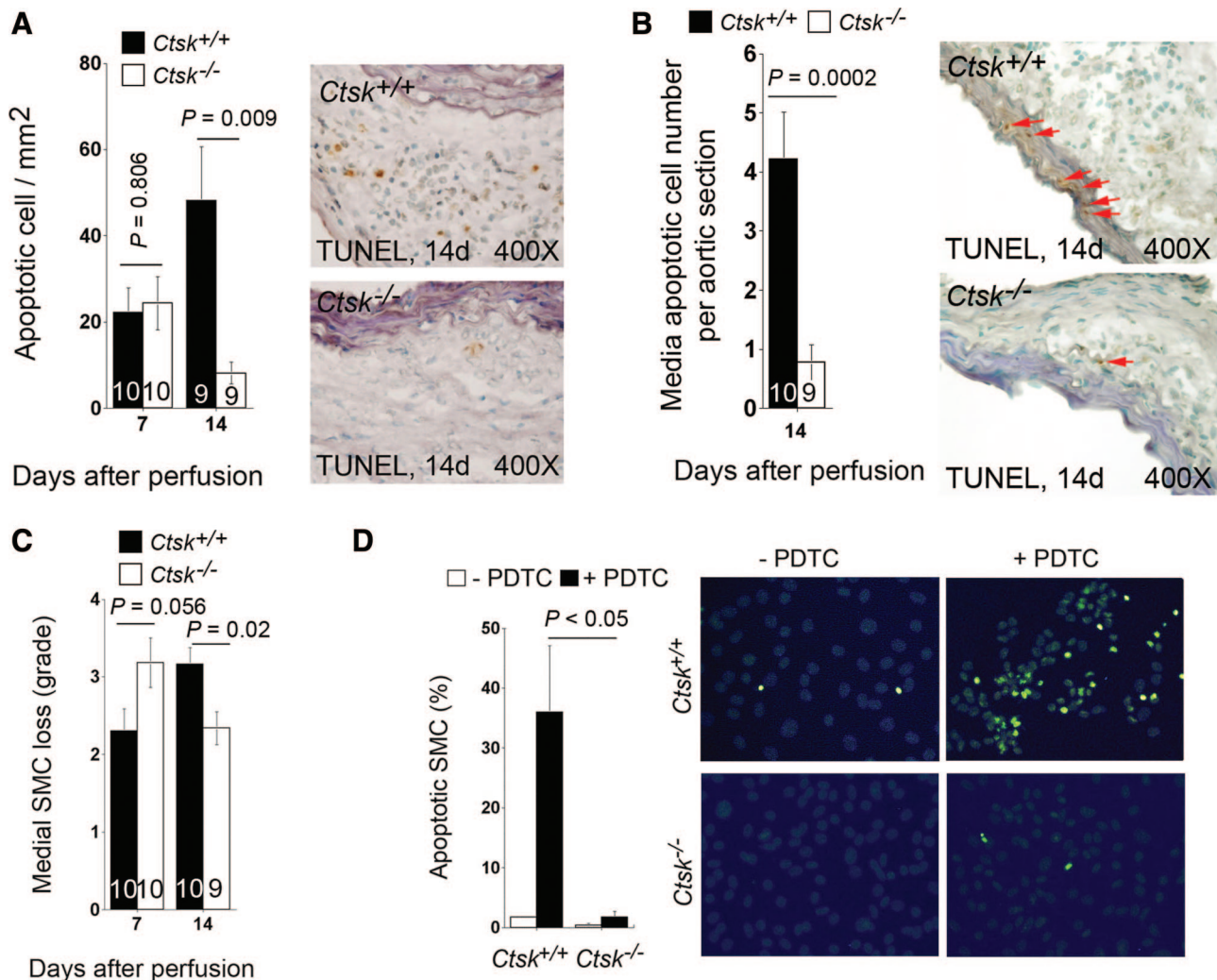
### Transmigration Assay

T-cell and monocyte transmigration assay was performed on a type I collagen (100 ng/25  $\mu$ L per well in a pH7.0 HEPES buffer)–

precoated 96-well Chemotaxis plate, according to the manufacturer's instructions (Neuro Probe, Inc, Gaithersburg, MD). Stromal cell-derived factor-1 $\alpha$  (0, 10, 100, and 1000 ng/mL, PeproTech) in a plain RPMI containing 1% bovine serum albumin (Sigma) was used as chemokine. Although stromal cell-derived factor-1 $\alpha$  is an important chemokine for stem cells or progenitor cells, it has been widely used in inflammatory cell in vitro migration assays.<sup>21–23</sup> Briefly, T cells and monocytes (25 000 cells in 25  $\mu$ L of 1% bovine serum albumin RPMI) were seeded on the top of the filter, and 30  $\mu$ L of stromal cell-derived factor-1 $\alpha$  was added to the bottom chamber. After incubation (3 hours for monocytes; 1.5 hours for T cells), the remaining filter top cells were removed, and the bottom cells were collected (1500 rpm, 5 minutes), fixed with 1% paraformaldehyde, and counted by fluorescence-activated cell sorting analysis.

