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# Mast Cell Tryptase Deficiency Attenuates Mouse Abdominal Aortic Aneurysm Formation

Jie Zhang,\* Jiusong Sun,\* Jes S. Lindholt, Galina K. Sukhova, Mark Sinnamon, Richard L. Stevens, Roberto Adachi, Peter Libby, Robert W. Thompson, Guo-Ping Shi

**Rationale:** Mast cells (MCs) contribute to the formation of abdominal aortic aneurysms (AAAs) by producing biologically active mediators. Tryptase is the most abundant MC granule protein and participates in MC activation, protease maturation, leukocyte recruitment, and angiogenesis—all processes critical to AAA pathogenesis.

**Objective:** To test the hypothesis that tryptase participates directly in AAA formation.

**Methods and Results:** Immunohistochemistry demonstrated enhanced tryptase staining in media and adventitia of human and mouse AAA lesions. Serum tryptase levels correlated significantly with the annual expansion rate of AAA before ( $r=0.30$ ,  $P=0.003$ ) and after ( $r=0.29$ ,  $P=0.005$ ) adjustment for common AAA risk factors in a patient follow-up study, and associated with risks for later surgical repair or overall mortality before ( $P=0.009$ ,  $P=0.065$ ) and after ( $P=0.004$ ,  $P=0.001$ ) the adjustment. Using MC protease-6-deficient mice ( $Mcpt6^{-/-}$ ) and aortic elastase perfusion-induced experimental AAAs, we proved a direct role of this tryptase in AAA pathogenesis. Whereas all wild-type (WT) mice developed AAA at 14 or 56 days postperfusion,  $Mcpt6^{-/-}$  mice were fully protected. AAA lesions from  $Mcpt6^{-/-}$  mice had fewer inflammatory and apoptotic cells, and lower chemokine levels, than did those from WT mice. MC from WT mice restored reduced AAA lesions and lesion inflammatory cell content in MC-deficient  $Kit^{W-sh/W-sh}$  mice, but those prepared from  $Mcpt6^{-/-}$  mice did not. Mechanistic studies demonstrated that tryptase deficiency affected endothelial cell (EC) chemokine and cytokine expression, monocyte transmigration, smooth-muscle cell apoptosis, and MC and AAA lesion cysteinyl cathepsin expression and activities.

**Conclusions:** This study establishes the direct participation of MC tryptase in the pathogenesis of experimental AAAs, and suggests that levels of this protease can serve as a novel biomarker for abdominal aortic expansion. (*Circ Res.* 2011;108:1316-1327.)

**Key Words:** abdominal aortic aneurysm ■ tryptase ■ mMCP-6 ■ macrophage ■ T cell ■ apoptosis

Tryptase<sup>1</sup> is a mast cell (MC) restricted serine protease stored in abundance in the secretory granules, as an enzymatically active tetramer ionically bound to serglycin proteoglycan. The serum tryptase level is normally <1 ng/mL, but it increases in patients with systemic anaphylaxis and other inflammatory disorders when their tissue MCs become activated.<sup>2</sup> Mouse MC protease 6 (mMCP-6) is the ortholog of human tryptase.<sup>3</sup>

Tryptases have pathophysiologic functions pertinent to the development of abdominal aortic aneurysms (AAAs). Tryptase induces vascular leakage<sup>4</sup> and chemotaxis of eosinophils and neutrophils.<sup>5,6</sup> In support of these data, the injection of recombinant mMCP-6 in the mouse's perito-

neal cavity or lungs results in a marked infiltration of granulocytes at those tissue sites.<sup>7,8</sup> Moreover, mMCP-6-null ( $Mcpt6^{-/-}$ ) mice have a diminished ability to combat bacteria and helminth infections because of a defect in the rapid recruitment of granulocytes to infected tissue sites.<sup>7,9</sup> Mouse and human tryptases induce endothelial cells (ECs) and other cell types to increase their expression of numerous chemokines (eg, interleukin [IL]-8) and cytokines (eg, IL-1 $\beta$ )<sup>10</sup> by unknown mechanisms. Mouse and human tryptases also activate protease zymogens (eg, promatrix metalloproteinase-3 [pro-MMP-3]<sup>11</sup> and prourokinase<sup>12</sup>) that have been implicated in AAA formation.<sup>13,14</sup> In turn, these tryptase-activated proteases can trigger a more ex-

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Non-standard Abbreviations and Acronyms	
<b>AAA</b>	abdominal aortic aneurysm
<b>AP</b>	anteroposterior
<b>BMI</b>	body-mass index
<b>BMMC</b>	bone marrow–derived mast cell
<b>EC</b>	endothelial cell
<b>MC</b>	mast cell
<b>MCP-1</b>	monocyte chemoattractant protein-1
<b>mMCP</b>	mouse mast cell protease
<b>MMP</b>	matrix metalloproteinase
<b>PDTC</b>	pyrrolidine dithiocarbamate
<b>SDF-1<math>\alpha</math></b>	stromal cell derived factor-1 $\alpha$

tensive proenzyme activation cascade<sup>11</sup> that likely contributes to arterial wall remodeling.

MCs are numerous in the media<sup>15</sup> and adventitia<sup>16</sup> of human or murine AAA lesions. They often localize adjacent to the thrombosed vessels and neovessels,<sup>15,17</sup> and release factors that promote thrombolysis, prevent coagulation, and enhance neovessel growth.<sup>17,18</sup> The absence of MCs protects mice and rats from experimental AAA formation,<sup>16,18</sup> and the pharmacological activation or stabilization of MCs can differentially alter AAA growth in mice.<sup>18</sup> In addition to recruiting leukocytes and activating proenzymes, tryptase can act as an autocrine mediator, provoking the release of histamine and other mediators from nearby MCs.<sup>19,20</sup> Thus, tryptase and its mouse ortholog mMCP-6 might be important MC-derived effectors in AAA pathogenesis. We now report that the tryptase levels in the serum of AAA patients associate with aortic expansion. Using *Mcpt6*<sup>-/-</sup> mice, we also show that this tryptase participates in experimental AAA formation in vivo.

## Methods

See the Online Data Supplement at <http://circres.ahajournals.org> for details.

### Tryptase Detection in AAA Lesions

Paraffin-embedded human aortic sections were prepared from 10 AAA donors (5 females and 5 males; mean age, 78.80±2.05 years) and 10 non-AAA heart-transplant patients (5 females and 5 males; mean age, 41.90±4.19 years) without detectable vascular diseases, from the Department of Surgery, Washington University in St. Louis. These sections were evaluated for the presence of tryptase protein (mouse antihuman tryptase monoclonal antibody, 1:1500, Chemicon International, Inc., Billerica, MA) and elastin fiber (Verhoeff–Van Gieson staining). Human aortic tissue extracts were prepared from 3 female AAA patients and 3 female heart-transplant donors with no detectable vascular disease from the Department of Medicine, Brigham and Women's Hospital. Lysates of the resulting tissue samples were used for immunoblot analysis (30  $\mu$ g/lane) with the same tryptase antibody (1:1000). The same protein blot was reprobed using a  $\beta$ -actin antibody (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA) to affirm equal protein loading. Separate human protocols were preapproved by the Human Investigation Review Committees at Washington University in St. Louis and at Brigham and Women's Hospital.

**Table 1. Baseline Characteristics Concerning Cases and Controls: Dichotomous Variables**

Dichotomous Variables	AAA (%)	Control (%)	<i>P</i> Value*
Current smoking†	59/100 (59.0)	15/35 (42.9)	0.17
Acute myocardial infarction†	26/100 (26.0)	2/35 (5.7)	0.01
Angina pectoris	22/100 (22.0)	2/35 (5.7)	0.01
Stroke or transcatheter inhibition†	5/100 (5.0)	1/35 (2.9)	0.64
Lower limb ischemia†	7/100 (7.0)	0/35 (0.0)	0.12
Chronic obstructive pulmonary disease†	6/100 (6.0)	0/35 (0.0)	0.15
Hypertension†	15/100 (15.0)	3/35 (8.6)	0.39

AAA indicates abdominal aortic aneurysm.

\*Chi-square test or Fisher exact test.

†Hospital discharge diagnosis.

