


ORIGINAL RESEARCH

# Spermidine Suppresses Development of Experimental Abdominal Aortic Aneurysms

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**BACKGROUND:** The protective effects of polyamines on cardiovascular disease have been demonstrated in many studies. However, the roles of spermidine, a natural polyamine, in abdominal aortic aneurysm (AAA) disease have not been studied. In this study, we investigated the influence and potential mechanisms of spermidine treatment on experimental AAA disease.

**METHODS AND RESULTS:** Experimental AAAs were induced in 8- to 10-week-old male C57BL/6J mice by transient intra-aortic infusion of porcine pancreatic elastase. Spermidine was administered via drinking water at a concentration of 3 mmol/L. Spermidine treatment prevented experimental AAA formation with preservation of medial elastin and smooth muscle cells. In immunostaining, macrophages, T cells, neutrophils, and neovessels were significantly reduced in aorta of spermidine-treated, as compared with vehicle-treated elastase-infused mice. Additionally, flow cytometric analysis showed that spermidine treatment reduced aortic leukocyte infiltration and circulating inflammatory cells. Furthermore, we demonstrated that spermidine treatment promoted autophagy-related proteins in experimental AAAs using Western blot analysis, immunostaining, and transmission electron microscopic examination. Autophagic function was evaluated for human abdominal aneurysmal and nonaneurysmal adjacent aortae from AAA patients using Western blot analysis and immunohistochemistry. Dysregulated autophagic function, as evidenced by increased SQSTM1/p62 protein and phosphorylated mTOR, was found in aneurysmal, as compared with nonaneurysmal, aortic segments.

**CONCLUSIONS:** Our results suggest that spermidine supplementation limits experimental AAA formation associated with preserved aortic structural integrity, attenuated aortic inflammatory infiltration, reduced circulating inflammatory monocytes, and increased autophagy-related proteins. These findings suggest that spermidine may be a promising treatment for AAA disease.

**Key Words:** abdominal aortic aneurysm ■ autophagy ■ experimental animal models ■ inflammation ■ spermidine

**A**bdominal aortic aneurysm (AAA) is defined as a maximal infrarenal abdominal aortic diameter  $\geq 30$  mm or at least 1.5 times the normal diameter and is characterized by progressive dilatation and rupture of the aortic wall.<sup>1</sup> In people aged 75 to 79 years, global AAA age-specific prevalence and incidence rate were 2.75% and 0.2%, respectively.<sup>2,3</sup> Currently, patients with a large AAA with aortic diameter  $>55$  mm in men and 50 mm in women are usually treated clinically by surgical repair, whereas patients with a small AAA ( $<55$  mm in diameter) have to wait for the

aortic diameter to reach the size suitable for surgery.<sup>4</sup> However, no pharmacological treatments have been convincingly shown to limit AAA growth or reduce the risk of rupture. Thus, patients with small AAAs or those deemed unfit for AAA repair lack active treatment options.<sup>5,6</sup> Hence, safe and effective medications that restrict small AAA expansion are anticipated.<sup>7</sup>

Spermidine, a naturally occurring polyamine, is widely found in vegetables, cereals, legumes, cheese, mushrooms, and other foods and particularly enriched in Mediterranean and Asian diets.<sup>8,9</sup> In epidemiological

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## CLINICAL PERSPECTIVE

### What Is New?

- Our present study found that autophagic function was dysregulated in clinical abdominal aortic aneurysm tissues and that spermidine, as an autophagy inducer, limited experimental abdominal aortic aneurysm by attenuating aortic inflammatory infiltration and upregulating autophagy-related proteins.

### What Are the Clinical Implications?

- Given that spermidine is a natural polyamine present in daily diet with proven safety and tolerability, our study may have a potential translational significance for treating abdominal aortic aneurysm disease.

## Nonstandard Abbreviations and Acronyms

<b>AAA</b>	abdominal aortic aneurysm
<b>ACS</b>	aortic cross section
<b>CR</b>	calorie restriction
<b>CRM</b>	calorie restriction mimetic
<b>EVG</b>	Verhoeff's Van Gieson
<b>Keap1</b>	Kelch-like ECH-associated protein 1
<b>LC3</b>	microtubule-associated protein 1 light chain 3
<b>LPS</b>	lipopolysaccharide
<b>MPO</b>	myeloperoxidase
<b>mTOR</b>	mammalian target of rapamycin
<b>PPE</b>	porcine pancreatic elastase
<b>SMC</b>	smooth muscle cell

studies, higher intake of polyamines in Mediterranean and Asian diets increases longevity and lowers mortality.<sup>8,10</sup> Spermidine is rapidly resorbed from the intestine and distributed in the body without degradation,<sup>11</sup> resulting in its subsequent increased concentration in the blood.<sup>12</sup> Spermidine, as a histone acetyl transferase inhibitor, induces protein deacetylation and autophagy, and is involved in an array of biological events including autophagy induction, DNA stability, transcription, translation, and apoptosis.<sup>13</sup> Previous studies have shown that spermidine prolongs mouse lifespan and exerts multiple cardioprotective effects in an autophagy-dependent manner.<sup>14</sup> In mice, spermidine reversed arterial aging by increasing nitrogen oxide bioavailability, reducing oxidative stress, modifying structural factors, and enhancing autophagy.<sup>15</sup> In humans, high dietary intake of spermidine is associated

with reduced incidence of atherosclerosis and other cardiovascular disease.<sup>16</sup> Additionally, spermidine also activates adenosine monophosphate-activated protein kinase and inhibits mTORC1,<sup>17</sup> all involved in autophagy and AAAs.<sup>18</sup> However, whether exogenous spermidine supplementation alleviates AAAs has not been explored. In this study, we explored the autophagy function in human AAAs and further evaluated the influence and potential mechanisms of spermidine supplementation on the development of experimental AAAs.

## METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

### Clinical AAA Tissue and Aorta Tissue Specimens

The protocol for collection of clinical AAAs and nearby artery of AAAs in the same patient was approved by the Ethics Committee of the Xiangya Hospital, Central South University (IRB Approval Number: 201803480). Informed consent was obtained from all participants or their legal guardians. Fresh aortic wall tissue samples were collected from 11 patients with AAA who underwent open AAA repair surgery at Xiangya Hospital, Central South University. The demographic and clinical characteristics of AAA patients are shown in Table. After tissue harvest, attached intramural thrombi were removed and the specimens were immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for further

**Table. Main Characteristics of 11 Aneurysm Patients**

Variable	Result
Male/female	10/1
Age, y	63.9±14.3
Smoking	7 (63.6%)
Aortic diameter, mm	6.69±1.43
Luminal thrombus	8 (72.7%)
Hypertension	7 (63.6%)
Systolic pressure, mm Hg	141.1±23.6
Diastolic pressure, mm Hg	87.8±16.9
Hyperlipidemia	3 (27.3%)
COPD	1 (9.1%)
Diabetes mellitus	0
Antihypertension medication	7 (63.6%)
Lipid-lowering medication	2 (18.2%)
Diabetic medications	0

Categorical variables are presented as number (%). Continuous variables are presented as mean±SD. COPD indicates chronic obstructive pulmonary disease.

analysis, or fixed in 10% formalin for embedding and sectioning. The study conforms to the declaration of Helsinki.

## Experimental Animals

Eight- to 10-week-old male C57BL/6 mice were purchased from the Xiangya School of Medicine, Central South University. All animals were housed under specific pathogen-free conditions in a 12-hour light/dark cycle with free access to food and water *ad libitum*. All experiments were carried out in accordance with the animal care guidelines and regulation of the Department of Laboratory Animals in Central South University and approved by the Animal Care and Use Committee of Ethics of the Xiangya Hospital, Central South University (IRB Approval Number: 201803481). All mouse experiments conform to the guidelines formulated by the European Community for experimental animal use (Directive 2010/63/EU).

## Experimental Aneurysm Creation

AAAs were created via intraluminal infusion of porcine pancreatic elastase (PPE) into the infrarenal aorta as previously described.<sup>19</sup> Briefly, mice were anesthetized with inhaled 2% isoflurane and considered as adequately anesthetized with no attempt to withdraw the limb after pressure. Under operative magnification, the infrarenal abdominal aorta was isolated and subjected to transient infusion of type I PPE (4.0 U/mL; E1250, Sigma-Aldrich, St. Louis, MO) dissolved in phosphate-balanced saline at the pressure of 150 mm Hg via a PE-10 polyethylene microcatheter. The 11-0 nylon sutures (Jinhuan, Shanghai, China) were used to close the aortotomy after infusion. Outer aortic diameter was measured under laparotomy at day 0 and day 14 after PPE infusion using S-Eye software (Hayear, Shenzhen, China.). AAAs were defined as a  $\geq 50\%$  increase in maximal outer aortic diameter over the baseline level. At the end of the study, mice were anesthetized with ketamine/xylazine (100 and 20 mg/kg *ip*, respectively) and euthanized by exsanguination before aorta tissue collection at day 14.