### Blood Cell and Splenocyte Fluorescence-Activated Cell Sorting Analysis

Blood cells and splenocytes from *Ctsk*<sup>+/+</sup> mice, *Ctsk*<sup>-/-</sup> mice, and those treated with angiotensin II (Ang II) were analyzed for contents of Ly6G<sup>+</sup>, CD11b<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cells. To treat *Ctsk*<sup>+/+</sup> and *Ctsk*<sup>-/-</sup> mice with Ang II, mice received Alzet osmotic minipumps (model 2004, Alzet, Durect Corp, Cupertino, CA) filled with Ang II (Sigma, 1000 ng/kg/min). Blood samples were collected after 28 days. Total blood cells were isolated by cardiac puncture, and splenocytes were prepared as described previously.<sup>20</sup> Blood cells and splenocytes were stained with rat anti-mouse Ly6G-FITC (RB6–



**Figure 3.** Cathepsin K (CatK) activities on lesion cell apoptosis. Abdominal aortic aneurysm (AAA) lesion adventitia (A) and medial (B) terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)-positive cell numbers were significantly lower in *Ctsk*<sup>-/-</sup> mice than in *Ctsk*<sup>+/+</sup> mice at 14 days postperfusion. At the same time point, lesion medial smooth muscle cell (SMC) loss was reduced in *Ctsk*<sup>-/-</sup> mice (C). The number of mice per group is indicated in each bar. Aortic SMCs from *Ctsk*<sup>-/-</sup> mice were resistant to pyrrolidine dithiocarbamate (PDTC)-induced apoptosis (D). All data are mean±SE.  $P < 0.05$  is considered statistically significant; Mann-Whitney *U* test. Representative images are shown to the right in A, B, and C.

8C5, eBioscience), rat anti-mouse CD11b-PE (M1/70, eBioscience, San Diego, CA), rat anti-mouse CD4-PE (GK1.5, Biologend, San Diego, CA), and rat anti-mouse CD8-Alexa 610 (5H10, Invitrogen). Ly6G<sup>+</sup>, CD11b<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cell percentages were determined by fluorescence-activated cell sorting analysis (Cytomics FC500, Beckman Coulter, Brea, CA).

### Statistical Analysis

Because of relatively small sample sizes and data distribution abnormality, we selected the nonparametric Mann-Whitney *U* test for nonpaired data sets and the Wilcoxon signed-rank test for paired data sets to examine statistical significance throughout this study.  $P < 0.05$  was considered statistically significant.

## Results

### Reduced AAA in CatK-Deficient Mice

Both *Ctsk*<sup>-/-</sup> mice and *Ctsk*<sup>+/+</sup> mice underwent aortic elastase perfusion to induce AAA. In mice used for the 7-day time point, there were no significant differences in aortic diameters between *Ctsk*<sup>+/+</sup> mice and *Ctsk*<sup>-/-</sup> mice before and immediately after

elastase perfusion. At 7 days postperfusion, *Ctsk*<sup>+/+</sup> mice and *Ctsk*<sup>-/-</sup> mice also showed no significant differences in aortic diameters, and none of these mice developed AAA, as defined by a 100% increase in aortic diameter (Figure 1A).<sup>14</sup> In *Ctsk*<sup>-/-</sup> mice used for the 14-day time point, both preperfusion ( $0.55 \pm 0.01$  versus  $0.48 \pm 0.01$  mm,  $P < 0.001$ ) and immediate postperfusion ( $0.87 \pm 0.03$  versus  $0.79 \pm 0.02$  mm,  $P = 0.02$ ) aortic diameters were larger than those from *Ctsk*<sup>+/+</sup> mice, although we cannot explain this observation. At 14 days postperfusion, however, aortic diameters from *Ctsk*<sup>-/-</sup> mice were not significantly different from those immediately postperfusion ( $P = 0.562$ ) but were significantly smaller than those from *Ctsk*<sup>+/+</sup> mice ( $P = 0.001$ ) (Figure 1B). At this time point, all *Ctsk*<sup>+/+</sup> mice (10 of 10) developed AAA, but none of the *Ctsk*<sup>-/-</sup> mice did.

### Inflammatory Cells in AAA Lesions

Inflammatory cell infiltration is an important event following AAA initiation in humans and animals.<sup>24</sup> Although we detected no significant differences in lesion macrophage

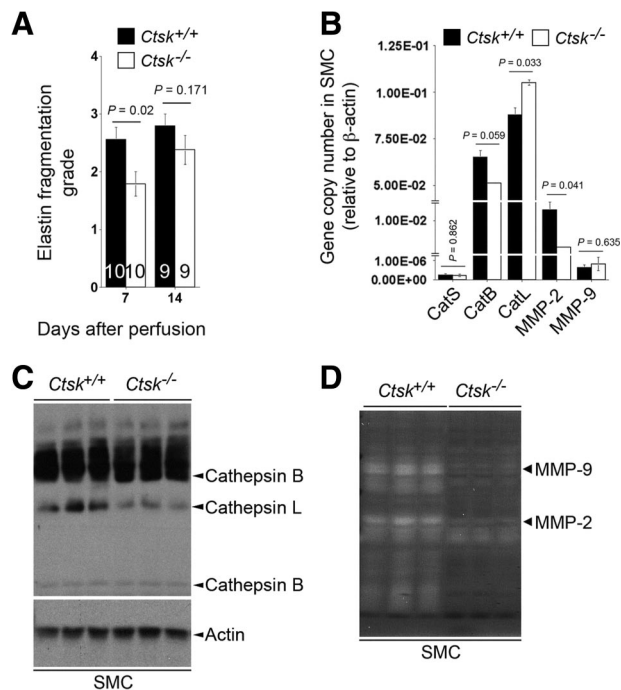
contents between *Ctsk*<sup>+/+</sup> and *Ctsk*<sup>-/-</sup> mice at either time point (Figure 2A), *Ctsk*<sup>+/+</sup> mouse AAA lesions contained significantly higher numbers of CD4<sup>+</sup> T cells than did *Ctsk*<sup>-/-</sup> AAA lesions at 14 days postperfusion (Figure 2B). The reduced numbers of CD4<sup>+</sup> T cells in *Ctsk*<sup>-/-</sup> mouse lesions suggest that CatK activity contributed to T-cell migration or proliferation. To test these possibilities, we performed Boyden chamber cell transmigration assay using stromal cell-derived factor-1 $\alpha$  as a chemoattractant. When either monocytes or CD4<sup>+</sup> T cells were used, we did not detect significant differences in their transmigration through collagen-coated Transwells (Neuro Probe Inc.) with or without CatK (data not shown). In contrast, CD4<sup>+</sup> T cells (Figure 2C), but not monocytes (data not shown), proliferated differently between the 2 genotypes. CD4<sup>+</sup> T cells from *Ctsk*<sup>+/+</sup> mice proliferated much faster than those from *Ctsk*<sup>-/-</sup> mice. These data may explain the unchanged macrophage content but reduced CD4<sup>+</sup> T-cell content in *Ctsk*<sup>-/-</sup> mouse AAA lesions (Figure 2A and 2B). In AAA lesions, Ki67<sup>+</sup> proliferating cells were significantly reduced in *Ctsk*<sup>-/-</sup> mice, compared with those in *Ctsk*<sup>+/+</sup> mice at 14 days postperfusion (Figure 2D). Anti-CD4 and anti-Ki67 monoclonal antibody coimmunostaining examined proliferating CD4<sup>+</sup> T cells in AAA lesions. Among all Ki67<sup>+</sup> proliferating cells, the percentages of CD4<sup>+</sup> T cells were significantly lower in *Ctsk*<sup>-/-</sup> mice than in *Ctsk*<sup>+/+</sup> mice (Figure 2E), suggesting a role for CatL in T-cell proliferation in vivo.