## Results

### Association of Serum Tryptase Level With Aneurysmal Progression

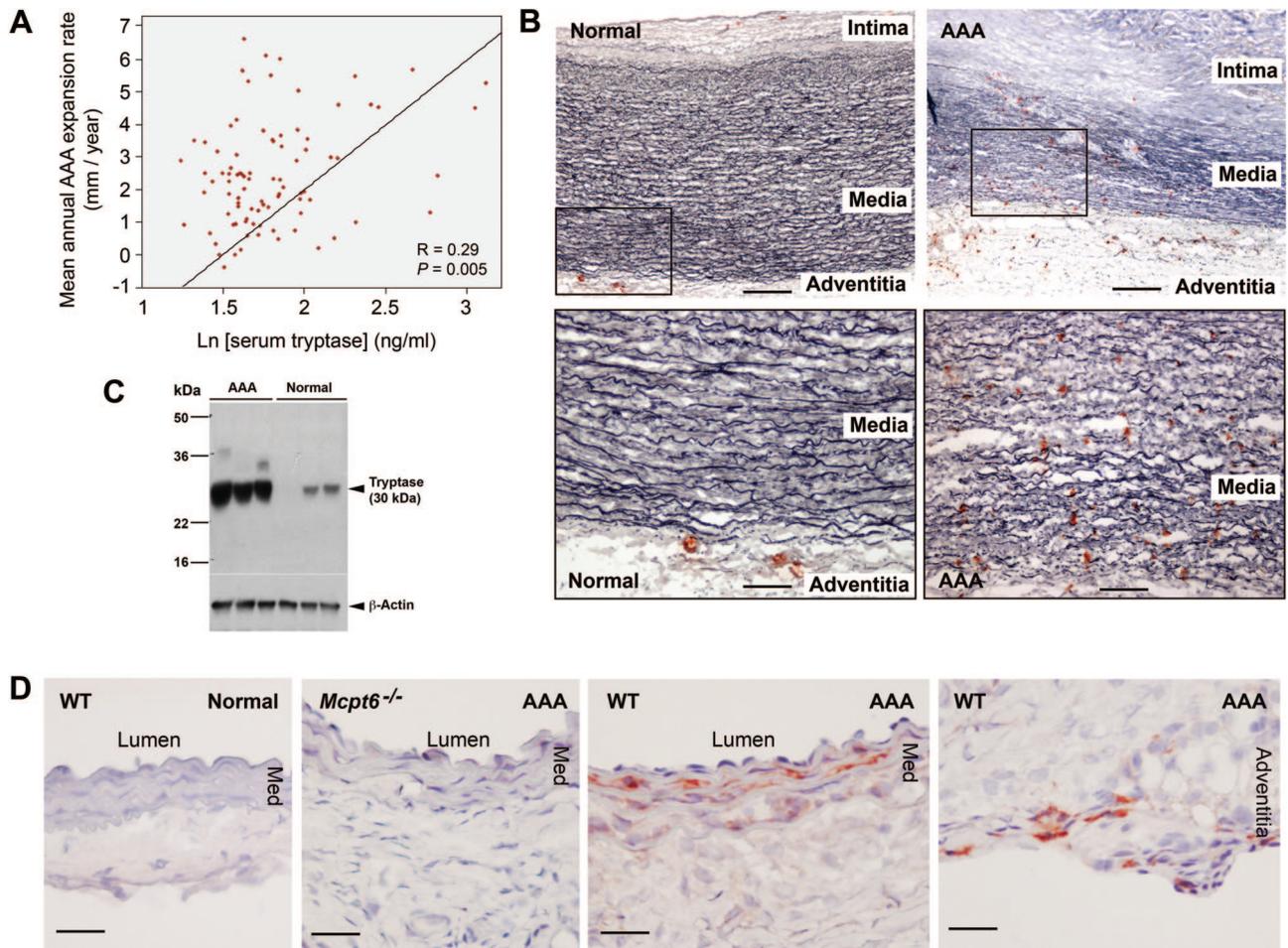
MC degranulation and inflammatory mediator release associate with murine AAA formation.<sup>18</sup> We hypothesized that patients with AAA have elevated serum MC tryptase levels. To test this hypothesis, we developed an enzyme-linked immunosorbent assay to measure human serum tryptase levels. In this study, cases of AAA and controls are from the Viborg Study—a population-based randomized screening trial of men 65 to 73 years of age.<sup>21</sup> Of the patients in this study, 100 had defined AAA, and 35 age-matched men did not. Characteristics of cases and controls are listed in Tables 1 and 2. Controls had a maximal diameter below 25 mm, with an average of 17 mm, as the background population. In comparison with controls, AAA patients had more coexisting atherosclerotic manifestations, including previous acute myocardial infarction and angina pectoris. AAA patients also had lower ankle–brachial blood pressure indices, more frequent smoking, higher body mass index (BMI), and lower pulmonary function than did control subjects. Serum tryptase levels (ng/mL) had a left-skewed distribution in these populations, and were therefore log-transformed. The mean transformed serum tryptase levels of the AAA group and the control group, and those with and without coexisting cardiovascular and pulmonary diseases, were compared by the Student *t* test. The mean log-transformed serum tryptase levels in men with and without AAA were 1.80±0.35 and 1.69±0.20 (mean ± SD, ng/mL), respectively (*P*=0.041) (Table 2). High tryptase

**Table 2. Baseline Characteristics Concerning Cases and Controls: Continuous Variables**

Continuous Variables	AAA, Mean (SD)	Control, Mean (SD)	<i>P</i> Value*
Serum tryptase (ng/mL)	103.6 (188.1)	56.2 (55.6)	0.03
Log-transformed serum tryptase (ng/mL)	1.80 (0.35)	1.69 (0.20)	0.04
Age (years)	67.9 (2.96)	67.9 (2.68)	0.74
AAA size (mm)	33.9 (4.61)	17.3 (2.14)	<0.01

AAA indicates abdominal aortic aneurysm.

\*Student *t* test.



**Figure 1. Tryptase in human and mouse abdominal aortic aneurysms (AAAs).** **A**, Correlation of serum tryptase level with annual AAA expansion rate, before ( $r=0.30$ ,  $P=0.003$ ) or after ( $r=0.29$ ,  $P=0.005$ ) adjustment for AAA confounders, Pearson's correlation test. **B**, Tryptase and elastin immunostaining in normal (left 2 panels) and aneurysmal (right 2 panels) human aortas. Bar: 400  $\mu\text{m}$ ; in inset (bottom panels), bar: 100  $\mu\text{m}$ . **C**, Human AAA and normal aortic tissue extract immunoblot for human tryptase. Tryptase monomer (30 kDa) is indicated.  $\beta$ -Actin serves as a protein loading control. **D**, Mouse mMCP-6 polyclonal antibody immunostaining in frozen sections from normal mouse aorta, AAA lesions from *Mcpt6*<sup>-/-</sup> (as negative control) and wild-type (WT) mice. Lumen, media, and adventitia are indicated. Bar: 100  $\mu\text{m}$ .

levels in the control group may be due to the high percentage of current smokers (42.9%), and because many in the control group had acute myocardial infarction, angina pectoris, stroke, or hypertension (total 25.7%), although these subjects did not have AAA (Tables 1 and 2). Patients with coexisting cardiovascular and pulmonary diseases, however, had levels of mean log-transformed serum tryptase similar to those without coexisting cardiovascular and pulmonary diseases ( $1.78 \pm 0.37$  ng/mL versus  $1.76 \pm 0.26$  ng/mL,  $P=0.714$ ).

Elevated serum tryptase levels in AAA patients suggest an association of this MC protease with AAA development. We performed a Pearson correlation analysis to test whether serum tryptase levels associate with initial AAA size at baseline or the mean annual expansion rate. Initial AAA size and tryptase levels correlated weakly and insignificantly ( $r=0.14$ ,  $P=0.106$ ). The mean annual expansion rate also correlated weakly, but significantly, with serum tryptase levels ( $r=0.30$ ,  $P=0.003$ ) (Figure 1A). These findings did not change after adjustment for other potential AAA risk factors, including AAA size, use of glucocorticoids, BMI, diastolic blood pressure, use of

low-dose aspirin, current smoking, lowest ankle-brachial blood pressure index, coexisting cardiovascular and pulmonary diseases, age, and aneurysm wall calcification of more or less than 50% of the circumference<sup>22</sup> at the maximal diameter. Serum tryptase levels still associated weakly and insignificantly with initial AAA size ( $r=0.12$ ,  $P=0.299$ ), but significantly with AAA expansion rate ( $r=0.29$ ,  $P=0.005$ ).

Because of our relatively small sample sizes, we classified serum tryptase levels into tertiles and performed Cox regression analysis to assess whether serum tryptase levels associated with subsequent AAA surgical repair or overall mortality. The crude relative risk for later surgical repair increased 1.74 times between the tertiles (hazard ratio [HR], 1.74; 95% confidence interval [CI], 1.15–2.62,  $P=0.009$ ), and further increased to 2.15 (95% CI, 1.27–3.62,  $P=0.004$ ) after adjustment for AAA size, use of glucocorticoids, BMI, diastolic blood pressure, use of low-dose aspirin, current smoking, lowest ankle-brachial blood pressure index, coexisting cardiovascular and pulmonary diseases, age, and AAA wall calcification (Table 3). When the subgroups were

**Table 3. Cox Regression Analysis for Later Need for Surgery**

Variables	B	SE	Wald	df	Sig	Exp(B)	(95.0% CI)
Before adjustment							
Tryptase (ng/mL)	0.551	0.211	6.800	1	0.009	1.735	(1.147–2.624)
After adjustment							
Tryptase (ng/mL)	0.718	0.321	5.004	1	0.004	2.151	(1.273–3.618)
MAPD (cm)	0.264	0.061	18.614	1	0.000	1.302	(1.155–1.467)
Use of glucocorticoids	–2.446	1.239	3.897	1	0.048	0.087	(0.008–0.983)
Body mass index (kg/mm <sup>2</sup> )	–0.028	0.060	0.209	1	0.648	0.973	(0.864–1.095)
Diastolic blood pressure (mm Hg)	–0.001	0.019	0.002	1	0.968	1.001	(0.964–1.039)
Low-dose aspirin	–0.709	0.501	2.002	1	0.157	0.492	(0.184–1.314)
Current smoking	0.089	0.471	0.036	1	0.850	1.093	(0.434–2.753)
Abi	0.094	1.136	0.007	1	0.934	1.098	(0.118–10.181)
Coex	0.715	0.517	1.911	1	0.167	2.044	(0.742–5.633)
Age (years)	0.122	0.096	1.632	1	0.201	1.130	(0.937–1.364)
AAA wall calcification	–0.656	0.458	2.051	1	0.152	0.519	(0.212–1.273)

B indicates unstandardized regression coefficient; SE, standard error of B; Wald, Wald test significance value; df, degrees of freedom; Sig, the significance value of the coefficient; Exp(B), the predicted change in the hazard for each unit increase in the covariate; CI, confidence interval; MAPD, maximal aneurysmal anteroposterior diameter; Abi, ankle-brachial blood pressure index (sensitive marker for coexisting lower limb atherosclerosis); Coex, hospital-recorded coexisting hypertension, pulmonary obstructive disease, and cardiovascular disease; AAA wall calcification, degree of wall calcification at the maximal circumference of the abdominal aortic aneurysm.

based on the median, quartiles, or quintiles, the significant association between serum tryptase levels and the need for later repair persisted: median (HR, 1.94; 95% CI, 1.001–3.741,  $P=0.049$ ), quartiles (HR, 1.43; 95% CI, 1.07–1.94,  $P=0.017$ ), and quintiles (HR, 1.39; 95% C.I., 1.09–1.765,  $P=0.008$ ). We also found that serum tryptase levels correlated with overall mortality in this population, although less profoundly. The crude relative risk for dying increased 43% between the tertiles (HR, 1.43; 95% CI, 0.98–2.10,  $P=0.065$ ), but further increased to more than 3 times between the tertiles (HR, 3.17; 95% C., 1.60–6.30,  $P=0.001$ ) after adjustment for the mentioned potential AAA risk factors (Table 4).