## Spermidine Treatment

C57BL/6 mice were randomly divided into 3 groups: the sham group mice infused with normal saline receiving regular drinking water, experimental AAA vehicle group mice receiving regular drinking water, and spermidine group mice receiving spermidine (S0266, Sigma-Aldrich, St. Louis, MO) via drinking water at a concentration of 3 mmol/L. Spermidine at this dose has been proven to be effective in cardioprotection and reversing arterial aging.<sup>14,15</sup> SPD treatment started on the day for, or 3 days after, PPE infusion, and continued

until euthanasia. Drinking water was replaced every other day by diluting 1 mol/L stock (spermidine/HCl pH 7.4) solution with drinking water as previously described.<sup>14,15</sup>

## Determination of Plasma Spermidine Concentration in Mice

SPD concentration was determined using liquid chromatography-mass spectrometry (LC/MS).<sup>14,20</sup> To measure spermidine, blood samples were centrifuged at 4°C, 3000g for 10 minutes. The supernatant (30  $\mu$ L) was mixed with acetonitrile (120  $\mu$ L) and 10  $\mu$ mol/L 1,6-diaminohexane (H11696, Sigma) solution in acetonitrile (30  $\mu$ L), vortexed for 30 s, and centrifuged again at 3000g for 5 minutes. Then the solvent of supernatant was evaporated with nitrogen. The residue was redissolved in 150  $\mu$ L of 0.1 mol/L sodium tetraborate (229946, Sigma) and then reacted with an equal volume of 40 mmol/L 4-(*N,N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F) (A5595, J&K Scientific Ltd., Beijing, China) in acetonitrile at 60°C for 30 minutes. Thereafter, the reaction mixtures were analyzed by Triple Quad 6500+ LC-MS/MS System (AB Sciex, Framingham, MA) coupled to Analyst software 1.6.3.

## Histochemical and Immunohistochemical Staining

Mouse aortae were harvested at 14 days after PPE infusion, fixed in 10% formalin, embedded in paraffin, and horizontally cut into sections (4- $\mu$ m thickness) from the middle of the AAA. Medial elastin retention, collagen, and smooth muscle cell (SMC) destruction were evaluated using Verhoeff's Van Gieson (EVG) solution (Servicebio, G1005, China) and Masson (Servicebio, G1006, China), respectively. Standard 3-step biotin-streptavidin-peroxidase tissue immunostaining was performed to evaluate medial SMC destruction, angiogenesis, mural leukocyte infiltration, and autophagy. Primary antibodies for immunostaining are summarized in Table S1. Staining specificity for each antibody has been documented in previous studies. Other reagents, including biotinylated anti-rabbit secondary antibodies, streptavidin-peroxidase conjugates, aminoethylcarbazole or diaminobenzidine peroxidase substrate kit were purchased from the ZSGB-biotechnology Co., Ltd., Beijing, China. Based on EVG, Masson, and SMC  $\alpha$  actin staining, destruction of medial elastin and SMCs was graded as I (mild) to IV (severe) as previously described.<sup>21,22</sup> Data about mural T cells, macrophages, neutrophils and angiogenesis were quantified as CD3<sup>+</sup> cells, CD68<sup>+</sup> cells, myeloperoxidase (MPO)-positive cells, and CD31<sup>+</sup> blood vessels per aortic cross section (ACS), respectively. Images of sections from the middle

of the AAA were captured under uniform settings using an upright brightfield microscope.

### Western Blot Analysis

Tissues were lysed for 30 minutes at 4°C in RIPA lysis buffer (P0013B, Beyotime, Shanghai, China). Total protein concentration was determined using bicinchoninic acid reagent. Then 50 µg of total protein was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to a polyvinylidene fluoride membrane, and subjected to immunoblot analysis. The membranes were incubated with primary antibodies followed by incubation with horseradish peroxidase–conjugated secondary antibody (1:5000 dilution, Santa Cruz). Primary antibodies for Western blot are summarized in Table S1. The bands were visualized using enhanced chemiluminescence reagents and analyzed with a gel documentation system (Bio-Rad Gel Doc1000 and Multi-Analyst version 1.1). After visualizing the band of mammalian target of rapamycin (mTOR), the membrane was incubated by stripping buffer (P0025B; Beyotime, Shanghai, China) to wash out antibodies, blocked and incubated with a rabbit anti-mTOR (7C10) monoclonal antibody.

### Transmission Electron Microscopic Examination

Mouse aortae were harvested at 14 days after PPE infusion and sliced into 1×1×3 mm<sup>3</sup> in size. Then the specimens were fixed in 2.5% glutaraldehyde with Millonig's phosphate buffer (pH=7.3) for transmission electron microscopic laboratory examination. Briefly, specimens were washed with Millonig's phosphate buffer and incubated for 1 hour in 1% osmium tetroxide. Then dehydration of the samples was carried out in a graded series of acetone and followed by a sample resin soaking, embedding, and solidifying process. Ultrathin sections (50 to 100 nm) of specimens were made with an ultramicrotome and a diamond knife. After double staining of 3% uranyl acetate and lead nitrate, the specimens were examined and photographed on a Hitachi HT-7700 electron microscope.

### Flow Cytometric Analysis

Flow cytometry was used to quantify macrophages and other leukocytes in aneurysmal aorta and peripheral blood. Single cell suspensions from peripheral blood and aneurysmal aortae were prepared at euthanasia as previously described.<sup>23,24</sup> Cells were incubated with appropriate antibody cocktail for 20 minutes at room temperature and followed by lysis of erythrocytes with red blood cell lysis buffer. All antibodies were purchased from Biolegend (San Diego,

CA) and summarized in Table S1. Staining data were acquired on a Cytex Athena Dxp flow cytometer with Flowjo CE software and analyzed using FlowJo software (Ver X.0.7; FlowJoLLC, Eugene, OR).

### Statistical Analysis

GraphPad Prism 8.0.1 (GraphPad Prism Software, Inc., San Diego, CA) software was used for all statistical analysis. All continuous data were reported as the mean±SD. The paired *t* test, unpaired *t* test, and nonparametric Mann–Whitney test were used to determine differences between groups according to data characteristics with an *P*<0.05 considered statistically significant. Differences in feed intake, body weight, and percentage of p62-positive area in mice aorta were determined by 2-way ANOVA, and the differences in aneurysm incidence were determined by Fisher exact test.

## RESULTS

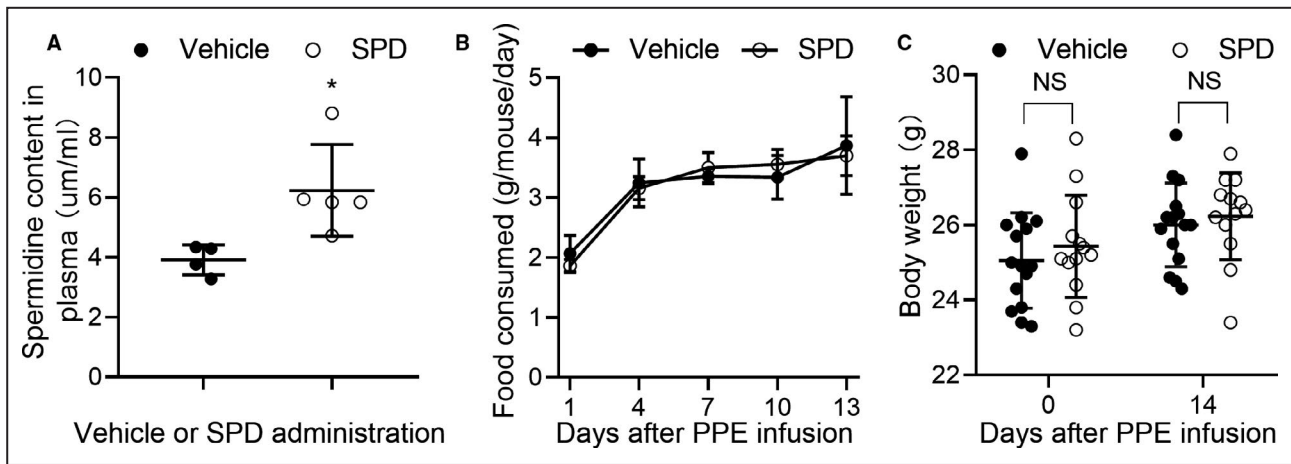
### Spermidine Oral Administration Does Not Alter Mouse Feed Intake and Body Weight Gain

To verify whether spermidine oral administration increases plasma spermidine levels in mice, we performed liquid chromatography/mass spectrometry (LC/MS) analysis. Anticipatively, plasma spermidine concentration was significantly increased in spermidine supplemented, as compared with vehicle, mice (Figure 1A). In addition, food consumption was calculated by weighing each cage of food every 3 days. As demonstrated in Figure 1B, food consumption decreased significantly after PPE infusion, recovered at day 4, and then gradually increased. Neither food consumption (Figure 1B) nor body weight (Figure 1C) were affected by spermidine treatment.