### CatK Function in Vascular Cell Apoptosis

SMC apoptosis determines tunica media thinning and characterizes both human and animal AAA lesions,<sup>25–27</sup> whereas infiltrating leukocytes release apoptotic stimuli to promote vascular cell apoptosis.<sup>26,28</sup> The absence of CatK reduced AAA lesion apoptotic cells significantly. Terminal deoxynucleotidyl transferase dUTP nick-end labeling–positive cells were reduced in whole AAA lesions (Figure 3A) and in the media (Figure 3B, mainly SMCs) from *Ctsk*<sup>-/-</sup> mice, compared with those in *Ctsk*<sup>+/+</sup> mice at 14 days postperfusion. In correlation with this observation, medial SMC loss in AAA lesions from *Ctsk*<sup>-/-</sup> mice also was significantly impaired at this time point (Figure 3C), although both lesion cell apoptosis and medial SMC loss were not significantly different between the phenotypes at the 7-day time point (Figure 3A and 3C). To examine the contribution of CatK to SMC apoptosis, we induced SMC apoptosis with pyrrolidine dithiocarbamate. When SMCs from *Ctsk*<sup>+/+</sup> mice underwent apoptosis in the presence of pyrrolidine dithiocarbamate, those from *Ctsk*<sup>-/-</sup> mice were protected (Figure 3D). Although the mechanisms behind these observations merit further investigation, these results suggest an important role of CatK in SMC apoptosis.

### CatK Deficiency Protected Medial Elastin Fragmentation

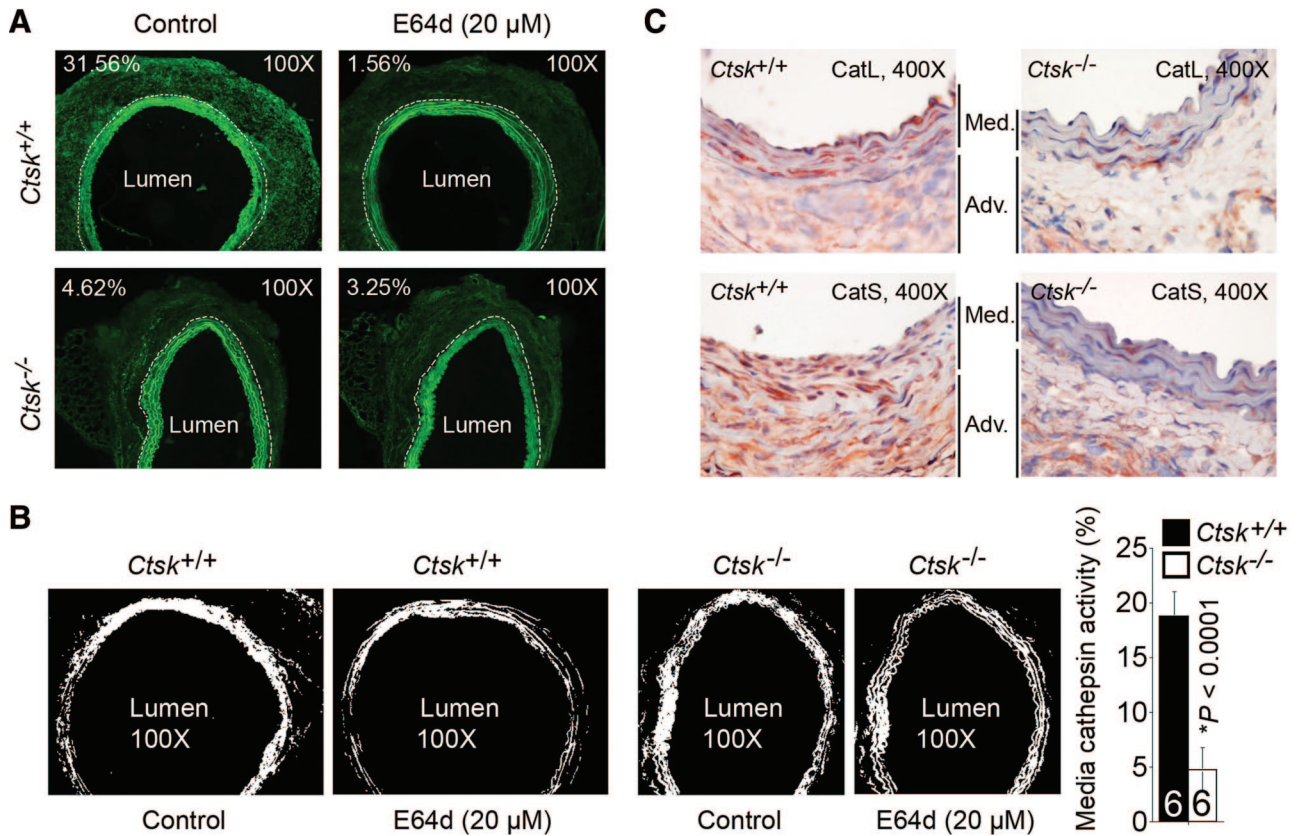
Elastin fragmentation is an important hallmark of human AAA<sup>29</sup> and is mediated by elastases in the media. As one of the most potent elastases, CatK may participate directly in medial elastin degradation. Verhoeff–van Gieson staining demonstrated significantly reduced elastin fragmentation in



