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ORIGINAL ARTICLE

Kunming mouse strain is less susceptible to elastase-induced abdominal aortic aneurysms

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Abstract

Background: Porcine pancreatic elastase (PPE) is successfully used to induce abdominal aortic aneurysm (AAA) in mice. However, differences between mouse strains in susceptibility to PPE induction have been reported. Kunming mouse is one of the most frequently used strains in China but whether it is suitable for induction of AAA by PPE application remains unclear.

Methods: PPE infusion (1.5 units/ml) in temporary controlled aorta was performed to induce AAAs in both C57BL/6J and Kunming mice. Phosphate-buffered saline (PBS) application was used as vehicle control. The aorta diameters of all mice were measured at days 0 and 14 after surgery to evaluate the AAA formation.

Results: After 14 days of PPE or PBS infusion, all mice were sacrificed and aorta tissues were collected for histological staining analysis. At the 14th day after infusion, PPE successfully induced aortic dilation in Kunming mice and typical AAA in C57BL/6J mice. The aorta diameter increased by 0.23 mm in Kunming mice after PPE infusion, while it was 0.72 mm in the C57BL/6J strain. PPE induced mild elastin degradation, smooth muscle cell (SMC) depletion and mural leucocyte infiltration in Kunming mice, but in PPE-sensitive C57BL/6J mice, it induced total loss of SMCs, elastin disappearance and diffused infiltrated leucocytes in aortic aneurysmal segments. The effects of PPE in inducing angiogenesis and upregulating matrix metalloproteinase 2 and 9 expression in Kunming mice were also weaker than that in C57BL/6J mice.

Conclusion: At the reported dose of PPE, Kunming mouse is not as susceptible to AAA formation as C57BL/6J mice. The failure of PPE to induce AAA formation in Kunming mice may be associated to its inability to boost a strong inflammatory response.

KEYWORDS
abdominal aortic aneurysms, C57BL/6J, histology, Kunming, strain

Haole Liu and Kangli Tian contributed equally to this work.

1 | INTRODUCTION

Abdominal aortic aneurysm (AAA) is a life-threatening vascular disease with up to 80% mortality in the case of rupture.[1,2] Effective management needs to be developed to lower the incidence or slow its progression. AAA is a multifactorial disease with both genetic and environmental risk factors.[3] Traditional risk factors for AAA include male sex, advanced age, cigarette smoking, chronic obstructive pulmonary disease and obesity.[1,3–5] Genetic and environmental factors, including familial, racial, and ethnic background are also involved in the progression of aneurysms.[6–9] Several animal models have been created and used to clarify the mechanism of AAA, and to test potential therapeutics. The porcine pancreatic elastase (PPE)-induced AAA animal model is one of the most important models in aneurysmal disease research.[10–12] It has been reported that, like humankinds, the sex and genetic background of experimental mouse also have effects on the outcome of aneurysms.[13–15] Therefore, testing the susceptibility of different animal strains to AAA formation remains an important issue for understanding the complex mechanism of aneurysmal disease.

Pressurized infusion of PPE into a surgically isolated aortic segments to induced aneurysm was first reported in rat.[16] Subsequently, this method has been widely used in mice to create AAA and explore its mechanism.[10] Use of PPE to induce AAA has been tested in C57BL/6J, BALB/c, SvJ, CBA, and B6129/SvEv strains, and C57BL/6J proved to be highly susceptible to aneurysm formation.[13–15,17] Kunming mouse, one of the most widely used outbred strains in China, is derived from Swiss mice and was introduced to China from the Hoffkine institute in India. Since the place where the mouse was first introduced and bred to form a new outbred strain was Kunming, it was named Kunming mouse.

The differences between strains in AAA formation have been compared in many other strains, but no study has been conducted in Kunming mice. The aim of this study was to test the differences in formation of AAA after PPE infusion between C57BL/6J and Kunming mice.

2 | MATERIALS AND METHODS

2.1 | Animals

Six- to nine-week-old male C57BL/6J and Kunming mice, (body weight range 26–30 g) were obtained from the Laboratory Animal Center of Xi'an Jiaotong University. All mice were housed and maintained in a specific pathogen free grade animal facility (Xi'an, China) with a 12 h/12 h light-dark cycle. The animal experimental protocols were approved by the Laboratory Animal Administration Committee of Xi'an Jiaotong University and were performed according to the Guidelines for Animal Experimentation of Xi'an Jiaotong University and the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 2011).