### Spermidine Treatment Prevents Development of Experimental AAAs

Maximal outer aortic diameters were significantly smaller in spermidine-treated than those in vehicle-treated mice (1.26±0.12 mm versus 1.67±0.29 mm) (Figure 2A and 2B). Within 14 days following PPE infusion, AAAs developed in all vehicle-treated mice (100%, 16/16) and 13 mice (72.22%, 13/18) in the spermidine-treated group (Figure 2C). As shown in Figure 2D and 2E, both elastin destruction and SMC depletion, as evaluated by histological scores, were significantly attenuated in mice treated with spermidine as compared with mice treated with vehicle. Similar results were noted when the positive areas for both medial elastin and SMC were quantified with





**Figure 1. Spermidine oral administration does not alter mouse feed intake and body weight gain.**

C57BL/6 male mice (8–10 weeks old) were administered 3 mmol/L spermidine in drinking water or normal drinking water as vehicle before PPE infusion to day 14 after PPE infusion. **A**, Plasma spermidine concentrations were measured by LC/MS. Unpaired *t* test, \**P*<0.05 vs vehicle group. *n*=4 to 5 mice/group. **B**, No difference in food intake in 2 treatment groups during whole experimental process. Two-way ANOVA with the Bonferroni correction, *P*=0.879 vs vehicle group. **C**, No difference in body weight at the baseline level (*P*=0.665) and 14 days (*P*=0.857) following treatment with vehicle (*n*=15) or spermidine (*n*=13). Two-way ANOVA. NS for nonsignificance, vs vehicle group. All data are means±SD. LC/MS indicates liquid chromatography-mass spectrometry; PPE, porcine pancreatic elastase; and SPD, spermidine.

ImageJ software, and were consistent with the above results (Figure S1). In Masson staining, spermidine treatment promoted the presence of collagen (blue stain) in aneurysmal aortic media (Figure 2D and 2E). These results histologically confirmed AAA suppression by spermidine treatment.

### Spermidine Treatment Reduces Aortic Inflammatory Cell Infiltration and Angiogenesis

Mural inflammatory cell infiltration and angiogenesis are the histopathologic features of abdominal aortic degeneration. As shown in Figure 3, immunohistological analysis revealed that spermidine attenuated mural accumulation of CD3<sup>+</sup> T cells and CD68<sup>+</sup> macrophages and MPO-positive neutrophils. A reduction in mural neo-angiogenesis was also present, as indicated by reduced CD31<sup>+</sup> mural capillary vessels in spermidine-treated mice. Meanwhile, flow cytometric analysis further revealed that spermidine treatment markedly reduced aortic accumulation of CD45<sup>+</sup> leukocytes, myeloid cells (CD45<sup>+</sup>CD11b<sup>+</sup>), and macrophages (CD45<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup>) (Figure 4). These results suggest that reduced mural inflammatory cell infiltration and angiogenesis are partly involved in AAA inhibition by spermidine.

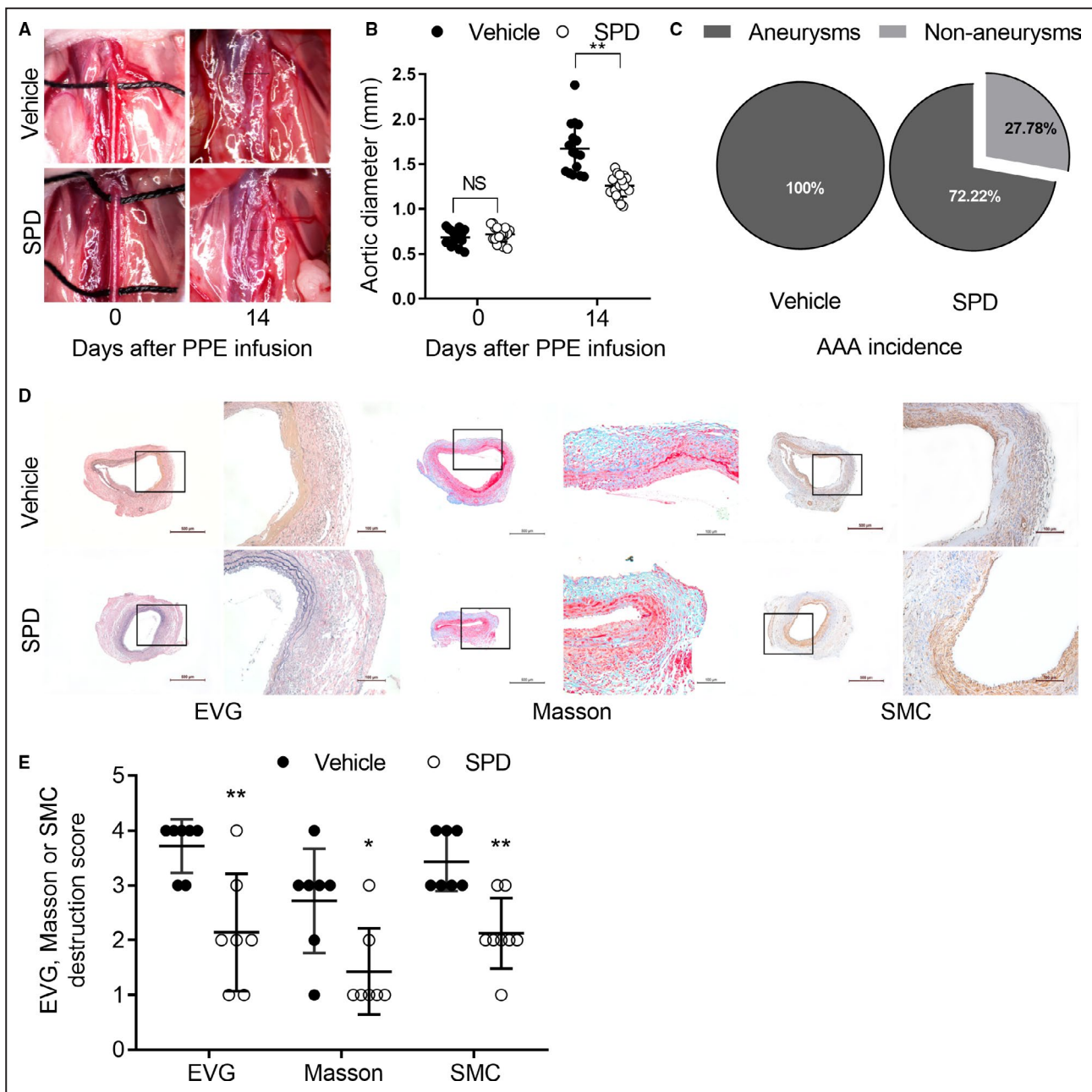
### Spermidine Treatment Reduces Circulating Inflammatory Monocytes and Neutrophils

Since circulating inflammatory monocytes are precursors of tissue macrophages, flow cytometric analysis

was used to quantify the relative number of circulating inflammatory monocytes (CD11b<sup>+</sup>Ly-6C<sup>high</sup> cells) and neutrophils (CD11b<sup>+</sup>Ly-6G<sup>+</sup> cells) in PPE-infused mice treated with either spermidine or vehicle. As shown in Figure 5, spermidine treatment reduced circulating inflammatory monocytes and neutrophils as compared with vehicle treatment. Since neutrophils and monocyte/macrophages participate in AAA formation and inflammatory cell infiltration, these results suggest that spermidine-mediated aneurysm inhibition may be partially associated with reduced circulating inflammatory monocytes and neutrophils.

### Spermidine Treatment Increases Autophagy-Related Protein in Experimental AAA

The SQSTM1/p62 protein, a link between LC3 (microtubule-associated protein 1 light chain 3) and ubiquitinated substrates, serves as an index of autophagic degradation.<sup>15</sup> To address whether spermidine promotes autophagy, we evaluated autophagy-related protein levels of LC3-I, LC3-II, and p62 in sham and 2 experimental AAA groups. As shown in Figure 6A and 6B, the ratio of LC3 II/I was upregulated in spermidine-treated as compared with vehicle-treated mice (*P*<0.05). In addition, compared with the sham group, p62 protein was increased in PPE-perfused mice with vehicle treatment (*P*<0.05). Furthermore, Keap1 (Kelch-like ECH-associated protein 1), a substrate adaptor protein binding to the Keap1 interacting region of p62 and degrading together with p62 through the autophagy pathway, showed an increased tendency in the expression