**Figure 4.** Cathepsin K (CatK) activities on medial elastin fragmentation and aortic smooth muscle cell (SMC) protease expression. At both time points (7 and 14 days postperfusion), medial elastin fragmentation in abdominal aortic aneurysm (AAA) lesions from *Ctsk*<sup>-/-</sup> mice were lower than in *Ctsk*<sup>+/+</sup> mice (A). The number of mice per group is indicated in each bar. Data are mean  $\pm$  SE. Cathepsin and matrix metalloproteinase (MMP) transcripts in aortic SMCs were examined by real-time polymerase chain reaction (B). Data are mean  $\pm$  SE of 4 independent experiments.  $P < 0.05$  is considered statistically significant; Mann-Whitney  $U$  test. CysteinyI cathepsin activities in SMCs were examined by active site JPM labeling (C). Actin immunoblot was used as protein loading control. MMP activities were assessed by gelatin gel zymogram assay (D).

AAA lesions from *Ctsk*<sup>-/-</sup> mice compared with those from *Ctsk*<sup>+/+</sup> mice at 7 days postperfusion. Elastin degradation was enhanced at the later time point and remained lower in *Ctsk*<sup>-/-</sup> mice than in *Ctsk*<sup>+/+</sup> mice at 14 days postperfusion, although it did not reach statistical significance ( $2.39 \pm 0.26$  versus  $2.79 \pm 0.22$ ,  $P = 0.171$ ) (Figure 4A). Insignificance in elastin degradation between the groups at this time point was due to an outlier aorta in each group of mice. Significant differences in AAA expansion (Figure 1B) but insignificant medial elastin fragmentation (Figure 4A) between *Ctsk*<sup>-/-</sup> mice and *Ctsk*<sup>+/+</sup> mice at 14 days postperfusion suggest that medial elastin degradation is merely part of the mechanism of murine AAA formation. Other mechanisms, such as inflammatory cell infiltration, medial cell apoptosis, and SMC loss, also contribute to the pathogenesis.

CatK deficiency can alter expression or activities of other elastases—and consequently impair elastin fragmentation—in AAA lesions from *Ctsk*<sup>-/-</sup> mice. Real-time polymerase chain reaction showed increased CatL transcript and decreased MMP-2 transcripts in SMCs from *Ctsk*<sup>-/-</sup> mice, as compared with *Ctsk*<sup>+/+</sup> mice (Figure 4B). Although SMCs routinely express MMP-2, they also express MMP-9 at much lower levels,<sup>30,31</sup> and CatK deficiency did not affect MMP-9 mRNA levels significantly (Figure 4B). CysteinyI cathepsin



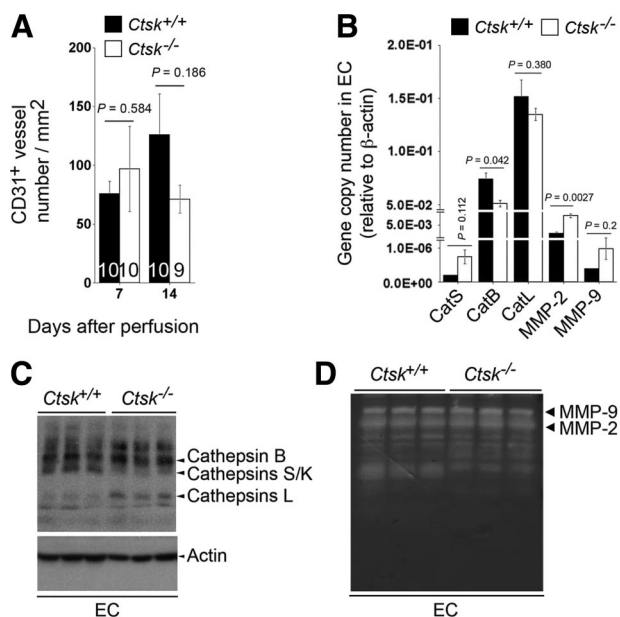
**Figure 5.** Elastinolytic cysteinyl cathepsins in abdominal aortic aneurysm (AAA) lesions at 14 days postperfusion. **A**, In situ cysteinyl elastinolytic activity fluorescent zymogram assay. High cathepsin activities in the adventitia of AAA lesions in *Ctsk*<sup>+/+</sup> mice (top left) could be largely inhibited by E64d (top right). Elastinolytic cathepsin activities were reduced in AAA lesion adventitia from *Ctsk*<sup>-/-</sup> mice (bottom left), and E64d further reduced cathepsin activities (bottom right). The percentages of adventitia fluorescence yielded from fluorogenic elastin degradation represented cysteinyl cathepsin activities. The white dotted lines separate medial elastin autofluorescence from the adventitia. **B**, Medial fluorescence subtractions between E64d-treated and untreated sections determined medial elastinolytic cathepsin activities, which were significantly reduced in the absence of cathepsin K (CatK). The number of mice per group is indicated in each bar. **C**, Immunostaining detected medial CatL and CatS expression in AAA lesions from *Ctsk*<sup>+/+</sup> and *Ctsk*<sup>-/-</sup> mice. Med. indicates media; Adv., adventitia.