2.2 | Surgical method for creating AAA in mice

Type I PPE solution was freshly prepared in phosphate-buffered saline (PBS; 1.5 units/ml, Cat #, E-1250; Sigma-Aldrich, St. Louis, MO). PPE-induced AAAs were created in both C57BL/6J and Kunming strains using a previously reported surgical method.[12,18–20] Briefly, after induction of anesthesia with isoflurane, the abdominal cavities of mice were opened and the infrarenal aorta was exposed and isolated from the level of the left renal vein to the iliac bifurcation. All small branches arteries were ligated to avoid leakage during PPE solution infusion. As shown in Figure 1A, three sutures were placed under the aorta for subsequent temporary ligation. After temporary ligature of the abdominal aorta, a 30-gauge needle was advanced into the aortic bifurcation to make an aortotomy. A P-10 tubing, connected to a syringe attached to a continuous pump, was inserted through the aortotomy into the controlled segment and fixed by temporary ligation. Under constant pressure (approximately 100 mmHg), about 30–50 μl of PPE solution was infused for 5 min. After PPE infusion, the PE-10 tubing was withdrawn, the aortotomy was closed with 11-0 sutures and aortic flow to the lower limbs was restored. After approximately 2 h recovery, all mice were moved to the animal facility and were housed for 14 days. In total, sixteen C57BL/6J mice received surgery and twelve of them survived (PPE group, $n = 6$; PBS group, $n = 6$), while twelve out of fourteen Kunming mice survived after the surgery (PPE group, $n = 6$; PBS group, $n = 6$). Fourteen days after PPE or PBS infusion, all mice were sacrificed by carbon dioxide inhalation.

2.3 | Aortic diameter measurement

Baseline aortic diameter was measured during the surgery. The exposed abdominal aorta was photographed using a surgical microscope equipped with a digital camera (ProS5 Lite, Motic, China) and aorta were measured with the image analysis software (Images Plus 3.0 ML, Motic, China). On the 14th day after surgery, the mouse abdominal aorta was photographed again immediately after sacrifice to determine whether aneurysm had been successfully induced. An AAA was defined when the infused aorta segment dilated more than 50% compared to the baseline level.[21]

2.4 | Histological analysis

Abdominal aortae were collected and immediately embedded in Tissue-Tek® O.C.T. Compound (Cat #,4583, Sakura, USA) for subsequent frozen tissue sectioning. To compare the histological characteristics of the AAA in the two mice stains, all aorta blocks were conducted on serial sections (6 μm) with Hematoxylin and Eosin (H&E), elastic van Gieson (EVG) and Masson's trichrome staining. Elastin degradation grading followed our previously reported criteria: (I) elastin break or degradation was limited to one outer medial elastin layer; (II) elastin degradation was seen in more than two