### Increased Tryptase Expression in Human AAA Lesions

Significantly higher serum tryptase levels in AAA patients, in comparison with non-AAA subjects, suggested that AAA lesions might have higher tryptase expression than normal aortas. Immunostaining of aortic sections from AAA lesions and from non-AAA donors showed high tryptase immunoreactivity in the adventitia and media (as determined by Verhoeff–Van Gieson elastin staining) of human AAA lesions. The adventitia of healthy aortas contained only a few tryptase-positive MCs (Figure 1B). Immunoblot analysis of aortic tissue extracts revealed similar tryptase expression patterns. AAA tissue extracts contained more of the 30-kDa human tryptase than those prepared from normal aortas (Figure 1C). These observations agree with the elevated serum tryptase levels in AAA patients and their significant correlations with subsequent AAA surgery (Table 3) or death (Table 4).

### Tryptase Deficiency Reduced AAA Formation in Mice

As discussed, tryptases trigger MC activation, stimulate inflammatory cell infiltration, and activate AAA-pertinent proteases, compatible with their participation in AAA formation. Experiments in mice subjected to aortic elastase perfusion showed increased expression of mMCP-6 and an essential role of this MC tryptase in AAA development. At 14 days postperfusion, all wild-type [WT] mice developed AAA, but no *Mcpt6*<sup>–/–</sup> mice did. Aortic sections from healthy mice or those from *Mcpt6*<sup>–/–</sup> mice 14 days postperfusion, immunostained with a rabbit anti-mMCP-6 polyclonal antibody,<sup>9,23</sup> showed no mMCP-6 protein. The same antibody, however, detected mMCP-6 expression in both the media and adventitia in aortic sections from WT mice 14 days post—elastase perfusion (Figure 1D) as in human AAA lesions (Figure 1B). Tryptase mMCP-6-positive mast cells in the media in aortic sections from WT mice were further confirmed with anti-mouse c-Kit (CD117) monoclonal antibody-mediated immunostaining (data not shown). We extended the time point to 56 days. Aortic expansion increased by >150% in WT mice, but the aortas of *Mcpt6*<sup>–/–</sup> mice showed less ectasia (Figure 2A). In comparison with WT mice, at 7 days, 14 days, and 56 days, *Mcpt6*<sup>–/–</sup> mice had significantly reduced AAA lesion accumulation of macrophages (Figure 2B) and T cells (Figure 2C). Lesions of *Mcpt6*<sup>–/–</sup> mice contained more media smooth-muscle cells (SMC) than those of WT mice at the 7-day time point (Figure 2D). Reduced inflammatory cell numbers correlated with low MHC class II–positive areas in *Mcpt6*<sup>–/–</sup> mouse lesions at all 3 time points (Figure 2E). Thus, tryptase deficiency impaired inflammatory cell accumulation in AAA lesions. This conclusion agrees with the

**Table 4. Cox Regression Analysis for Relative Risk of Dying Later**

Variables	B	SE	Wald	df	Sig	Exp(B)	(95.0% CI)
Before adjustment							
Tryptase (ng/mL)	0.359	0.195	3.406	1	0.065	1.432	(0.978–2.097)
After adjustment							
Tryptase (ng/mL)	1.154	0.350	10.854	1	0.001	3.172	(1.596–6.303)
MAPD (cm)	0.158	0.054	8.454	1	0.004	1.171	(1.053–1.302)
Use of glucocorticoids	–1.860	1.219	2.330	1	0.127	0.156	(0.014–1.696)
Body mass index (kg/mm <sup>2</sup> )	–0.002	0.028	0.003	1	0.956	0.998	(0.945–1.055)
Diastolic blood pressure (mm Hg)	–0.040	0.021	3.465	1	0.063	0.961	(0.921–1.002)
Use of aspirin	0.987	0.588	2.815	1	0.093	2.682	(0.847–8.494)
Current smoking	1.119	0.554	4.079	1	0.043	3.063	(1.034–9.074)
ABI	–2.134	1.641	1.692	1	0.193	0.118	(0.005–2.950)
Coex	0.049	0.509	0.009	1	0.924	1.050	(0.387–2.845)
Age (years)	0.258	0.098	6.883	1	0.009	1.295	(1.068–1.570)
AAA wall calcification	–1.114	0.483	5.328	1	0.021	0.328	(0.127–0.845)

B indicates unstandardized regression coefficient; SE, standard error of B; Wald, Wald test significance value; df, degrees of freedom; Sig, the significance value of the coefficient; Exp(B), the predicted change in the hazard for each unit increase in the covariate; CI, confidence interval; MAPD, maximal aneurysmal anteroposterior diameter; Abi, ankle–brachial blood pressure index (sensitive marker for coexisting lower limb atherosclerosis); Coex, hospital-recorded coexisting hypertension, pulmonary obstructive disease, and cardiovascular disease; AAA wall calcification, degree of wall calcification at the maximal circumference of the abdominal aortic aneurysm.

finding of significantly reduced levels of the chemokine MCP-1 in AAA lesions from *Mcpt6*<sup>−/−</sup> mice in comparison with WT mice (Figure 2F). Elastin fragmentation characterizes human or murine AAA.<sup>24</sup> Although elastin fragmentation increased over time in WT mice, it did not in *Mcpt6*<sup>−/−</sup> mice. At 56 days postperfusion, aortas from WT mice showed more elastin fragmentation than did those from *Mcpt6*<sup>−/−</sup> mice (Online Figure IA).

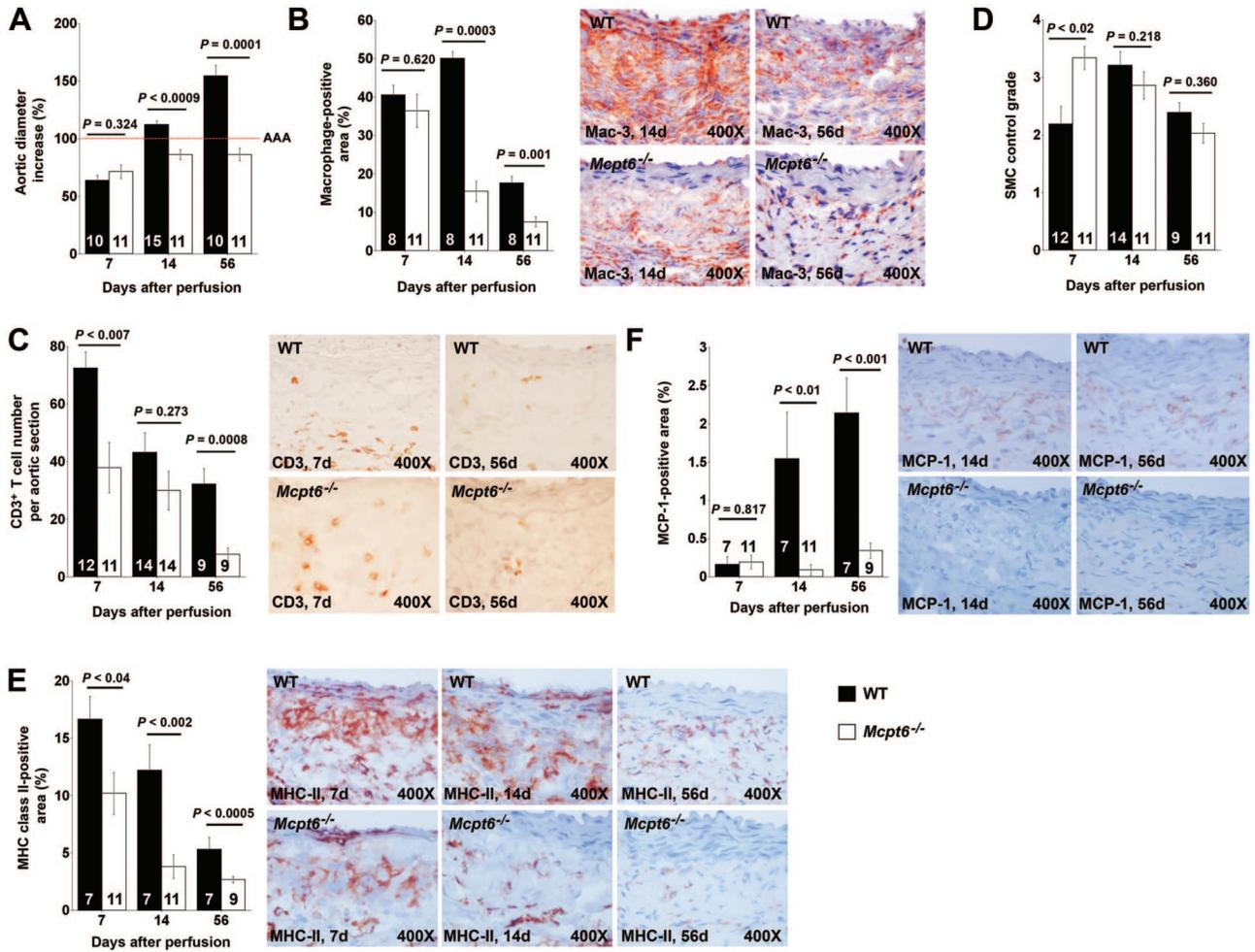
We have previously shown that MCs participate in AAA formation by promoting aortic SMC apoptosis.<sup>18</sup> At 7 days postperfusion, lesions of *Mcpt6*<sup>−/−</sup> mice had fewer total apoptotic cells (Online Figure IB, mainly infiltrated inflammatory cells) and media apoptotic cells (Online Figure IC, mainly SMC) than did lesions in WT mice. These data agree with the increase of lesional SMC content in *Mcpt6*<sup>−/−</sup> mice at the same time point (Figure 2D), suggesting that most of these medial apoptotic cells were SMC, as previously described in human AAA lesions.<sup>25</sup> The 2 genotypes did not have significant differences in lesion cell apoptosis or SMC content at the 14-day or 56-day time points (Figure 2D and Online Figure IB).