active site labeling with JPM in SMCs from *Ctsk*<sup>-/-</sup> mice, however, revealed reduced activity of CatL (Figure 4C), another potent elastase that is abundant in human AAA.<sup>32</sup> In a separate study, we proved that the absence of CatL prevented mice from elastase perfusion-induced AAA, with concomitant reduction of media elastin fragmentation (G.-P. Shi, unpublished data). Furthermore, MMP-2 and MMP-9 activities were reduced in SMCs from *Ctsk*<sup>-/-</sup> mice, as demonstrated in a gelatin gel zymogram assay (Figure 4D). MMP-2 and MMP-9 may play essential roles in murine experimental AAA formation.<sup>14,33</sup> Reduced MMP activities in *Ctsk*<sup>-/-</sup> mouse SMCs may indirectly contribute to the protection of elastase perfusion-induced AAA.

Impaired elastinolytic cathepsin activities in AAA lesions from *Ctsk*<sup>-/-</sup> mice was further illustrated in an in situ elastase zymogram assay to detect lesion elastinolytic cathepsin activity<sup>3,15,16</sup> and immunostaining to detect 2 common elastinolytic CatL and CatS in the media. When 8- $\mu$ m unfixed cross sections from AAA lesions were used to digest fluorogenic DQ-elastin,<sup>5</sup> AAA lesions from *Ctsk*<sup>+/+</sup> mice contained elastinolytic cathepsin activities in the adventitia regions that could be inhibited by 20  $\mu$ mol/L of a nonselective cathepsin inhibitor E64d (Figure 5A, top

panels). Reduced elastinolytic cathepsins in adventitia from *Ctsk*<sup>-/-</sup> AAA lesions may result from impaired lesion inflammatory cell infiltration and proliferation (Figure 2B to 2E). We focused on elastinolytic cathepsin activities in the adventitia, separated from media by dotted lines in Figure 5A, mainly because of the interference of internal elastica autofluorescence. In contrast, AAA sections from *Ctsk*<sup>-/-</sup> mice contained much lower adventitia elastinolytic cathepsin activities that were further inhibited by E64d (Figure 5A, bottom panels). To examine elastinolytic cathepsin activities in the media, we subtracted the percentages of media fluorescence of E64d-treated sections from those of untreated sections. Using this method, we found that the percentages of medial cathepsin activity from *Ctsk*<sup>+/+</sup> mice were significantly higher than those from *Ctsk*<sup>-/-</sup> mice ( $P < 0.0001$ , Figure 5B). To confirm further altered medial cathepsin expression and activity differences between the groups, we performed immunostaining for CatL and CatS, 2 potent mammalian elastases. Medial expressions of CatL and CatS were reduced in AAA lesions from *Ctsk*<sup>-/-</sup> mice compared with those from *Ctsk*<sup>+/+</sup> mice (Figure 5C), which is consistent with reduced medial elastase activity (Figure 5B) and elastinolysis in these AAA lesions (Figure 4A).





**Figure 6.** Cathepsin K (CatK) activities on angiogenesis and endothelial cell (EC) protease expression. CD31<sup>+</sup> microvessel numbers were not significantly different in abdominal aortic aneurysm (AAA) lesions between the genotypes at both time points (A). EC cathepsin and matrix metalloproteinase (MMP) transcription levels were assessed by real-time polymerase chain reaction (B). Cysteine protease active site labeling with JPM (C) and gelatin gel zymogram (D) were used to assess EC cathepsin and MMP activities, respectively.

### CatK Expression and Activities in ECs and Angiogenesis

Increased angiogenesis associates with AAA growth.<sup>34</sup> Neovascularization provides paths for inflammatory cell accumulation in human AAA lesions.<sup>35</sup> Reduced AAA growth in *Ctsk*<sup>-/-</sup> mice might be caused by impaired angiogenesis. To test this hypothesis, we immunostained aortic sections with anti-mouse CD31 antibodies and found lower CD31<sup>+</sup> microvessel numbers in lesions from *Ctsk*<sup>-/-</sup> mice than in those from *Ctsk*<sup>+/+</sup> mice at 14 days postperfusion, but the difference did not reach statistical significance (Figure 6A). These observations suggest that CatK activities in angiogenesis contribute partially to pathogenesis in this experimental AAA. In vitro aortic ring assays yielded similar results. When aortic rings from *Ctsk*<sup>+/+</sup> mice and *Ctsk*<sup>-/-</sup> mice were used, we did not see significant differences in microvessel growth area in the absence ( $0.44 \pm 0.39$  versus  $0.35 \pm 0.32$  mm<sup>2</sup>,  $P > 0.05$ ) or presence ( $5.34 \pm 1.86$  versus  $5.34 \pm 2.59$  mm<sup>2</sup>,  $P > 0.05$ ) of proangiogenic factor basic fibroblast growth factor. A mild effect of CatK in angiogenesis is consistent with nearly “unchanged” EC protease activities. Although real-time polymerase chain reaction revealed decreased CatB transcript and increased MMP-2 transcript in ECs from *Ctsk*<sup>-/-</sup> mice, cathepsin active site labeling with JPM demonstrated that CatK deficiency impaired EC CatS activity, with compensatory increase of CatL activity (Figure 6C). Gelatin gel zymogram assay showed that CatK deficiency did not affect EC MMP-9 or MMP-2 activities (Figure 6D).