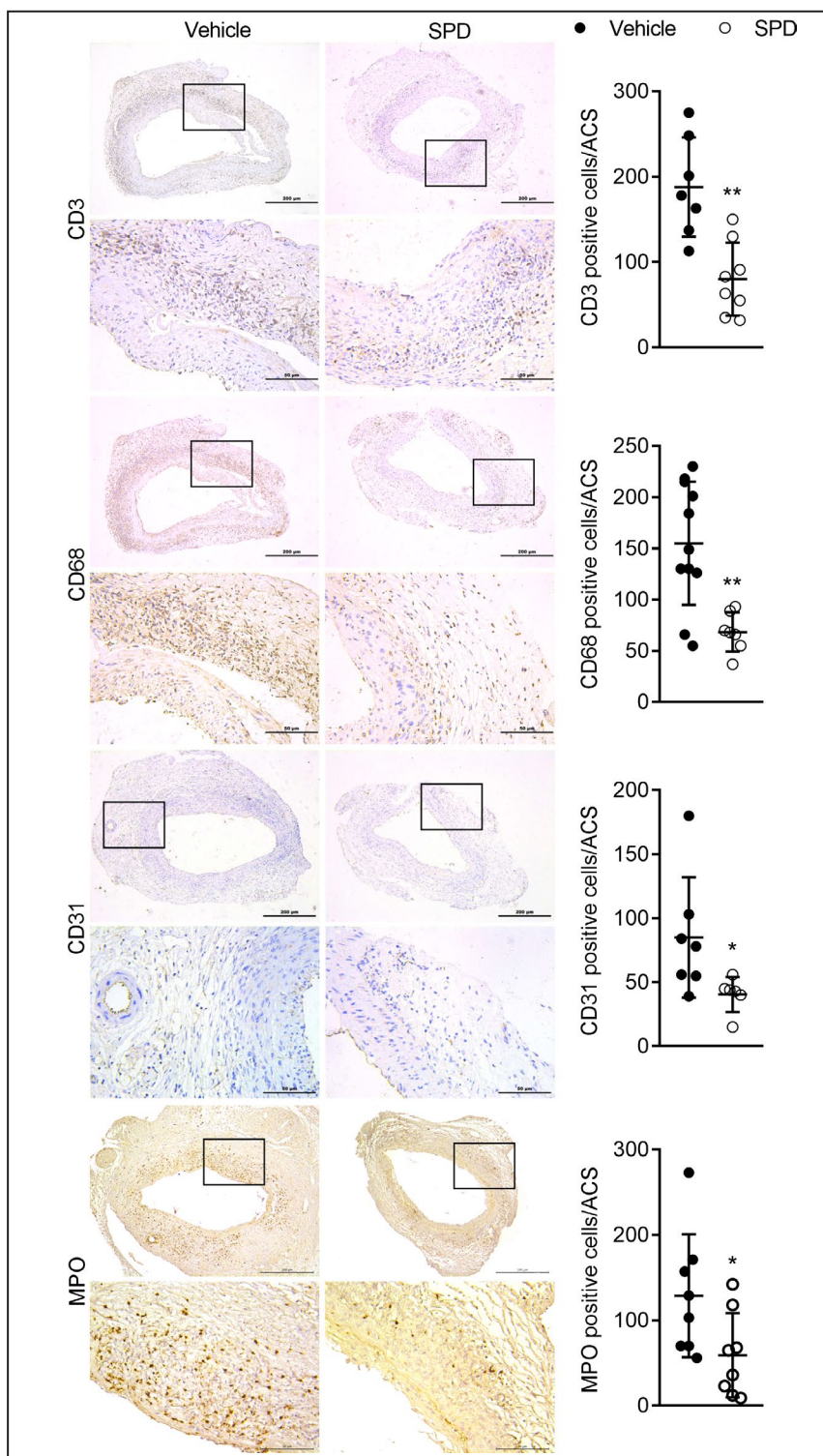


**Figure 2. Spermidine treatment prevents development of experimental AAA.**

External aortic diameter was measured by computer software under laparotomy. **A**, Representative aortic images for the baseline (day 0) and 14 days after PPE infusion. **B**, Mean and SD of aortic diameter (mm) in mice treated with vehicle (n=16) or SPD (n=18) at day 0 and day 14. Unpaired t test,  $**P<0.01$  vs vehicle group. **C**, AAA incidence. Fisher exact test,  $P=0.046$  vs vehicle group. **D** and **E**, Representative images as well as mean±SD of destruction scores for EVG ( $P=0.004$ ), Masson's ( $P=0.017$ ), and SMC  $\alpha$  actin staining ( $P=0.001$ ). Scale bar=100 and 500  $\mu$ m. Medial elastin and SMC destruction were graded as I (mild) to IV (severe). Nonparametric Mann-Whitney test,  $**P<0.01$  and  $*P<0.05$  vs vehicle group, n=7 to 8 mice/group. AAA indicates abdominal aortic aneurysm; EVG, Verhoeff's Van Gieson; PPE, porcine pancreatic elastase; SMC, smooth muscle cell; and SPD, spermidine.

in PPE-infused mice. While those tendencies can be stopped by spermidine compared with the vehicle group, spermidine treatment affected neither p62 nor Keap1 compared with the sham group. In immunohistochemical staining, less p62 protein accumulated in aortic media and adventitia in spermidine-treated as compared with vehicle-treated mice (Figure 6C and

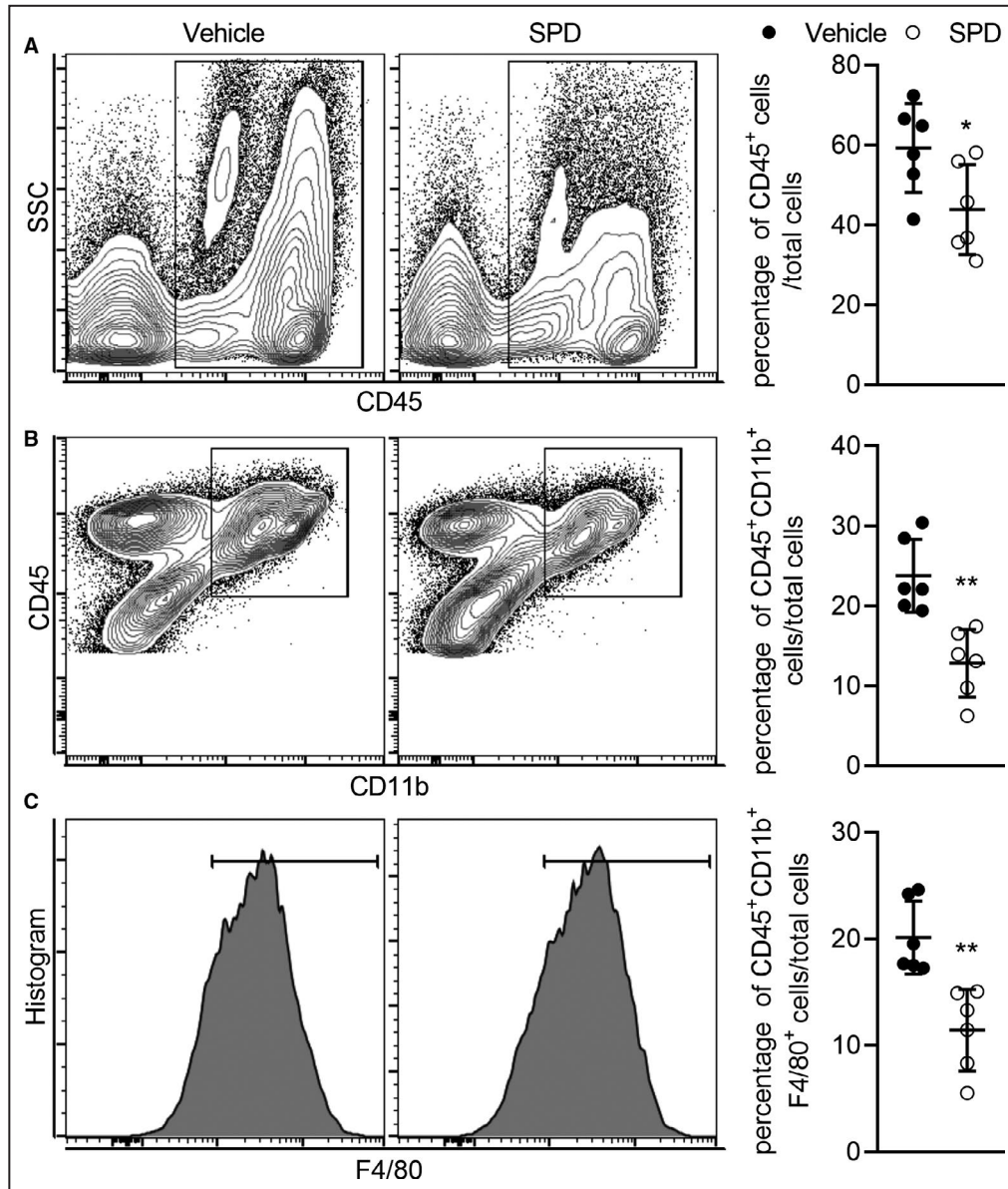
6D). Furthermore, 3-color immunofluorescent staining revealed that p62 protein accumulation was reduced in vascular SMCs. In electron microscopic examination, more autophagosomes were noted in medial SMCs and adventitial macrophages in spermidine-treated as compared with vehicle-treated mice (Figure 6E). Collectively, these data demonstrated that spermidine



**Figure 3. Spermidine treatment reduces aortic inflammatory cell infiltration and angiogenesis.**

Aortic sections from mice 14 days after PPE infusion were stained with antibodies against CD3, CD68, MPO, and CD31. Representative immunostaining images for CD3<sup>+</sup> T cells, CD68<sup>+</sup> macrophages, MPO<sup>+</sup> neutrophils, and CD31<sup>+</sup> blood vessels. Scale bar=50  $\mu$ m and 200  $\mu$ m. Mean and SD of aortic T cells ( $P=0.001$ ), macrophages ( $P=0.001$ ), neutrophils ( $P=0.041$ ), and angiogenesis ( $P=0.048$ ) quantified as the positive stain cells or vessels per ACS. Unpaired *t* test, \*\* $P<0.01$  and \* $P<0.05$  vs vehicle group,  $n=7$  to 12 mice/group. ACS indicates aortic cross section; MPO, myeloperoxidase; and PPE, porcine pancreatic elastase.