Tryptase can stimulate endothelial tube formation in vitro and promote angiogenesis,<sup>26</sup> which may contribute to reduced AAA formation in *Mcpt6*<sup>−/−</sup> mice. Immunostaining AAA lesion sections for CD31 to visualize microvascular endothelial cells did not show significant differences in CD31-positive microvessel numbers between WT and *Mcpt6*<sup>−/−</sup> mice at any time point tested (Online Figure ID), suggesting a negligible role of mMCP-6 in angiogenesis in this AAA model. An in vitro aortic ring angiogenesis assay yielded similar observations. Bone marrow–derived MCs (BMMC) from WT mice or *Mcpt6*<sup>−/−</sup> mice, used as angiogenic stimuli, showed similar microvessels sprouting from the aortic rings (data not shown).

Reduced AAA formation, lesion inflammatory cell infiltration, or cell apoptosis in *Mcpt6*<sup>−/−</sup> mice did not result from less MC accumulation. Enumeration of CD117<sup>+</sup> MCs showed no significant differences between the genotypes from all 3 time points (Online Figure IE), suggesting that reduced AAA formation in *Mcpt6*<sup>−/−</sup> mice resulted from the absence of mMCP-6 from MCs. To examine this hypothesis further, we reconstituted MC-deficient *Kit*<sup>W-sh/W-sh</sup> mice with BMMC from WT and *Mcpt6*<sup>−/−</sup> mice. We have previously shown reduced AAA in *Kit*<sup>W-sh/W-sh</sup> mice in comparison with WT mice in this preparation, and reconstitution of *Kit*<sup>W-sh/W-sh</sup> mice with BMMC from WT mice restored AAA phenotypes.<sup>19</sup> Reconstitution of *Kit*<sup>W-sh/W-sh</sup> with BMMC from *Mcpt6*<sup>−/−</sup> mice conferred protection from AAA formation at the 56-day postperfusion time point (Figure 3A). Macrophage and T-cell content also increased when *Kit*<sup>W-sh/W-sh</sup> mice received BMMC from WT mice but not from *Mcpt6*<sup>−/−</sup> mice at the 14-day time point, although this difference between WT mice and *Mcpt6*<sup>−/−</sup> mice subsided at the 56-day time point (Figure 3B and 3C). In contrast, BMMC from WT and *Mcpt6*<sup>−/−</sup> mice behaved similarly in regulating CD31<sup>+</sup> microvessel growth in AAA lesions at both time points (Figure 3D).

### MCs Interact With Inflammatory and Vascular Cells

The finding of reduced leukocytes in *Mcpt6*<sup>−/−</sup> mouse AAA lesions suggested a role of mMCP-6 in leukocyte homing. To test this hypothesis in vitro, we first examined whether the absence of mMCP-6 affects MC chemokine and cytokine expression. Real-time polymerase chain reaction (RT-PCR) analysis showed no significant differences in TNF- $\alpha$ , IL-6,



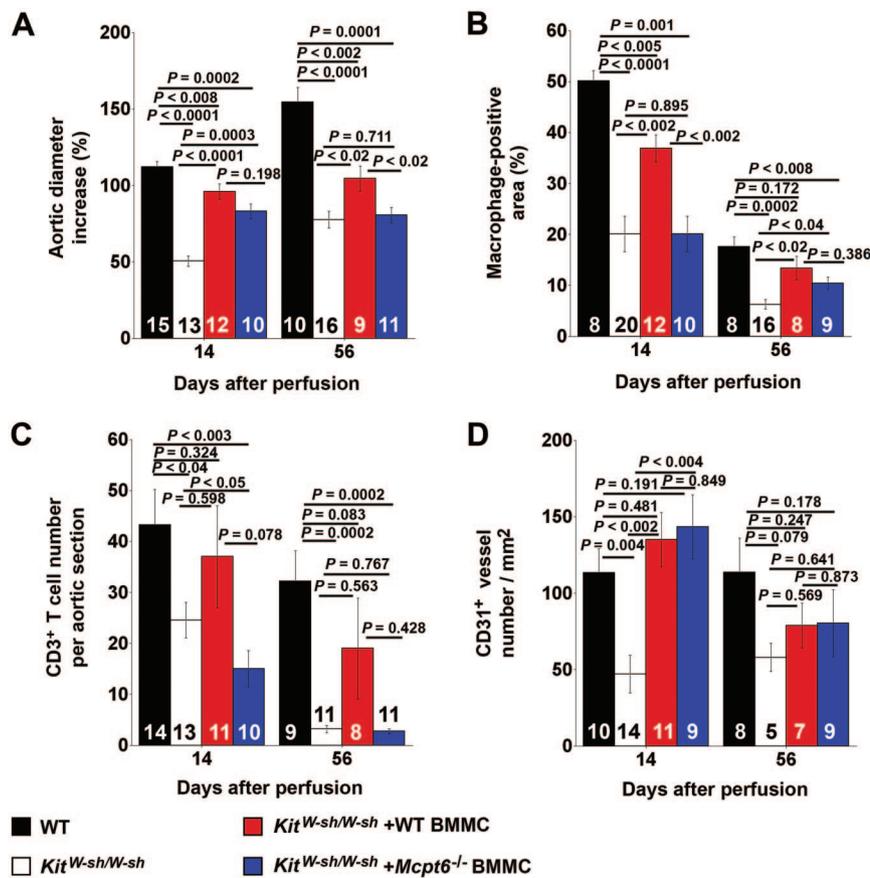
**Figure 2. Characterizations of abdominal aortic aneurysm (AAA) lesions from wild-type (WT) and *Mcpt6*<sup>-/-</sup> mice.** **A**, Aortic diameter increase (percentage). **B**, Mac-3<sup>+</sup> macrophage area (percentage). **C**, CD3<sup>+</sup> T-cell numbers. **D**,  $\alpha$ -Actin-positive smooth-muscle cell (SMC) grade. **E**, MHC class II-positive area (percentage). **F**, MCP-1-positive area (percentage). Representative images for **B**, **C**, **E**, and **F** are shown on the right. The number of mice in each experimental group is shown within each bar. Data are mean  $\pm$  SEM.  $P < 0.05$  was considered statistically significant, Mann-Whitney *U* test.

and MCP-1 expression between BMMC from WT and *Mcpt6*<sup>-/-</sup> mice (not shown). We then examined whether BMMC from WT and *Mcpt6*<sup>-/-</sup> mice behaved differently in inducing cytokine or chemokine expression in T cells, macrophages, monocytes, or neutrophils. Degranulated BMMC supernatants from WT and *Mcpt6*<sup>-/-</sup> mice again showed no significant differences in promoting TNF- $\alpha$ , IL-6, and MCP-1 expression in all aforesaid leukocytes (not shown). Yet, EC from WT mice expressed significantly higher levels of the cytokines TNF- $\alpha$  and IL-6, and the chemokines CXCL1/KC, CXCL2/MIP-2 (macrophage inflammatory protein-2), and CXCL5/LIX after stimulation with degranulated BMMC from WT mice than did BMMC from *Mcpt6*<sup>-/-</sup> mice (Figure 4A), a finding that agrees with the reduced leukocyte content of *Mcpt6*<sup>-/-</sup> AAA lesions (Figures 2B, 2C, 3B, and 3C).

To test further whether the absence of mMCP-6 directly affected monocyte transmigration, we assayed transmigration through a collagen-coated Boyden chamber cell using monocytes from WT and *Mcpt6*<sup>-/-</sup> mice. Monocytes from *Mcpt6*<sup>-/-</sup> mice transmigrated more slowly than did those from WT mice under different concentrations of the

chemokine SDF-1 $\alpha$  (Figure 4B). Reduced transmigration of monocytes from *Mcpt6*<sup>-/-</sup> mice suggested altered protease expression. Although monocytes do not express tryptase, these cells are rich sources of cysteinyl cathepsins involved in leukocyte transmigration.<sup>27,28</sup> Monocytes from *Mcpt6*<sup>-/-</sup> mice expressed significantly less mRNA encoding in all tested cathepsins—including cathepsins B, S, L, and K—than did those from WT mice (Figure 4C), as determined by RT-PCR.

We have previously shown that MCs induce aortic SMC apoptosis.<sup>18</sup> This study revealed reduced AAA media cell apoptosis in *Mcpt6*<sup>-/-</sup> mice (Online Figure IC), with concurrent increase of lesion SMC content (Figure 2D). Therefore, mMCP-6 may promote SMC apoptosis. We used postdegranulation supernatants of BMMC from WT and *Mcpt6*<sup>-/-</sup> mice to test this hypothesis. Live or degranulated WT BMMC enhanced PDTC-induced SMC apoptosis.<sup>18</sup> In contrast, the supernatants of degranulated mMCP-6-deficient BMMC showed significantly reduced induction of aortic SMC apoptosis under the same conditions (Figure 4D), supporting the contribution of tryptase mMCP-6 in this process.



**Figure 3. MC reconstitution in *Kit<sup>W-sh/W-sh</sup>* mice.** Wild-type (WT), mast cell (MC)-deficient *Kit<sup>W-sh/W-sh</sup>* mice, and those receiving bone marrow-derived MCs from WT and *Mcpt6<sup>-/-</sup>* mice were induced to produce abdominal aortic aneurysms. Aortic diameter increase (percentage) (A), Mac-3<sup>+</sup> macrophage areas (B), CD3<sup>+</sup> T-cell numbers (C), and CD31<sup>+</sup> microvessel numbers (D) were analyzed at 14 days and 56 days post-perfusion. Numbers of mice for each experimental group are shown in each bar. Data are mean ± SEM. P < 0.008 was considered statistically significant, Mann-Whitney U test.