### Discussion

Cathepsins have been implicated in human AAA formation despite a lack of direct proof. This study provides the first

evidence that cathepsins participate directly in AAA pathogenesis. Although many more mechanisms remain unknown, we demonstrated that CatK contributed to AAA formation by regulating lesion T-cell proliferation, SMC apoptosis, and medial vascular cell protease expression or activities. CatK deficiency protected mice from elastase perfusion-induced experimental AAA.

An earlier study from Bai et al, using apolipoprotein E and CatK double-deficient (*ApoE*<sup>-/-</sup>*Ctsk*<sup>-/-</sup>) mice, showed that the absence of CatK did not affect AAA progression in Ang II-induced experimental AAA.<sup>36</sup> These prior observations did not support a role for CatK in mouse AAA pathogenesis. Compensatory induction of other cathepsins in *ApoE*<sup>-/-</sup>*Ctsk*<sup>-/-</sup> mice, although not tested in the study by Bai et al, may explain their findings; different experimental models also may contribute to the insignificant effect of CatK in Ang II-induced AAA between *ApoE*<sup>-/-</sup> mice and *ApoE*<sup>-/-</sup>*Ctsk*<sup>-/-</sup> mice. Bai et al discovered unexpectedly that Ang II infusion enhanced peripheral active CD4<sup>+</sup>CD25<sup>+</sup> T cells and Ly6G<sup>+</sup> leukocytes in *ApoE*<sup>-/-</sup>*Ctsk*<sup>-/-</sup> mice. Such Ang II-induced peripheral inflammatory cell population changes may contribute in part to increased AAA lesion CD45<sup>+</sup> leukocytes and Mac-3<sup>+</sup> macrophages, thereby increasing expression of CatS and CatC in AAA lesions from *ApoE*<sup>-/-</sup>*Ctsk*<sup>-/-</sup> mice.<sup>36</sup> The absence of a mutant *ApoE* allele and Ang II treatment in this study may eliminate these confounding factors and therefore reflect the true function of CatK in AAA pathogenesis.

To assess whether Ang II treatment alone may alter the peripheral inflammatory cell population, we treated both *Ctsk*<sup>+/+</sup> and *Ctsk*<sup>-/-</sup> mice with Ang II for 28 days and then analyzed peripheral blood and spleen leukocyte populations. Blood total Ly6G<sup>+</sup> neutrophils and Ly6G<sup>+</sup>CD11b<sup>+</sup> activated neutrophils<sup>37</sup> were not different between untreated *Ctsk*<sup>+/+</sup> mice and *Ctsk*<sup>-/-</sup> mice. Although Ang II infusion did not yield appreciable AAA in either *Ctsk*<sup>+/+</sup> mice or *Ctsk*<sup>-/-</sup> mice, it significantly increased blood total neutrophils and activated neutrophils in *Ctsk*<sup>+/+</sup> mice, while showing no effects on *Ctsk*<sup>-/-</sup> mice. Blood CD11b<sup>+</sup> cells (eg, monocytes, macrophages, and natural killer T cells) were significantly fewer in *Ctsk*<sup>-/-</sup> mice than in *Ctsk*<sup>+/+</sup> mice, and Ang II treatment did not affect these populations in blood (Supplemental Figure IA, available online at <http://atvb.ahajournals.org>). Blood CD4<sup>+</sup> and CD8<sup>+</sup> T-cell populations were also affected by Ang II. Before Ang II infusion, *Ctsk*<sup>+/+</sup> mouse blood contained significantly fewer CD4<sup>+</sup> cells and CD4<sup>+</sup>CD25<sup>+</sup> activated CD4<sup>+</sup> cells, but more CD8<sup>+</sup> cells and CD4<sup>+</sup>CD8<sup>+</sup> cells. After 28 days of Ang II infusion, all these differences were impaired at different levels (Supplemental Figure IB and IC). In spleen, Ly6G<sup>+</sup> cells, CD11b<sup>+</sup> cells, and Ly6G<sup>+</sup>CD11b<sup>+</sup> cells were similar between *Ctsk*<sup>-/-</sup> mice and *Ctsk*<sup>+/+</sup> mice. Ang II infusion increased Ly6G<sup>+</sup> and Ly6G<sup>+</sup>CD11b<sup>+</sup> cells and reduced CD11b<sup>+</sup> cells similarly in both *Ctsk*<sup>-/-</sup> mice and *Ctsk*<sup>+/+</sup> mice (Supplemental Figure IIA). Furthermore, more CD4<sup>+</sup> cells and fewer CD8<sup>+</sup> cells in *Ctsk*<sup>+/+</sup> spleen were reversed after Ang II treatment (Supplemental Figure IIB), which also reduced spleen activated CD4<sup>+</sup>CD25<sup>+</sup> cells in both *Ctsk*<sup>-/-</sup> mice and *Ctsk*<sup>+/+</sup> mice (Supplemental Figure IIC). Altered peripheral inflammatory

cell profiles in *Apoe*<sup>-/-</sup> mice after Ang II infusion therefore may have confounded AAA progression, thus obscuring the contribution of CatK.<sup>36</sup>