**Figure 4. Spermidine attenuates infiltration of leukocytes and macrophages into aneurysmal aorta.** Aortae were collected 14 days after PPE infusion. After digestion into single cells, the aortae were stained with monoclonal antibodies against CD45, CD11b, and F4/80. Leukocytes (CD45<sup>+</sup>) (A), myeloid cells (CD45<sup>+</sup>CD11b<sup>+</sup>) (B), and macrophages (CD45<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup>) (C) were analyzed by flow cytometry. Data presented as mean and SD of the percentages of leukocytes ( $P=0.039$ ), myeloid cells ( $P=0.002$ ), and macrophages ( $P=0.002$ ) in total aortic cells. Unpaired *t* test with Welch's correction, \*\* $P<0.01$  and \* $P<0.05$  vs vehicle-treated group,  $n=6$  mice/group. PPE indicates porcine pancreatic elastase; and SPD, spermidine.

treatment increases autophagy-related protein in experimental AAAs.

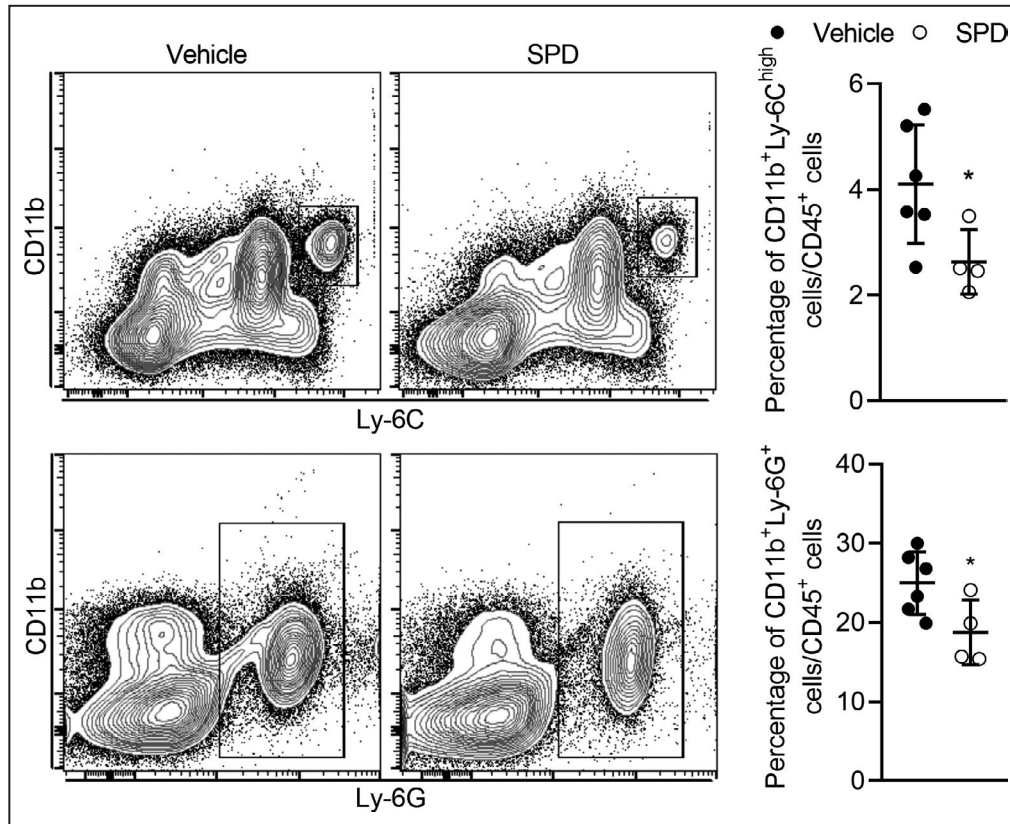
To further explore how spermidine treatment modulates autophagy in experimental AAAs, we analyzed total mTOR, p-mTOR, and Beclin1 by Western blot analysis. Spermidine treatment reduced mTOR phosphorylation, which is crucial in autophagic flux inhibition, and increased expression of Beclin1, a partner involved in the initial step of autophagosome formation (Figure 6A and

6B). These data suggest that the SPD alters autophagy in experimental AAAs, probably via mTOR and Beclin1 pathways.

### Evidence for Dysregulated Autophagy in Clinical AAAs

Finally, to see whether autophagy is altered in clinical AAAs, we analyzed autophagy-related protein levels





**Figure 5. Spermidine reduces circulating inflammatory cells in experimental abdominal aortic aneurysms.**

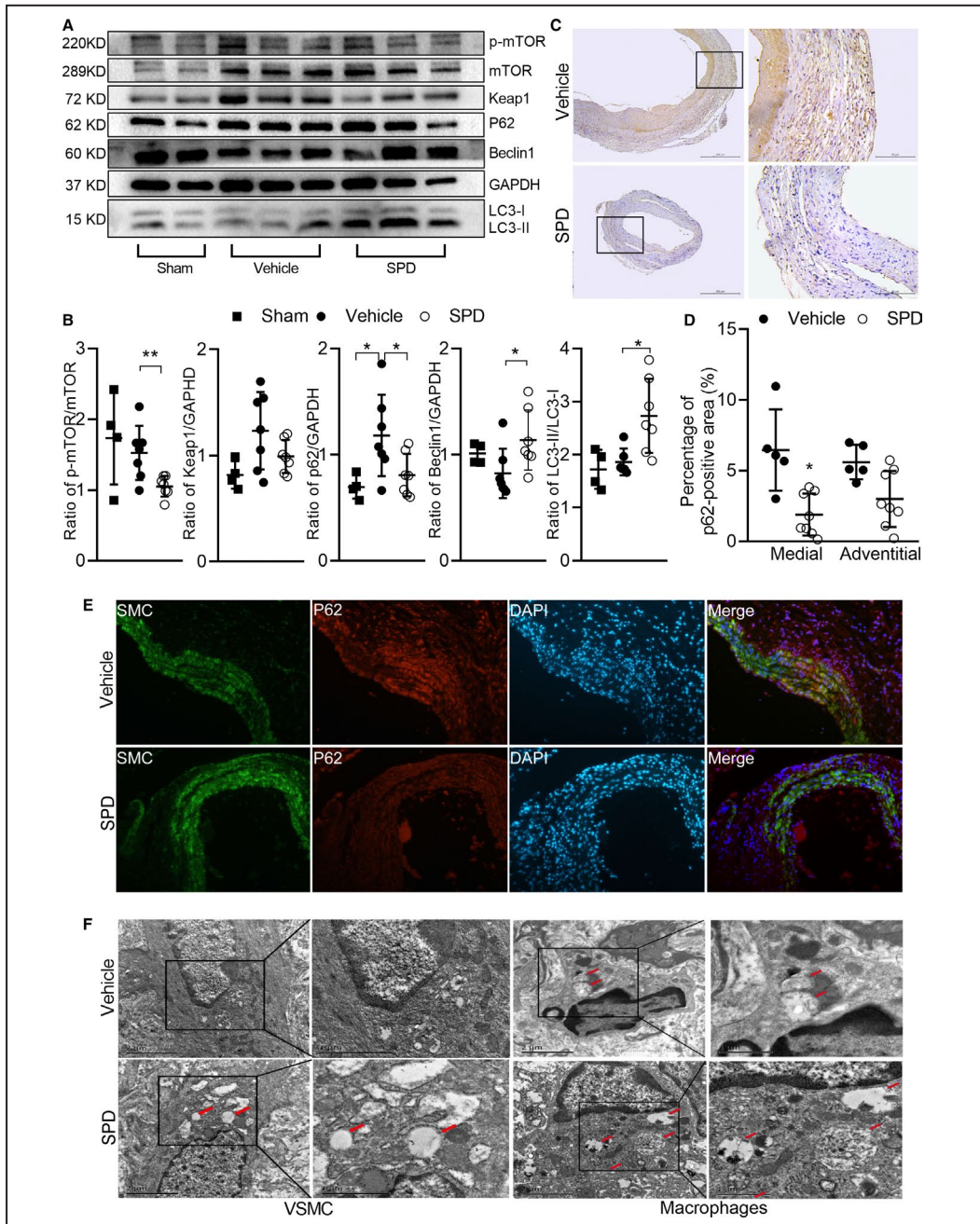
Whole blood samples collected from heart 14 days after PPE infusion were stained with monoclonal antibodies against CD45, CD11b, Ly-6C, and Ly-6G. Inflammatory monocytes ( $CD11b^+Ly-6C^{high}$ ) and neutrophils ( $CD11b^+Ly-6G^+$ ) in blood samples in SPD or vehicle group were analyzed by flow cytometry. Data presented as mean and SD of the percentages of inflammatory monocytes ( $P=0.046$ ) and neutrophils ( $P=0.043$ ) in total leukocytes gated by forward scatter and CD45. Unpaired  $t$  test with Welch's correction,  $*P<0.05$  vs vehicle-treated group,  $n=4$  to 6 mice/group. PPE indicates porcine pancreatic elastase; and SPD, spermidine.

of LC3-I, LC3-II, p62, p-mTOR, and mTOR in aneurysmal and nonaneurysmal aortic segments obtained from the same patients. As shown in Figure 7A and 7B, we found p62 protein ( $P=0.0102$ ) and phosphorylated mTOR ( $P=0.0155$ ) levels were increased in aneurysmal, as compared with nonaneurysmal, aortic segments. Immunohistochemical staining also revealed that p62 protein accumulation was increased in the medial and adventitial segments of aneurysmal, as compared with nonaneurysmal, aortic segments (Figure 7C and 7D). Although the reduced ratio of LC3-II to LC3-I was noted in some aneurysmal aortic segments, no statistical difference was seen between aneurysmal and nonaneurysmal aortic segments ( $P=0.14$ ) (Figure 7A and 7B). To clarify whether this difference in autophagy was caused by the different cellular composition of the specimens, Masson staining and immunohistochemical staining for CD3, CD68, and CD31 were performed. No significant difference in muscle fiber and infiltrated inflammatory cells was noted between

clinical aneurysmal and nonaneurysmal aortic segments (Figure S2), suggesting that the difference in autophagy might not be caused by the different cellular composition of the specimens. Collectively, these results indicate that autophagy is dysregulated in clinical AAAs.