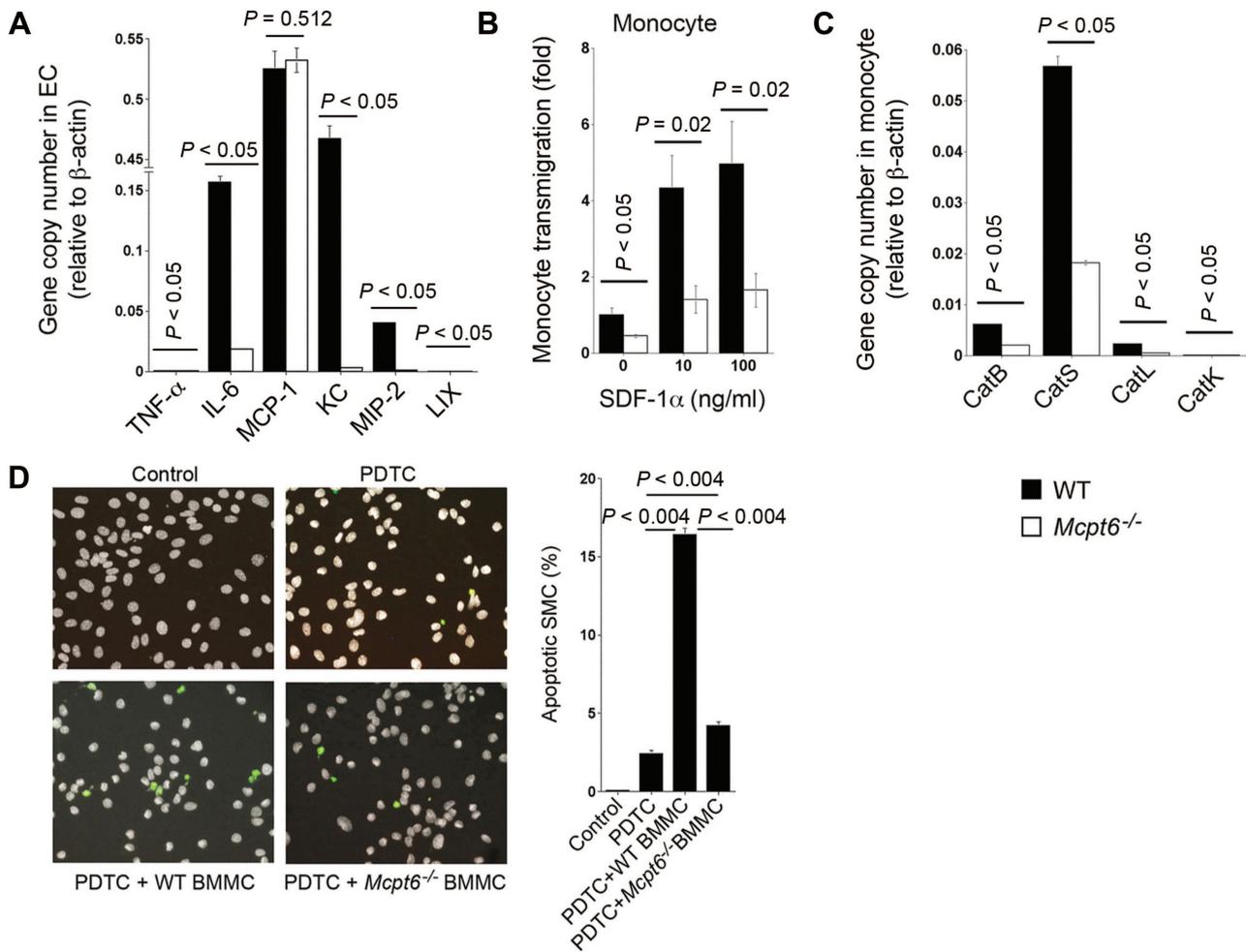
**Trypsin Deficiency Affects Cysteine Protease Cathepsin Expression and Activities**

Cysteine protease cathepsins and MMPs contribute to aortic wall remodeling. Mice lacking cysteine proteases—such as cathepsins S, K, or L—resist diet-induced atherosclerosis,<sup>27–29</sup> and MMP-9-deficient, MMP-2-deficient, or chymase mMCP-4-deficient mice show reduced experimental AAA formation.<sup>30–32</sup> As discussed, MC trypsin participates in MMP and serine protease activation,<sup>11,12</sup> processes pertinent to AAA formation. Absence of mMCP-6 may affect the expression or activation of these proteases in MCs, thereby providing an additional mechanism underlying reduced AAA formation in *Mcpt6<sup>-/-</sup>* mice. BMMC from *Mcpt6<sup>-/-</sup>* mice expressed significantly less mRNA encoding of cathepsins B, S, L, and K, but did not affect the expression of serine proteases cathepsin G, chymase mMCP-4, MMP-2, and MMP-9 mRNAs, as assessed by RT-PCR (Figure 5A). To test whether reduced cathepsin mRNA levels corresponded to altered enzymatic activities, we performed active site labeling for cysteine proteases with biotinylated JPM. BMMC cell lysates from *Mcpt6<sup>-/-</sup>* mice showed reduced cathepsin activities, in comparison with those from WT mice (Figure 5B). AAA lesions from *Mcpt6<sup>-/-</sup>* mice also had reduced activities of cysteinyl cathepsins. Using frozen AAA sections and fluorescein-conjugated elastin as substrate, we performed in situ cathepsin zymography in a buffer that was optimized for cysteine protease cathepsin activities (pH 5.5).<sup>18</sup> AAA lesions from WT mice 56 days postperfusion showed elastolytic activity in the adventitia (green fluorescence), sensitive to the

nonselective cathepsin inhibitor E64d (20 μmol/L, Sigma-Aldrich, St. Louis, MO). In contrast, the adventitia in AAA sections from *Mcpt6<sup>-/-</sup>* mice 56 days postperfusion contained much less elastolytic activity (green fluorescence), and E64d days showed negligible effect on this activity (Figure 5C), a finding consistent with reduced cathepsin activities in AAA lesions from *Mcpt6<sup>-/-</sup>* mice. Although in situ cathepsin zymography experimental and photograph shuttering conditions were the same between AAA lesions from WT and *Mcpt6<sup>-/-</sup>* mice, media green fluorescence was brighter in WT mice than in *Mcpt6<sup>-/-</sup>* mice (Figure 5C), likely from increased media inflammatory cells in WT AAA lesions (Figure 1D). To confirm increased cathepsin activities in AAA lesions from WT mice in comparison with those from *Mcpt6<sup>-/-</sup>* mice, we performed cysteinyl cathepsin active site labeling with JPM using AAA tissue extracts. AAA tissue lysates from *Mcpt6<sup>-/-</sup>* mice had reduced cathepsin activities in comparison with those from WT mice 56 days postperfusion (Figure 5D), consistent with less medial elastin degradation in AAA lesions from *Mcpt6<sup>-/-</sup>* mice than in those from WT mice at this time point (Online Figure IA).

**Discussion**

The release of undefined granular content from activated MCs contributes to arterial remodeling.<sup>18,33</sup> The granules in mouse and human MCs contain substantial amounts of different types of neutral proteases. In particular, the