Although we did not examine them in this study, cysteinyl cathepsins induce cell apoptosis by cleaving the antiapoptotic protein Bcl-2 member Bid and creating proapoptotic signal for mitochondrial cytochrome C release.<sup>38</sup> For example, CatB-deficient mice showed reduced cerebral cell losses, whereas a mutation on cystatin resulted in increased apoptosis of cerebellar granule cells.<sup>39</sup> Osteoclasts from *Ctsk*<sup>-/-</sup> mice showed increased growth due to reduced apoptosis and senescence.<sup>40</sup> Reduced apoptosis in AAA lesions from *Ctsk*<sup>-/-</sup> mice may use the same mechanism. Cysteinyl cathepsins also regulate lymphocyte proliferation with complicated mechanisms. Prior studies have suggested that cathepsins promote T-cell proliferation by degrading matrix proteins in the lymphoid organs,<sup>41</sup> activating transcription factor nuclear factor- $\kappa$ B,<sup>42</sup> and regulating  $\beta$ 2 integrin receptor lymphocyte function-associated antigen-1 activity.<sup>43,44</sup> Consistent with these prior studies, our study showed impaired proliferation of CD4<sup>+</sup> T cells isolated from *Ctsk*<sup>-/-</sup> splenocytes compared with those from *Ctsk*<sup>+/+</sup> splenocytes, which helped to explain reduced CD4<sup>+</sup> T cells in aortic tissues from *Ctsk*<sup>-/-</sup> mice (Figure 2B and 2C). Total CD4<sup>+</sup> cells in spleen, however, were fewer in *Ctsk*<sup>+/+</sup> mice than in *Ctsk*<sup>-/-</sup> mice. Ang II infusion reversed splenic CD4<sup>+</sup> cell profiles. Similar mechanisms may occur in the aortic wall during the progression of AAA. All such speculations merit further investigation.

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### Disclosures

None.

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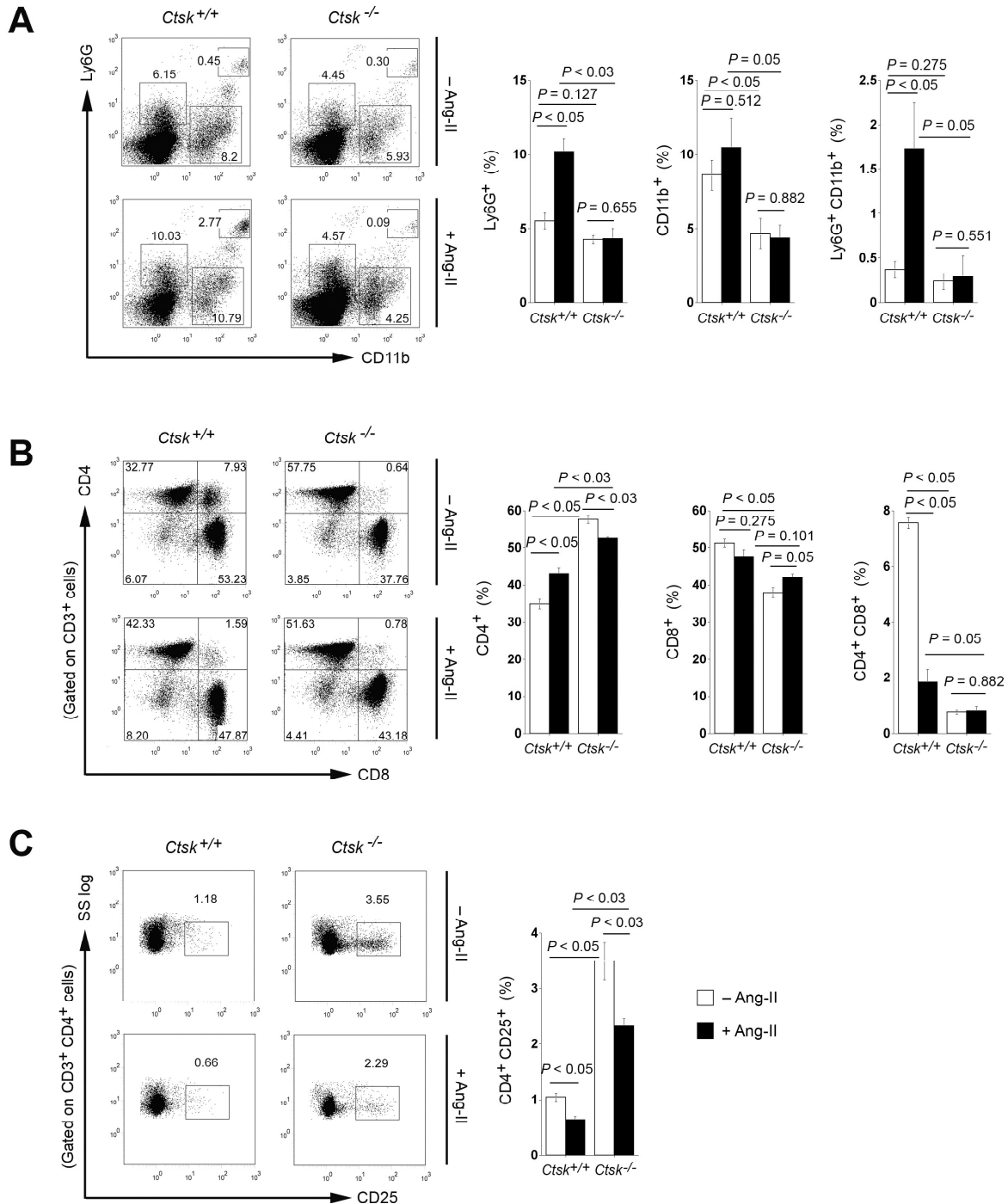
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## Supplement Material