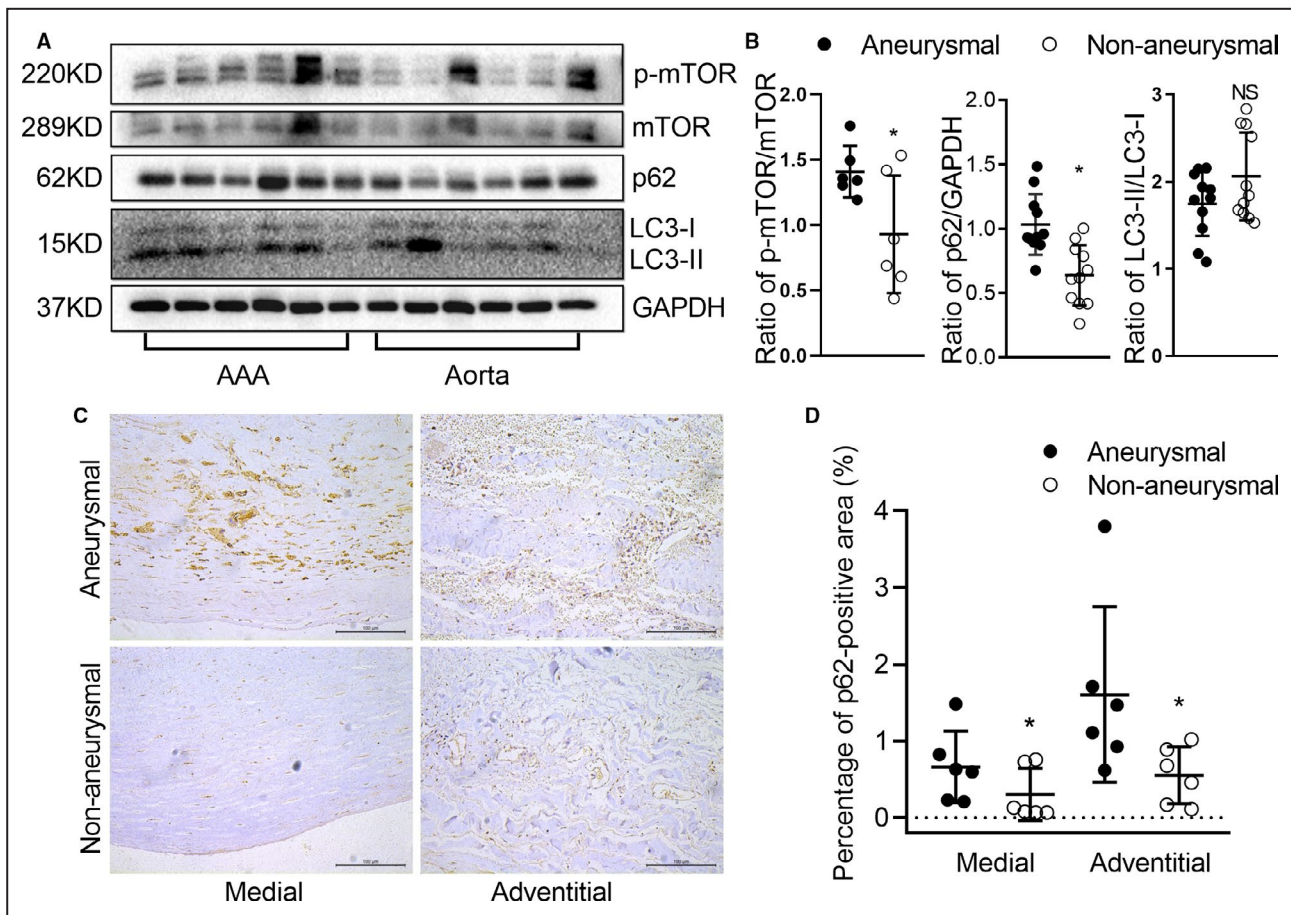
### Spermidine Treatment Reduces Aortic Remodeling in Established Experimental AAA

To investigate the translational potential of SPD treatment in existing AAAs, treatment of SPD or vehicle alone was initiated 3 days after PPE infusion and continued until euthanasia. As shown in Figure S3A and S3B, delayed treatment with SPD was effective in reducing aneurysm enlargement in established AAAs, although lack of significance was likely because of large variations within the responses of individual mice. On histologic analysis, spermidine treatment preserved



**Figure 6. Spermidine treatment increases autophagy-related proteins in experimental AAAs.**

Aortae were collected 14 days after PPE infusion. Western blot, immunohistochemical staining and transmission electron microscopic examination were performed to evaluate the autophagy in experimental AAA. Protein levels of p-mTOR, mTOR, Keap1, p62, Beclin1, GAPDH, LC3-I, and LC3-II were detected by Western blot (A) and quantified by densitometry (B). Data are presented as mean±SD. Unpaired *t* test, \*\**P*<0.01 and \**P*<0.05 vs vehicle group, *n*=4 to 7 in each group. C, Representative immunohistochemical images for p62 protein in vehicle (*n*=5) and SPD (*n*=8) groups. Scale bar=50 μm, 100 μm. D, p62 protein in medial (*P*=0.001) and adventitial (*P*=0.054) segments of aneurysm was quantified as p62-positive area. Two-way ANOVA, \**P*<0.05 vs vehicle group. E, Representative immunofluorescence staining with antibodies against p62 protein conjugated with Alexa Fluor 594 (red color), and antibodies against SMA conjugated with Alexa Fluor 488 (green color), with the nucleus counterstained with DAPI (blue color). F, Representative transmission electron microscopy of aorta in two groups. AAA indicates abdominal aortic aneurysm; DAPI, 4',6-diamidino-2-phenylindole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Keap1, kelch-like ECH associated protein 1; LC3, microtubule-associated protein 1 light chain 3; mTOR, mammalian target of rapamycin; PPE, porcine pancreatic elastase; and SPD, spermidine.



**Figure 7. Evidence for dysregulated autophagy in clinical AAAs.**

Western blot and immunostaining were performed to evaluate the autophagy of AAA and adjacent normal aortas from patients who underwent open AAA repair surgery. Protein levels of p-mTOR, mTOR, p62, LC3-I, and LC3-II were detected by Western blot (A) and quantified by densitometry (B). Data are presented as mean±SD. Paired *t* test, NS for nonsignificance, \**P*<0.05 vs AAA, n=6 to 11. C, Representative aortic histology images of immunohistochemical staining of p62 protein. D, Analysis of p62 protein in medial (*P*=0.025) and adventitial (*P*=0.036) in adjacent normal and aneurysmal human aortae. Scale bar=100 μm. Data are presented as mean±SD. Paired *t* test, \**P*<0.05 vs AAA, n=6 in each group. AAA indicates abdominal aortic aneurysm; LC3, microtubule-associated protein 1 light chain 3; and mTOR, mammalian target of rapamycin.

medial elastin and SMCs and diminished mural leukocyte infiltration (Figure S3C and S3D). Thus, these data suggest that spermidine might attenuate aortic remodeling and thus has therapeutic potential for established AAAs.

## DISCUSSION

In this study, we discovered that oral spermidine supplementation increased plasma spermidine levels and suppressed the development of experimental AAAs with preservation of aortic structural integrity. Spermidine-mediated aneurysm inhibition was accompanied by attenuation of mural angiogenesis, leukocytes infiltration, and circulating inflammatory cells. Furthermore, spermidine upregulated autophagy in experimental AAA with decreased p62 protein accumulation and

phosphorylated mTOR and increased Beclin1. We also demonstrated that the dysregulated autophagy in clinical AAAs was characterized by increased p62 protein accumulation and phosphorylated mTOR. These results underscore the specific role of autophagy in AAA pathogenesis and place spermidine as a promising translational pharmacological strategy for AAA disease suppression.