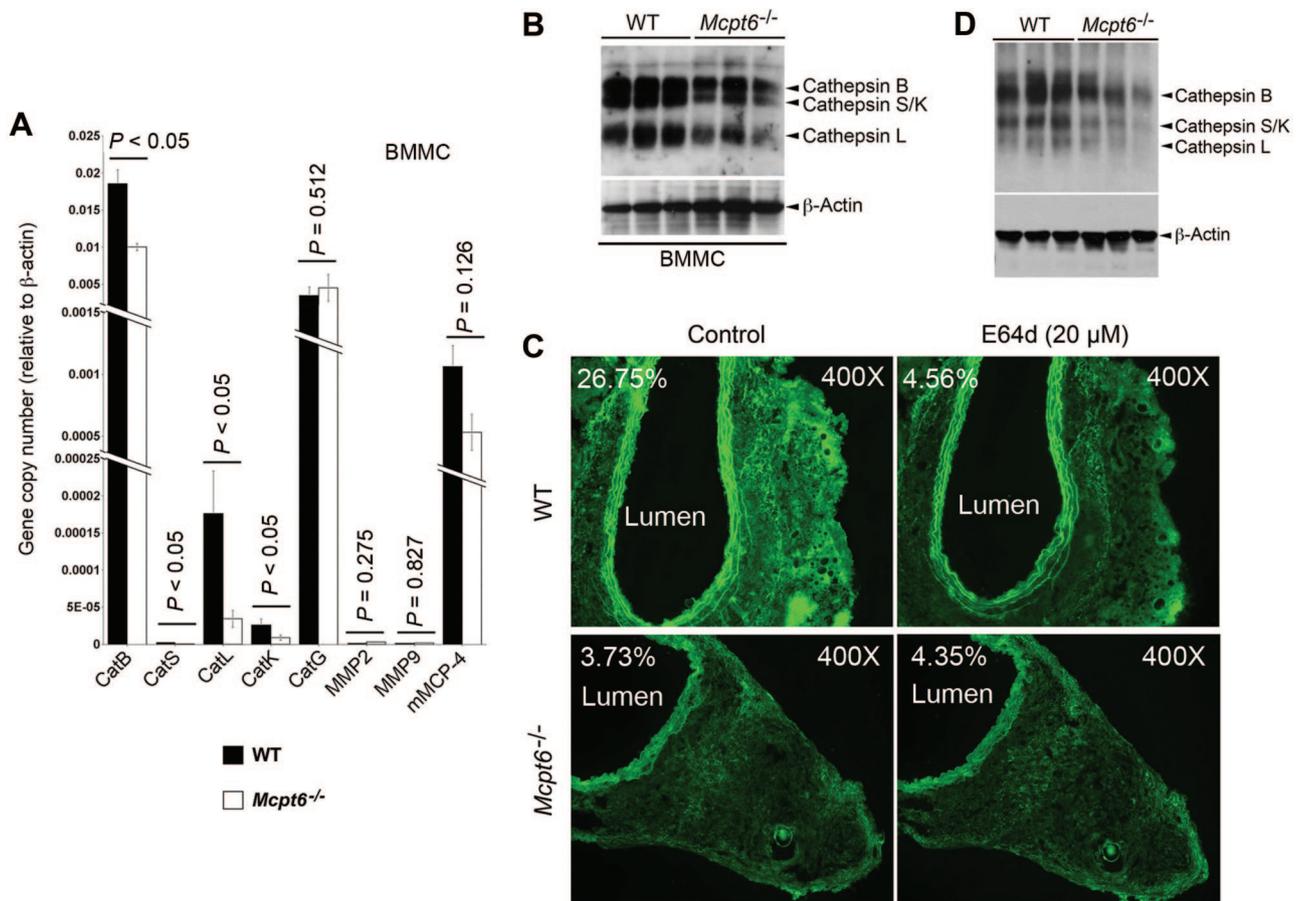


**Figure 4. EC cytokine and chemokine expression, monocyte migration, and smooth muscle cell apoptosis.** **A**, RT-PCR analysis of cytokine and chemokine expression in mouse aortic EC after incubation with degranulated bone marrow–derived mast cells (BMMC) from wild-type (WT) and *Mcpt6*<sup>-/-</sup> mice. Data are mean±SE of 3 experiments. **B**, Peripheral monocyte transmigration assay under different concentrations of SDF-1a. Data are mean±SE of 6 experiments. **C**, RT-PCR determines expression of cathepsins B, S, L, and K in peripheral blood monocytes from WT and *Mcpt6*<sup>-/-</sup> mice. Data are mean±SE of 3 experiments. *P*<0.05 was considered statistically significant, Mann–Whitney *U* test. **D**, Mouse aortic smooth muscle cell apoptosis after induction with PDTC with or without BMMC from WT or *Mcpt6*<sup>-/-</sup> mice. Representative panels are shown on the left. Green fluorescent cells indicate TUNEL-positive apoptotic cells. *P*<0.02 was considered statistically significant, Mann–Whitney *U* test.

chymase and trypsin families of MC-restricted serine proteases participate in pathological events, such as atherosclerosis,<sup>34</sup> that pertain to AAA pathogenesis. We have previously shown that the chymase mMCP-4 contributes to AAA by affecting lesion leukocyte infiltration, apoptosis, elastin degradation, and angiogenesis.<sup>31</sup> The absence of mMCP-4 (but not its chromosome 14C3 family member mMCP-5) protected mice from elastase perfusion-induced AAA. We now show that the chromosome 17A3.3 trypsin family member mMCP-6 also contributes critically to experimental AAA formation in mice, and biomarker studies also implicate this protease in human AAA.

Identifying novel biomarkers to predict arterial expansion—not only for the aorta but also for the coronary, cerebral, and peripheral arteries—could prove useful as an investigative tool and as a clinical tool. This study showed that human serum trypsin levels correlated significantly but weakly with the aneurysmal expansion rate (*P*=0.005, *R*=0.29) (Figure 1A), suggesting that serum trypsin is

probably not useful as a stand-alone biomarker in clinical decision making. Larger and broader clinical studies in the future, however, may yield different conclusions. Statistical significances and high Exp(B) values before and after adjusting for all available risk factors in predicting AAA expansion and subsequent aortic surgery and mortality in this small patient population follow-up study (Tables 3 and 4), and the independence from traditional AAA risk factors, make this protease a compelling and attractive biomarker candidate. The present data suggest that both trypsin and chymase participate in mouse AAA development. Our prior study demonstrated that serum chymase levels also correlated with AAA expansion rate,<sup>31</sup> but not with the need for surgical repair or with death (not shown), which suggests that there are mechanistic differences between the 2 types of MC proteases. For example, BMMC from *Mcpt4*<sup>-/-</sup> mice showed impaired activity in promoting microvessel growth in an aortic ring assay, and AAA lesions from *Mcpt4*<sup>-/-</sup> mice had reduced CD31<sup>+</sup> microvessels compared with those in WT



**Figure 5. Cysteine protease cathepsin expression and activity in mast cells (MCs) and abdominal aortic aneurysm (AAA) lesions.** **A**, RT-PCR to assess the expression of common MC proteases in bone marrow–derived MCs (BMMC) from wild-type (WT) and *Mcpt6*<sup>-/-</sup> mice. Data are mean±SE of 3 experiments.  $P < 0.05$  was considered statistically significant, Mann–Whitney *U* test. **B**, Cysteine protease cathepsin active site JPM labeling with cell lysate from WT and *Mcpt6*<sup>-/-</sup> BMMC. **C**, WT and *Mcpt6*<sup>-/-</sup> mouse frozen AAA cross-section in situ elastase activity zymograph in the presence or absence of cathepsin inhibitor E64d. Lumen and percentage of fluorescence intensity are indicated. Images were obtained with the same magnification and shutter speed, and all data are from the 56-day time point experiments. **D**, Cysteine protease cathepsin active site JPM labeling with aortic tissue lysates from WT and *Mcpt6*<sup>-/-</sup> mice from the 56-day time point experiments. In **B** and **D**, active cathepsins B, S, K, and L are indicated with arrowheads. The  $\beta$ -actin blot was used to ensure equal protein loading.

mouse AAA lesions.<sup>31</sup> Although tryptase may participate in angiogenesis,<sup>26</sup> we did not find an effect of this protease in promoting microvessel growth in the aortic ring assay (data not shown), and we did not detect significant differences in CD31-positive microvessel numbers in AAA lesions from WT mice and *Mcpt6*<sup>-/-</sup> mice (Online Figure ID). MC tryptase therefore may not influence angiogenesis in this AAA preparation.

This study suggests that tryptase regulates leukocyte recruitment to lesions in experimental AAA. AAA lesions from *Mcpt6*<sup>-/-</sup> mice had fewer macrophages and T cells (Figure 2B and 2C). Reconstitution of BMMC from *Mcpt6*<sup>-/-</sup> mice did not restore lesion macrophages (Figure 3B) or T cells (Figure 3C). Although other mechanisms may participate, our data suggest that tryptase mMCP-6 stimulates vascular EC expression of chemokines CXCL1/KC, CXCL2/MIP-2, and CXCL5/LIX. This mechanism may contribute to the observed reduction in lesion leukocyte content. Yet reduced MCP-1 expression in *Mcpt6*<sup>-/-</sup> mouse AAA lesions (Figure 2F) remained unexplained. Use of BMMC from *Mcpt6*<sup>-/-</sup> mice did not show any effect of this tryptase isoform on MCP-1

expression in any tested cell types, including EC, T cells, macrophages, monocytes, and neutrophils (not shown). Therefore, tryptase may exert indirect effects on lesion MCP-1 expression, including a reduced ability of *Mcpt6*<sup>-/-</sup> BMMC to induce EC expression of KC, MIP-2, and LIX (Figure 4A), impaired cathepsin expression, and transmigration of monocytes from *Mcpt6*<sup>-/-</sup> mice (Figure 4B and 4C), all which may result in fewer macrophages in AAA lesions and thus account for lower levels of MCP-1.

We have long been interested in why the absence of one protease affects the expression or activities of the others. All tested cathepsins showed lower expression in BMMC from *Mcpt6*<sup>-/-</sup> mice than in those from WT mice (Figure 5A and 5B). Although not tested in this study, tryptase is known to induce mitogen-activated protein kinase (MAPK) activation in human eosinophils. Phosphorylation of extracellular signal-regulated kinase-1 and -2 (ERK1/2), MAPK p38, and Jun N-terminal kinase-1 and -2 (JNK1/2) occur within 3 minutes after incubation with 50 ng/mL of recombinant human skin tryptase.<sup>35</sup> Conditional activation of ERK can induce the expression of CatB and CatL in 3T3 and K1735

cells.<sup>36</sup> The receptor activator of NF- $\kappa$ B ligand (RANKL)-induced CatK expression during osteoclastogenesis depends on p38 MAPK.<sup>37</sup> The N-terminal telepeptide of collagen type II enhances expression of cathepsins B, K, and L in articular chondrocytes, and also associates with the activation of p38 MAPK.<sup>38</sup> Tryptase may control cathepsin expression in BMMC (Figure 5A) and in other inflammatory cells and vascular cells by regulating MAPK activation. This study tested whether reduced cathepsins in tryptase-deficient MCs resulted from decreased chymase expression. Although BMMC from *Mcpt6*<sup>-/-</sup> mice expressed less mMCP-4 than did those from WT mice, this difference did not reach statistical significance. Cathepsin activities fell not only in MCs, but also in AAA lesions from *Mcpt6*<sup>-/-</sup> mice. Reduced cathepsin expression and activity may result in part from fewer leukocytes in AAA lesions; leukocytes are a rich source of proteases and inflammatory stimuli that are required for vascular cell protease expression.<sup>39,40</sup> The low MHC class II levels in *Mcpt6*<sup>-/-</sup> mouse lesions support this possibility (Figure 2E). Reduced cathepsin expression and activity in *Mcpt6*<sup>-/-</sup> mice BMMC and aortic tissue extracts (Figure 5) did not explain fully the aortic wall elastin fragmentation. Increased T-cell content at 7 days postperfusion and increased macrophage content after 14 days in WT mice relative to *Mcpt6*<sup>-/-</sup> mice did not affect elastin degradation grades. Indeed, *Mcpt6*<sup>-/-</sup> mice showed more elastin degradation than did WT mice at the 7-day time point (Online Figure 1A). We currently lack a good explanation for this finding, although absence of tryptase may lead to compensatory increase of other elastases at this time point, and this speculation merits further investigation. This study also demonstrated that the absence of mMCP-6 affects monocyte cathepsin expression (Figure 4C). This finding is perplexing, because mMCP-6 is a MC-specific protease and should not affect monocyte gene expression. These monocytes, isolated from peripheral blood, may have encountered a different environment. In humans, blood tryptase level ( $\approx 100$  ng/mL) is  $\approx 10$ -fold that of chymase ( $\approx 10$  ng/mL),<sup>31</sup> 20-fold that of cathepsin S ( $\approx 5$  ng/mL),<sup>41</sup> and  $>20$ -fold that of cathepsin L ( $< 5$  ng/mL).<sup>42</sup> A high blood tryptase level may affect blood cells, such as monocyte gene expression. Indeed, tryptase promotes human EC MCP-1 and IL-8 expression.<sup>43</sup> This study demonstrated that tryptase stimulated mouse EC expressions of IL6, KC, MIP-2, and LIX (Figure 4A). Tryptase in blood, therefore, may have more complex functions than previously appreciated.