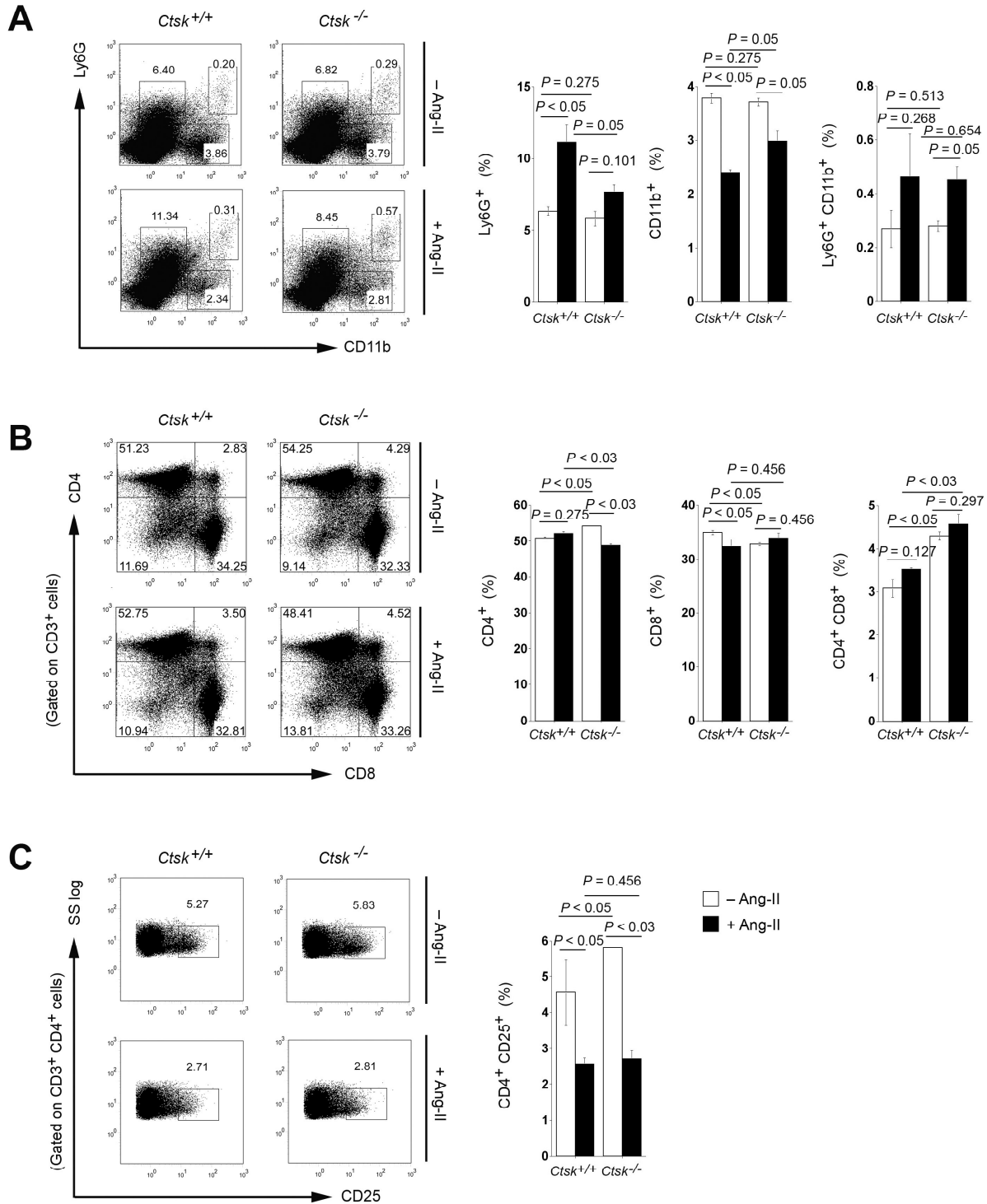
### **Cathepsin K Deficiency Reduces Elastase Perfusion-Induced Abdominal Aortic Aneurysms in Mice**

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**Supplemental Figure I.** FACS characterization of blood leukocytes from *Ctsk*<sup>+/+</sup> mice and *Ctsk*<sup>-/-</sup> mice treated with and without Ang-II. **A.** Ly6G<sup>+</sup>, CD11b<sup>+</sup>, and Ly6G<sup>+</sup>CD11b<sup>+</sup> cell percentages. **B.** CD3<sup>+</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup> cell percentages. **C.** CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> cell percentage. SS log: side scatter log value. Representative FACS for each genotype and treatment is shown to the left. Data are mean ± SE from 3~5 mice per group. *P* < 0.05 is considered statistically significant; Mann-Whitney *U* test.



**Supplemental Figure II.** FACS characterization of spleen leukocytes from *Ctsk*<sup>+/+</sup> mice and *Ctsk*<sup>-/-</sup> mice treated with and without Ang-II. **A.** Ly6G<sup>+</sup>, CD11b<sup>+</sup>, and Ly6G<sup>+</sup>CD11b<sup>+</sup> cell percentages. **B.** CD3<sup>+</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup> cell percentages. **C.** CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> cell percentage. SS log: side scatter log value. Representative FACS for each genotype and treatment is shown to the left. Data are mean ± SE from 3~5 mice per group. *P* < 0.05 is considered statistically significant; Mann-Whitney *U* test.