Main pathological features of AAA include extracellular matrix degradation and loss of vascular SMCs as a result of the inflammatory cells infiltration, and eventually lead to vascular remodeling and weakening of the aortic wall.<sup>25</sup> Chronic aortic inflammation plays a critical role in AAAs through the release of matrix-degrading enzymes, free radicals, and proinflammatory cytokines.<sup>26–28</sup> Previous studies demonstrated that SPD levels and autophagy function declined with aging and disease,<sup>29,30</sup> and our



present study also indicated a dysregulated autophagy function in clinical AAA specimens. Polyamines, primarily spermidine and spermine, regulate inflammatory responses.<sup>31</sup> Although the mechanism of anti-inflammatory and anti-oxidative effects has not been fully elucidated, spermidine has been shown to exert anti-inflammatory effects in several disease models.<sup>14,32–34</sup> In previous *in vivo* studies, spermidine suppressed subclinical inflammation and reduced circulating tumor necrosis factor in aged mice,<sup>14</sup> protected against oxidative stress and recruitment of neutrophils and macrophages in both zebrafish<sup>32</sup> and 12-*O*-tetradecanoylphorbol-13-acetate-induced skin inflammation in mice,<sup>33</sup> and inhibited the expression levels of pro-inflammatory cytokines and macrophage activation by targeting the NF- $\kappa$ B pathway with experimental autoimmune encephalomyelitis.<sup>34</sup> In previous *in vitro* studies, spermidine treatment reduced reactive oxygen species accumulation and the levels of inflammatory cytokines such as nitric oxide, tumor necrosis factor- $\alpha$ , and interleukin-1 $\beta$  in lipopolysaccharide (LPS)-stimulated RAW264.7 murine macrophages.<sup>32,33</sup> Moreover, spermidine inhibited the secretion of inflammatory cytokines such as tumor necrosis factor- $\alpha$  and MCP-1 in lipopolysaccharide-induced NR8383 macrophage<sup>35</sup> as well as inhibited lipopolysaccharide-stimulated inflammation in microglia by blocking the NF- $\kappa$ B, PI3K/Akt, and MAPKs signaling pathways.<sup>36</sup> In agreement with those studies, we found that oral spermidine supplementation attenuated mural inflammatory cell infiltration in experimental AAAs as presented by immunohistochemical staining and flow cytometric analyses. Spermidine treatment also reduced circulating inflammatory monocytes and neutrophils, thereby diminishing the population of inflammatory cells available for migration and aortic localization. Those results suggest that the inhibitory effect of spermidine on AAAs was attributed to reduced systemic inflammation and migration of immune cells. However, whether the effects of spermidine on retaining aortic structure and alleviating mural inflammation are associated with autophagic flux is not fully understood.

Autophagy is a conserved intracellular process of recycling damaged cytoplasmic constituents, the primary mechanism by which hosts maintain protein homeostasis and protect against stressful conditions. It has been shown that autophagy is associated with many basic biological processes and dysregulated autophagy is involved in a variety of chronic AAA diseases.<sup>37</sup> Moreover, basal autophagy, as an essential *in vivo* process, mediates proper vascular function and serves as an important survival pathway in endothelial cells and SMCs.<sup>38</sup> Despite the protective effects of autophagy in arterial aging, atherosclerosis,

and other cardiovascular diseases, the role of autophagy in AAA disease remains incompletely resolved.<sup>38</sup> In previous research, the expressions of LC3 protein and autophagy-related genes such as Beclin1/Atg6 were markedly upregulated in both clinical and experimental aneurysmal lesions,<sup>39,40</sup> suggesting autophagy was induced in these tissues. However, autophagic flux is a dynamic and multistep process that involves autophagosome formation, fusion with lysosomes and degradation, and its changes in AAA need to be further confirmed. In recent studies, defective autophagy in vascular SMCs has been shown to increase the susceptibility of SMCs to cell death and the severity of aortic disease with more obvious pathological changes such as aortic degradation and atherosclerosis lesions in mice with SMC-specific deletion of gene *atg7*<sup>41</sup> and *atg5*.<sup>42</sup> Mice with SMC-specific ATG7 deficiency also exhibited exacerbated adverse aortic remodeling and larger suprarenal aortic diameters following angiotensin II infusion.<sup>43</sup> Although no overt differences in the development of dissecting AAA are found in this study, it suggests an essential role of normal autophagy in maintaining normal structure of the aorta. However, chloroquine, a well-known autophagy inhibitor, was found to have no effect on angiotensin II-induced AAA formation and pathological features.<sup>44</sup> This may be because of anti-inflammatory effects of chloroquine, considering its anti-inflammatory effects as an immunomodulator for the treatment of autoimmune diseases. In addition, previous studies have reported that several anti-inflammatory agents, also confirmed as potent autophagy inducers, such as rapamycin,<sup>21</sup> resveratrol,<sup>45</sup> and everolimus,<sup>46</sup> attenuated experimental AAAs. These studies indicate that autophagy might be an adaptive response to pathogenic factors and a potential therapeutic target in AAA disease.

In our study, we discovered impaired autophagy in clinical aneurysmal segments characterized by increased p62 protein accumulation and phosphorylated mTOR as compared with nonaneurysmal segments. Inconsistent with the previous study,<sup>39</sup> there is no change in expression of LC3 protein, probably because we choose the adjacent normal aorta from the same patients instead of the normal aorta from organ donors as control. In addition, we found that autophagy-related protein in experimental AAAs was increased by spermidine as evidenced by the down-regulated p62 protein and upregulated LC3 II to LC3 I conversion following spermidine treatment. To further explore the potential mechanism of spermidine-induced autophagy in experimental AAAs, Western blot analysis revealed decreased phosphorylation of mTOR and increased Beclin1 in the spermidine group. Although spermidine-induced autophagy has been reported in an mTOR-independent manner,<sup>47</sup> our



results are consistent with previous studies in which spermidine treatment alleviated myocardial infarction via enhanced cardiac autophagic flux by targeting the adenosine monophosphate-activated protein kinase/mTOR signaling pathway<sup>48</sup> and promoted arterial autophagy in aged mice.<sup>15</sup>

SPD is also considered a calorie restriction mimetic (CRM) because of induction of deacetylation and autophagy. Calorie restriction (CR), which consists of decreasing caloric intake without causing malnutrition, protected experimental AAAs.<sup>49</sup> Along with CR and most CRMs, spermidine treatment mimics the effects of starvation with regard to acetyl coenzyme A depletion and consequently protein deacetylation. CR<sup>49</sup> and several CRMs such as rapamycin,<sup>21</sup> resveratrol,<sup>45</sup> metformin,<sup>50</sup> and curcumin,<sup>51</sup> also protected against experimental AAAs. In our study, spermidine supplementation has no effect on food consumption and body weight, suggesting spermidine-induced AAA inhibition was likely not mediated by altering calorie intake directly.

In current drug research on AAA disease, spermidine might be very promising in clinical applications for multiple reasons. First, spermidine is a natural compound present in the daily diet; thus its oral supplementation is an effective means for clinical use. Second, spermidine can be synthesized in mammalian cells<sup>52</sup> and mainly excreted through the kidneys after metabolism, suggesting that adverse reactions are unlikely to occur after exogenous supplementation. In fact, in a 3-month randomized Phase II clinical trial, supplementation with spermidine-rich plant extracts (equivalent to a daily spermidine dose of 1.2 mg) was safe and well tolerated in older adults.<sup>53</sup> Third, 2 prospective population-based studies (summarized in the same paper) reported for the first time that nutritional spermidine uptake is also linked to reduced overall, cardiovascular, and cancer-related mortality in humans.<sup>29</sup>

## CONCLUSIONS

In conclusion, our present study demonstrates that spermidine inhibited the development of experimental AAA, accompanied by preservation of aortic structural integrity, reduced inflammatory cell infiltration, and circulating inflammatory monocytes, as well as increased autophagy-related protein. These findings suggest that spermidine might have therapeutic potential for AAA diseases.

## ARTICLE INFORMATION

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### Disclosures

None.

### Supplementary Materials

Table S1

Figures S1–S3

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# **SUPPLEMENTAL MATERIAL**

**Table S1. Major Resources.****Animals (in vivo studies)**

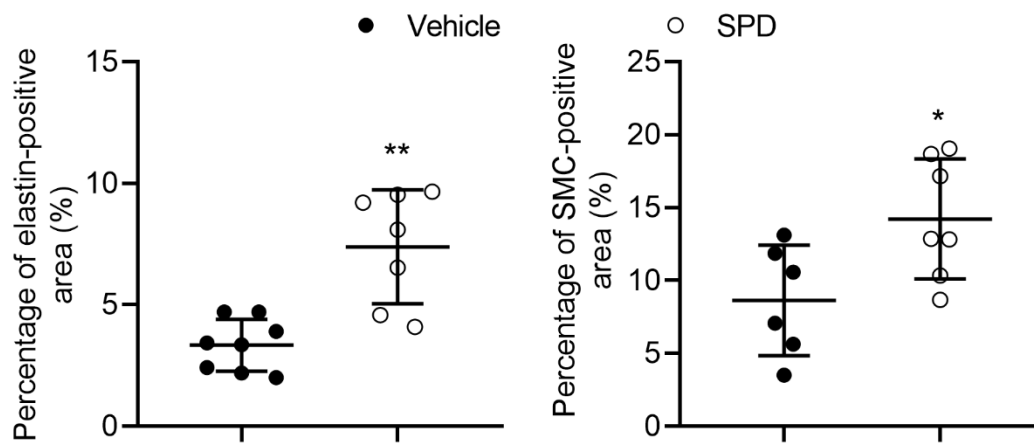
<b>Species</b>	<b>Vendor or Source</b>	<b>Background Strain</b>	<b>Sex</b>
Mice	Xiangya School of Medicine, CSU	C57BL/6	Male