Reduced apoptosis and enhanced SMC content in AAA lesions from *Mcpt6*<sup>-/-</sup> mice at the 7-day time point suggested that tryptase could promote SMC apoptosis (Figure 2D, Online Figure 1B and 1C). Study of cultured SMC affirmed this hypothesis (Figure 4D). At later time points (14 days and 56 days postperfusion), however, tryptase expression did not affect AAA lesion SMC loss and apoptosis. Our current data do not explain these discrepant changes. As discussed, tryptase induces cysteinyl cathepsin expression via the MAPK pathway.<sup>35-38</sup> These cathepsins induce cell apoptosis by cleaving the antiapoptotic protein Bcl-2 member Bid and creating a proapoptotic signal for mitochondrial cytochrome C release.<sup>44</sup> Observations in several cathepsin mutant mouse

cells support this hypothesis,<sup>45,46</sup> but tryptase inhibits Fas-induced fibroblast apoptosis in a concentration-dependent manner, likely via the Rho kinase pathway.<sup>47</sup> One mechanism may predominate over the other at different stages of AAA progression; this speculation requires experimental confirmation.

This study provided evidence from human AAA patients and mice with experimental AAA supporting the direct participation of MC tryptase in AAA pathogenesis. Although more mechanisms remain undiscovered, tryptase regulates EC cytokine and chemokine expression, leukocyte migration, SMC apoptosis, and AAA-pertinent cysteinyl cathepsin expression in lesions. All of these functions may contribute to AAA formation. Significant correlation of serum tryptase levels with subsequent surgery and overall mortality indicate that serum tryptase could serve as a biomarker of AAA expansion, and that pharmacological inhibition of tryptase activity might benefit AAA patients.

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### Disclosures

None.

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## Novelty and Significance

### What Is Known?

- Mast cells contribute to the pathogenesis of both atherosclerosis and abdominal aortic aneurysm (AAA) by releasing inflammatory mediators to affect neighboring inflammatory and vascular cells.
- The serine proteases chymase and tryptase both derive uniquely from mast cells.
- Mast cell chymase contributes to AAA formation, and the absence of chymase protects mice from AAA in an experimental model.

### What New Information Does This Article Contribute?

- Patients with AAA have significantly higher serum mast cell tryptase levels than controls, and tryptase levels correlate with subsequent need for surgery and overall mortality.
- The absence of tryptase protects mice from aortic elastase perfusion-induced experimental AAA.
- Mast cell tryptase contributes to AAA formation by regulating inflammatory cell infiltration, smooth muscle cell (SMC) apoptosis, cysteinyl cathepsin expression, and arterial remodeling.

Studies from human samples and experimental animals demonstrate that mast cells participate directly in cardiovascular diseases. Using mast cell–deficient mice, several studies have

shown that mast cells contribute to both atherosclerosis and AAA by releasing granular mediators to induce vascular cell protease expression, and consequently promoting vasculature remodeling. Tryptase constitutes one of the most abundant human mast cell granular proteins and is important in mast cell activation, inflammatory cell recruitment, SMC proliferation, protease activation, and angiogenesis. These properties are consistent with participation in AAA formation. This study demonstrates that serum tryptase may serve as a novel biomarker for AAAs. Increased serum tryptase concentrations in AAA patients correlated significantly with subsequent surgical repair and overall mortality in a follow-up study. In elastase perfusion-induced experimental AAA, absence of tryptase (mMCP-6) protected mice from AAA progression. Mechanistic studies demonstrated that tryptase contributes to AAA formation by regulating inflammatory cell (macrophage and T cell) recruitment, SMC apoptosis, cysteinyl cathepsin expression, and aortic wall elastin degradation. Although larger AAA population follow-up studies are required to confirm our observations, measurements of serum tryptase level may assist future clinical decision making in treating patients with AAA, and these protease inhibitors may be useful in treating AAA patients.

## On-Line Supplemental Materials and Methods

### Patients and Serum Tryptase Measurements

In 1994, half (n=4404) of all males in Viborg County, Denmark aged 65–73 years were invited to undergo B-mode-ultrasonographic screening for abdominal aortic aneurysms (AAA) at Viborg Hospital. All participants were informed, interviewed, and examined (including a rescan) by the trial doctors. An AAA was defined as an infrarenal aortic diameter of 30 mm or more, and patients with an AAA >50 mm were referred for surgery. Patients with an AAA of 30–49 mm were offered yearly follow-up examinations to check for expansion.<sup>1</sup> AAA expansion was calculated by individual linear regression analysis due to the existence of more than two observations and variability of the measurements. Every patient with an AAA consulted a trial doctor for information, examination, and a rescan. Two observers were used. Their arithmetic interobserver variability of the measurements was 1.4 mm.<sup>2</sup> To reduce the diurnal variations of the serological variables, all samples were taken between 9:00 a.m. and 12:00 p.m. by the screening team. Because it was impossible to do this during the daily screening sessions, sampling days were arranged within 10 days of the initial scan. Only subjects living less than 30 km from the hospital were asked to donate blood for analyses, and two of these patients refused sampling. A total of 13 subjects had no blood samples taken. In addition, blood samples were taken from a small group of age-matched men without an AAA (n=35), as the non-AAA control group. Existing cardiovascular and pulmonary comorbidities among all participants were identified through the national registry of hospital admission.<sup>3,4</sup> Of the 4404 invited men, 3344 (76%) underwent screening, and 141 (4.2%) were diagnosed with AAA; 19 had AP diameters >50 mm and therefore were referred for surgery. The remaining 122 subjects were offered annual control scans or were referred for surgical repair if the AP diameters expanded to >50 mm during the follow-up monitoring (n=64). Ten cases were lost to follow-up due to death or severe illness during the first year, and 12 did not complete the study, leaving 100 cases for analyses. Their ages, AAA sizes, and smoking statuses were not different from those not included in the study.

To investigate the correlation of AAA expansion rates to serum total tryptase levels (both  $\alpha$ - and  $\beta$ -tryptase), we developed a tryptase ELISA system. In brief, anti-tryptase polyclonal antibody (1:5000, Calbiochem, La Jolla, CA) was used for plate coating. After blocking the ELISA plate with 3% bovine serum albumin, purified human tryptase (Calbiochem) as standard and serum samples (1:1 in 1xPBS) were added to the plate, followed by incubation for 2 hours at room temperature. Mouse anti-human tryptase monoclonal antibody (1:3000, AbD Serotec, Raleigh, NC), which recognized both human  $\alpha$ -tryptase and  $\beta$ -tryptase, was used as the detecting antibody for 1 hour at room temperature. The plate was washed, incubated with HRP-conjugated goat anti-mouse IgG (1:2000, KPL, Gaithersburg, MD), developed in TMB solution (Thermo Scientific, Waltham, MA), and read at 450 nm. Serum tryptase levels may differ from those of plasma due to blood coagulation and cell lysis. Thus, we prepared both serum and plasma samples from 30 donors. Tryptase ELISA did not show significant differences between preparations (plasma vs. serum: 15.06 $\pm$ 6.66 ng/mL vs. 13.80 $\pm$ 8.23 ng/mL, mean $\pm$ SE,  $P>0.05$ ).

### Mouse AAA Production and Lesion Characterization

Eight- to ten-week-old male WT C57BL/6 mice (Jackson Laboratory, Cat# 000664) and *Mcpt6*<sup>-/-</sup> C57BL/6 mice (also in congenic C57BL/6 background, N>10)<sup>5</sup> underwent aortic perfusion with 0.411 U/ml type I porcine pancreatic elastase (Cat# E1250, Sigma, St. Louis, MO) to produce experimental AAA, as previously described.<sup>6</sup> Although the

MCs in most mouse strains express two tetramer-forming tryptases known as mMCP-6 and mMCP-7, WT C57BL/6 mice constitutively lack mMCP-7 because of a premature translation-termination codon.<sup>7</sup> Thus, mMCP-6 is the only MC-restricted, tetramer-forming tryptase in this mouse strain.<sup>8</sup> Mice were first anesthetized by intraperitoneal injection of pentobarbital (60 mg/kg). Pre-perfusion and immediate post-perfusion (5 minutes after perfusion restoration) aortic diameters were measured to a resolution of 0.1 mm. Analgesic buprenorphine (0.1 mg/kg) was applied by intraperitoneal injection at the end of the procedure and every 12 hours for 2 days. Aortic diameters were also measured before aorta harvesting at 7 days, 14 days, and 56 days post-perfusion. Aortic diameter expansion  $\geq 100\%$  of that before perfusion defined AAA.<sup>6</sup> Mouse protocol #03759 was pre-approved by the Standing Committee on Animals of Harvard Medical School. Each mouse aorta was isolated for frozen section preparation (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 mM sodium acetate, and 1 mM EDTA.<sup>11</sup> Frozen sections were used for immunostaining for macrophages (Mac-3), SMCs ( $\beta$ -actin), T cells (CD3), major histocompatibility complex class II (MHC class II), apoptotic cells (TUNEL), microvessels (CD31), MCs (Kit/CD117), elastin degradation (Verhoeff-van Gieson), and MCP-1 levels. SMC content and elastin fragmentation were graded as described previously.<sup>9,10</sup> T cells, apoptotic cells, MCs, and microvessels were counted blindly. Positive areas for macrophages, MHC class II, and MCP-1 were measured using computer-assisted image analysis software (Image-Pro Plus; Media Cybernetics, Bethesda, MD).