**Antibodies**

<b>Target antigen</b>	<b>Vendor or Source</b>	<b>Catalog #</b>	<b>Working concentration</b>
CD3	Abcam	ab16669	IHC 1:400
CD31	Abcam	ab28364	IHC 1:100
CD68	Abcam	ab125212	IHC 1:400
SMC $\alpha$ actin	Abcam	ab5694	IHC 1:800 IF 1:400
Myeloperoxidase	Abcam	ab208670	IHC 1:500
SQSTM1/p62	Abcam	ab91526	IHC 1:200 IF 1:100
SQSTM1/p62	Abcam	ab109012	WB 1:10000
LC3B	CST	3868T	WB 1:1000
Beclin1	proteintech	11306-1-AP	WB 1:1000
GAPDH	Abcam	ab181602	WB 1:10000
mTOR	CST	2983S	WB 1:4000
p-mTOR	Santa Cruz	sc-293133	WB 1:2000
Keap1	Abcam	ab227828	WB 1:1000
FITC anti-mouse CD45	Biolegend	103107	0.2ug/10 <sup>6</sup> cells
PE/Cy7 anti-mouse CD11b	Biolegend	101216	0.2ug/10 <sup>6</sup> cells
APC anti-mouse Ly-6G	Biolegend	127614	0.2ug/10 <sup>6</sup> cells
PE anti-mouse Ly-6C	Biolegend	128007	0.2ug/10 <sup>6</sup> cells
PE/Cy7 anti-mouse CD11b	Biolegend	101216	0.2ug/10 <sup>6</sup> cells
Violet 421™ anti-mouse F4/80	Biolegend	123137	0.2ug/10 <sup>6</sup> cells

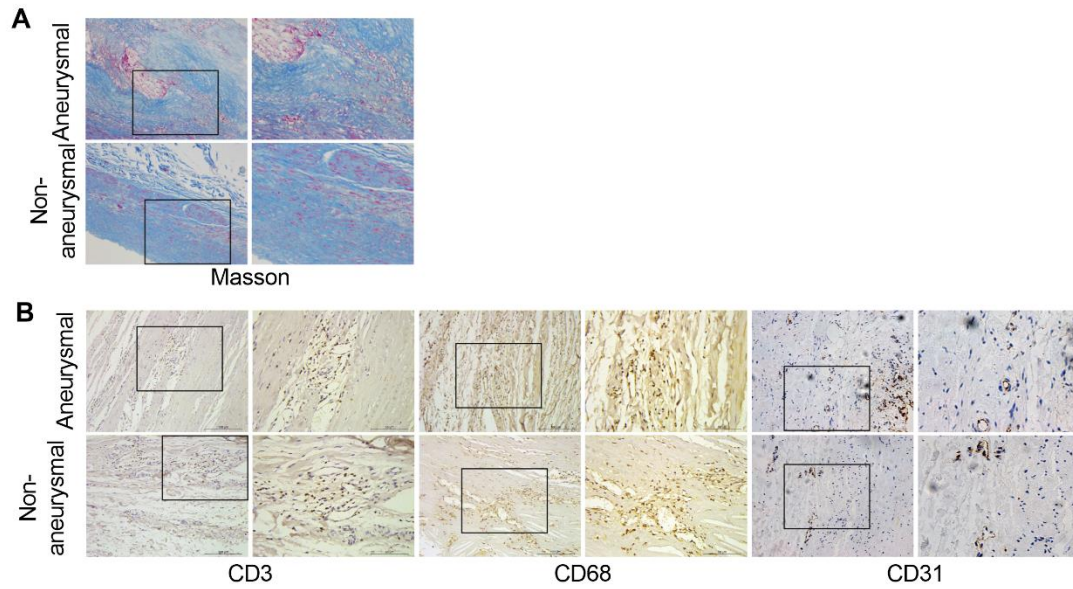


**Figure S1. Quantification of elastin and SMA in experimental AAA.**



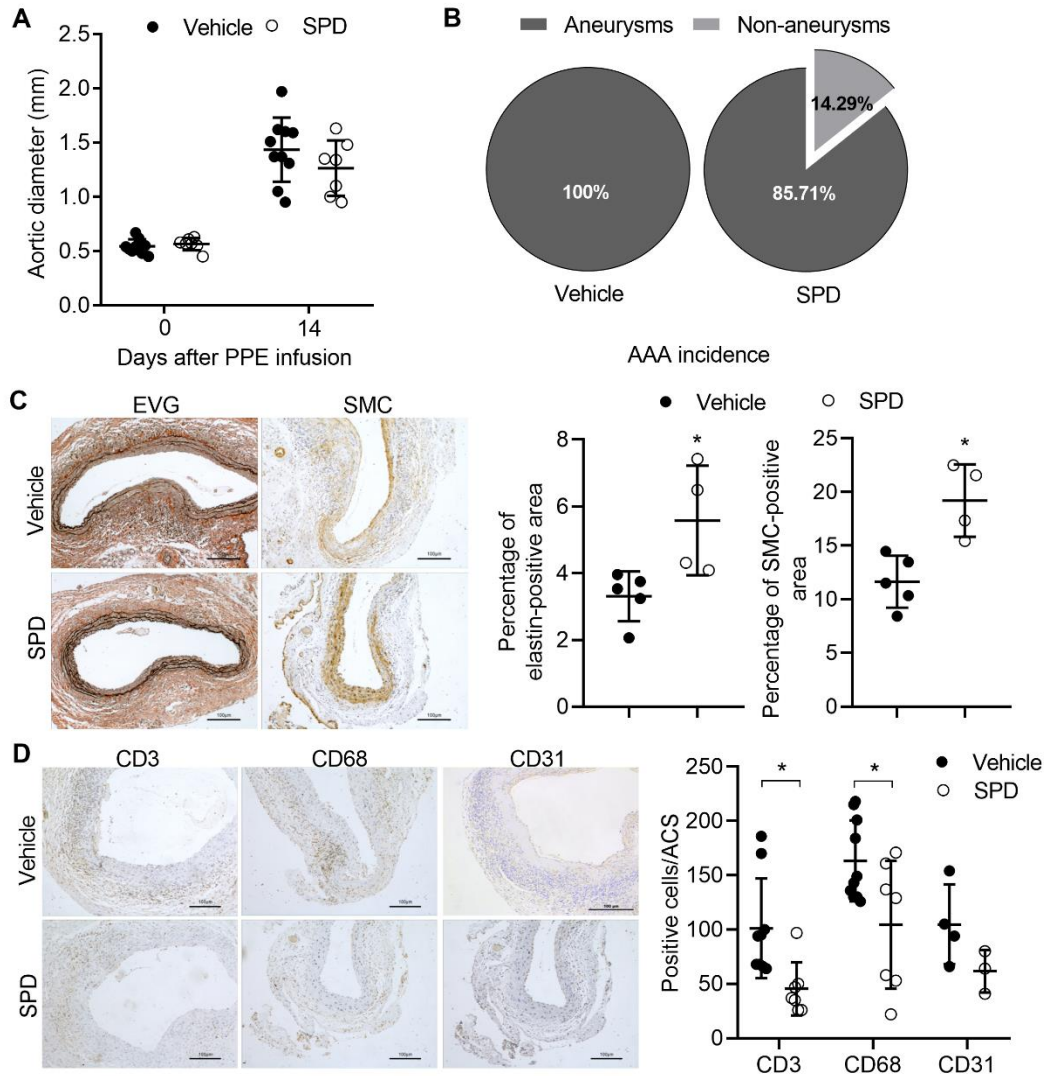
The percentage of elastin ( $P < 0.001$ ) and SMC ( $P = 0.028$ ) positive area in the total medial area was quantified with ImageJ software in each group. Data are presented as mean  $\pm$  SD. unpaired  $t$  test,  $*P < 0.05$  and  $**P < 0.01$  vs. vehicle group,  $n = 4-7$  in each group.

**Figure S2. Masson and IHC staining in human AAA and aorta.**



Human abdominal aneurysm and adjacent non-aneurysm aortic segments were conducted Masson and IHC staining. Representative images for muscle fiber (red),  $CD3^+$  T cells,  $CD68^+$  macrophages and  $CD31^+$  blood vessels in each group were shown above.

**Figure S3. Spermidine treatment reduces aortic remodeling in established experimental AAA.**



Vehicle and SPD treatment was performed in established experimental AAA from 3 days after PPE infusion to execution. (A) Aortic diameter (mm) of mice treated with vehicle (n= 10) or SPD (n=7) at day 0 and day 14. Data are presented as mean  $\pm$  SD. Unpaired *t* test,  $P = 0.237$  vs. vehicle group. (B) AAAs developed in 10 mice (100%, 10/10) within 14 days in the vehicle group, whereas AAAs developed in 6 mice (85.71%, 6/7) within 14 days in the SPD group. Fisher's exact test,  $P= 0.41$  vs. vehicle

group. (C) Representative images and positive area for elastin ( $P= 0.004$ ) and SMC ( $P= 0.017$ ) in each group. (D) Representative images for  $CD3^+$  T cells ( $P= 0.012$ ),  $CD68^+$  macrophages ( $P= 0.023$ ) and  $CD31^+$  blood vessels ( $P= 0.128$ ) and quantified as the positive stain cells per ACS and SMC in each group. Scale bar =100  $\mu\text{m}$ . Data are presented as mean  $\pm$  SD. Unpaired  $t$  test, \*\*  $P < 0.01$  and \* $P < 0.05$  vs. vehicle group,  $n= 3-8$  in each group.