### **BMMC Preparation and *Kit*<sup>W-sh/W-sh</sup> Reconstitution**

BMMCs were generated, as previously described, by culturing bone marrow cells in medium supplemented with IL-3.<sup>9,10</sup> For *Kit*<sup>W-sh/W-sh</sup> mice reconstitution, recipient mice at 5 weeks of age were given  $1 \times 10^7$  of BMMCs from WT mice or *Mcpt6*<sup>-/-</sup> mice, to the tail vein. Five weeks after BMMC reconstitution, mice underwent elastase perfusion. Mouse abdominal aortas were harvested after 14 or 56 days for lesion characterization.

### **Real Time qPCR Analyses**

qPCR analyses were used to determine the levels of varied protease transcripts in BMMCs. Total RNA was extracted from WT and mMCP-6-null mBMMCs using TRIzol reagent (GIBCO) and then treated with RNase-free DNase (Ambion) to remove residual genomic DNA contamination. Equal amounts of RNA were reverse-transcribed, and qPCR assays were performed in a single-color qPCR detection system (Stratagene). The level of each protease transcript was normalized to that of  $\beta$ -actin.

A qPCR approach also was used to evaluate the effects of MC products on chemokine and cytokine expression in treated ECs (TNF- $\alpha$ , IL-6, MCP-1, KC, MIP-2, and LIX), T cells, peritoneal macrophages, monocytes, and neutrophils (TNF- $\alpha$ , IL-6, MCP-1) at the mRNA level. BMMCs ( $3 \times 10^6$ /mL) from WT and *Mcpt6*<sup>-/-</sup> mice were incubated for 2 hours at 37 °C with compound 48/80 to trigger degranulation (Sigma, 100 ng/mL in RPMI-1640). The resulting supernatants from the activated BMMCs were diluted in cell culture medium (1:10) and used to culture mouse EC monolayer and T-cells, macrophages, monocytes, and neutrophils ( $3 \times 10^6$ ) in 6-well plates for 4 hours, followed by RNA preparation and qPCR analysis.

### **BMMC *In Vitro* Assays**

Frozen aortic tissue sections were subjected to *in situ* zymography to evaluate cathepsin activity levels as previously described.<sup>9,10</sup> BMMCs and aortic tissue extracts were evaluated for their cysteinyl cathepsin activities using biotin-conjugated JPM, an

affinity probe that specifically and irreversibly labels all active cathepsins. Briefly, BMMCs and pulverized aortic tissues were lysed into the pH5.5 buffer. Then, 5 µg of protein from each sample were incubated with 12 mM dithiothreitol and 1 µL of JPM in 30 µL of the pH5.5 buffer for 1 hour at 37°C. The treated protein samples were then separated on a 12% SDS-PAGE, followed by immunoblot detection with horseradish peroxidase-conjugated avidin.

BMMC pro-apoptotic activity for vascular SMC apoptosis was assessed using primary cultured mouse aortic SMCs on an 8-well chamber slide. Confluent SMCs were exposed overnight to 80 µM PDTC with or without 300 µL of degranulated BMMC supernatant in DMEM (1x10<sup>6</sup> BMMC supernatant/mL) from WT or *Mcpt6*<sup>-/-</sup> mice as described.<sup>9</sup> Apoptotic cells were detected with *In Situ* Cell Death Detection Kit, according to the manufacturer's instructions (Roche Diagnostics Co.).

### Monocyte Transmigration Assays

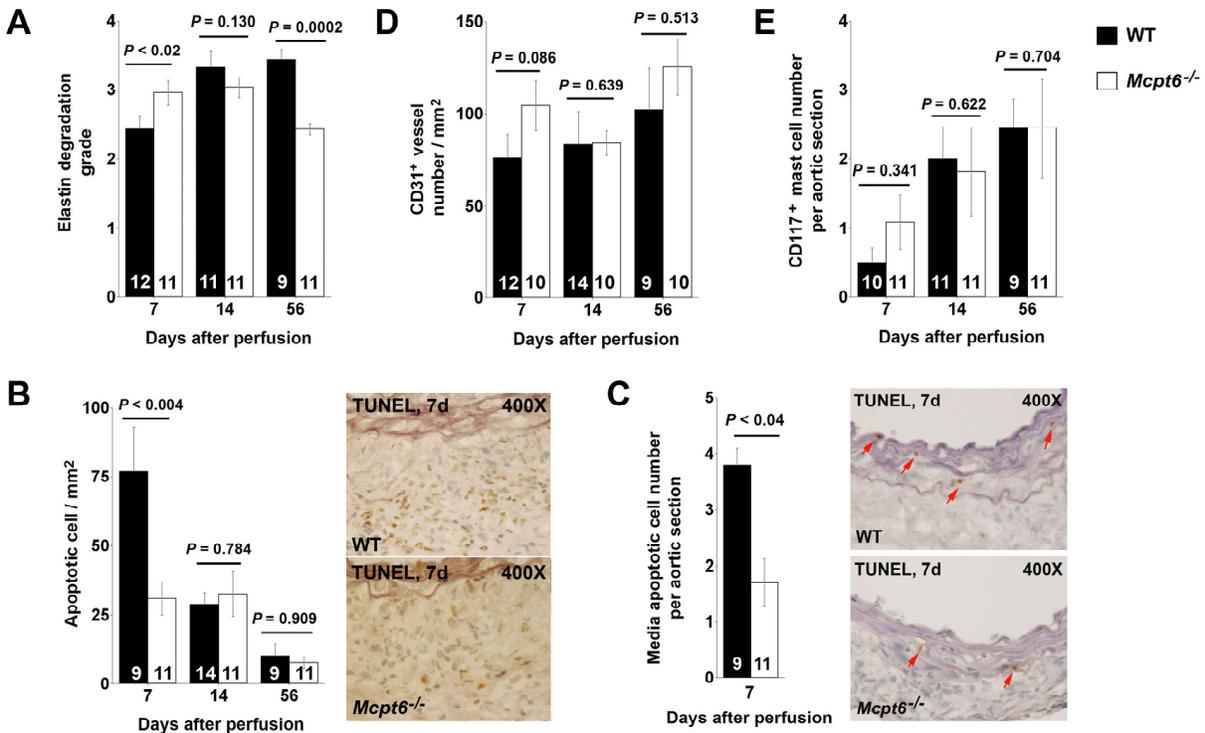
Mouse peripheral blood monocytes were prepared as previously described.<sup>11</sup> A 96-well chemotaxis plate (Neuro Probe, Inc., Gaithersburg, MD) was pre-coated with a mixture of type-IV collagen (100 ng/25µL per well) and type-I collagen (100 g/25 µL per well) (Sigma). Monocytes (25,000 in 25 µL of 1% BSA in RPMI) were then added to the collagen-pre-coated 96-well chemotaxis plate that contained 30 µL of 1% BSA (Sigma) RPMI with or without chemokine SDF-1α (0, 10, 100 ng/mL; PeproTech Inc., Rocky Hill, NJ) in the bottom chambers. After 3 hours of culture, non-translocated cells on the top transwell were removed, and transmigrated cells in the bottom chambers were mixed with CyQUANT GR (Molecular Probes) reagent and incubated at room temperature in dark for 2 hours, followed by reading of the plate with a fluorescence plate reader.

### Statistical analysis

Serum tryptase levels had a left-skewed distribution and had to be logarithmised in order to gain a normal distribution. The mean transformed serum tryptase levels of the AAA group and the control group, and those with and without coexisting cardiovascular and pulmonary disease, were compared by Student's t-test. The level of serum tryptase was correlated with initial AAA size at baseline, and the mean annual expansion rate by Pearson's correlation analysis, and adjusted for AAA size, use of glucocorticoids, BMI, diastolic blood pressure, use of aspirin, current smoking, lowest ankle-brachial blood pressure index, coexisting cardiovascular and pulmonary disease, and age in linear regression models. Finally, the serum tryptase levels were classified into tertiles, and the relative risks for later surgery or death were analysed by Cox regression analysis bivariately, and adjusted for AAA size, use of glucocorticoids, BMI, diastolic blood pressure, use of aspirin, current smoking, lowest ankle-brachial blood pressure index, coexisting cardiovascular and pulmonary disease, and age. Pearson's correlation analysis was used to test the association with serum tryptase levels, with the initial AAA size at baseline, and with the mean annual expansion rate. Cox regression analysis was used to assess serum tryptase level association with later need for AAA surgical repair or overall mortality. Non-parametric Mann-Whitney *U* test was used for all mouse data due to small sample sizes, and data were sometimes abnormally distributed. SPSS 15.0 was used as statistical software, and two-tailed *P* values below 0.05 were considered significant.

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**Supplemental Figure I.** WT and *Mcpt6*<sup>-/-</sup> mouse AAA lesion characterization. **A.** Elastin fragmentation grade. **B.** Lesion total TUNEL-positive cell numbers. **C.** Media TUNEL-positive cell numbers. Red arrows indicate media TUNEL-positive cells. **D.** CD31<sup>+</sup> microvessel numbers. **E.** CD117<sup>+</sup> MC numbers. Representative images for panels **B** and **C** are shown on the right. The number of mice in each experimental group is shown within each bar. Data are mean ± SEM.  $P < 0.05$  was considered statistically significant, Mann-Whitney *U* test.