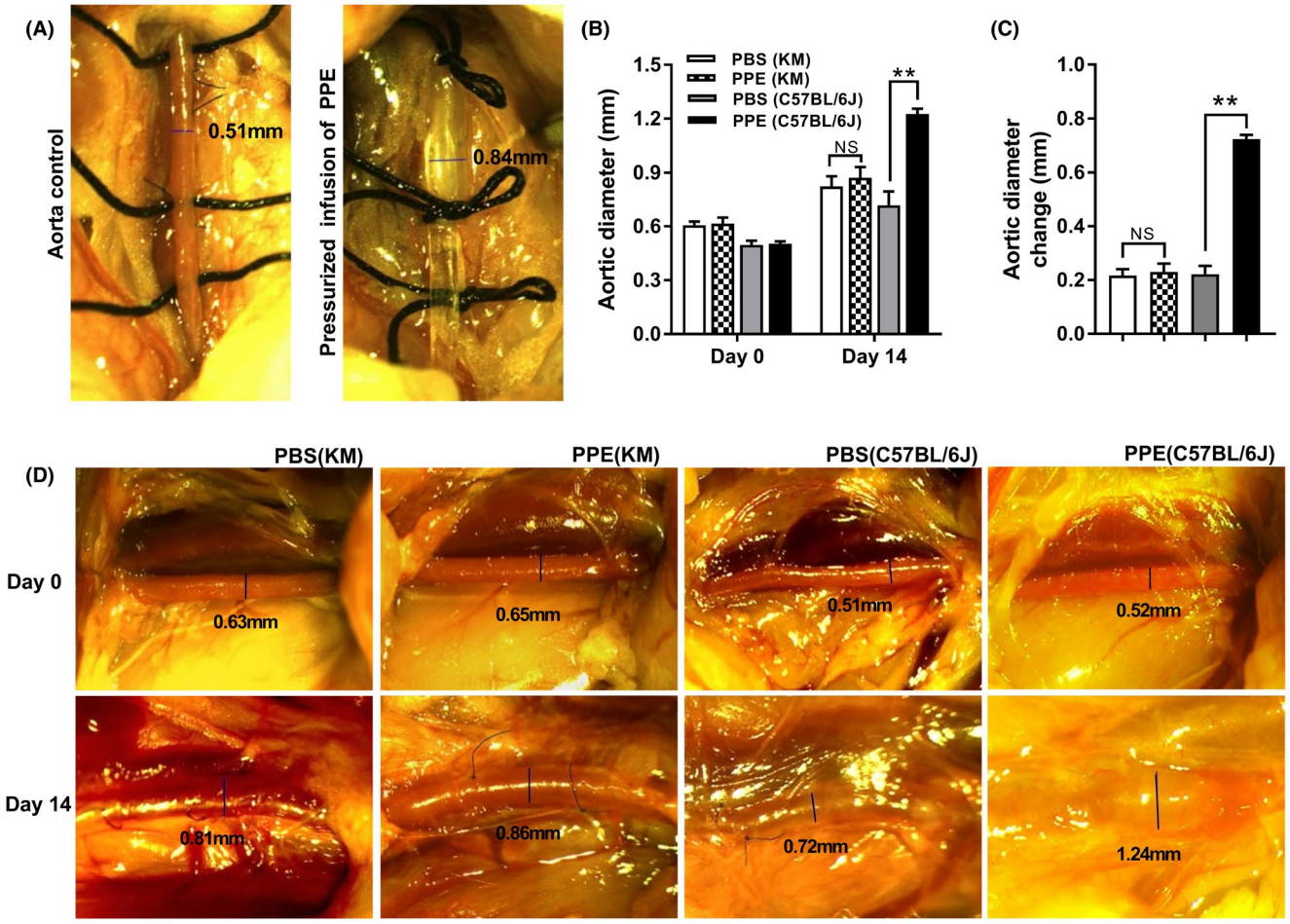

FIGURE 1 PPE infusion induced abdominal aortic dilation or aneurysm formation in Kunming or C57BL/6J mice. (A) All male mice were given PPE infusion in the controlled aorta segment. (B) Average aortic diameter at baseline level (day 0) and 14 days after PPE infusion; (C) Aortic diameters increased after PPE infusion between the two strains. (D) Representative abdominal aorta photographs at the 0 and 14th day in PPE-infused mice. One-way ANOVA followed by two group comparison, $^{**}p < .01$ between two groups. $n = 6$ mice in each group. NS, not significant difference

layers, or the entire medial elastin layer, but was limited to less than 1/4 of the aortic circumference (AC); (III) elastin degradation was seen in the entire medial elastin layer, but was limited to less than 1/2 the AC; (IV) elastin degradation was seen in all elastin layers and had expanded to more than 3/4 of AC.[12,19] For collagen degradation, the Masson's trichrome stained sections were photographed under a microscope equipped with a digital camera and measured with image analysis software (WinRoof 6.5, Mitani Co. Ltd., Tokyo, Japan).

2.5 | Immunohistochemical analysis

To compare the loss of smooth muscle cells (SMCs) in the two mouse strains 14 days after PPE or PBS infusion, frozen sections were stained with antibody against SMCs (α-actin) (Cat #, NB300-978; Novus Biologicals, Centennial, CO). Aortic medial SMC loss was graded on a scale of I to IV similar to the elastin grading method.[21] To analyse the infiltration of inflammatory cells into the AAA segments, antibodies against macrophages (CD68, Cat #,137002), $CD4^+$ T cells (Cat #,100402), $CD8^+$ T cells (Cat #,100702) and B cells (CD45R, Cat #,103202) were used for immunohistochemical (IHC) staining. CD31 IHC staining was used to show aortic mural angiogenesis (Cat #,100402)—neovessels were counted and reported as numbers per aortic cross-section. All the above leucocytes and CD31 primary antibodies were purchased from BioLegend Company and diluted 1:200 when used for IHC staining. Macrophage infiltration into AAA segments was also graded on a scale of I to IV based on mural infiltration patterns and proportionality of AC: (I) diffuse infiltration of less than 1/4 of AC; (II) infiltration of from 1/4 to 1/2 of AC; (III) infiltration of from 1/4 to 3/4 of AC; and (IV) aggregate infiltration of more than 3/4 of AC.[11] Mural infiltration of T and B cells was measured as the number of positive stained cells per aortic cross-section at 200× magnification. Matrix metalloproteinase 2 (MMP2; 1:200; Cat #, AF1488, R&D Systems, Minneapolis, MN) and MMP9 (1:200; Cat #, AF909, R&D Systems) expression levels were also checked using the IHC method. After the IHC stained sections were photographed under a microscope, the positive stained areas

were calculated using the above-mentioned image analysis software (WinRoof 6.5). The secondary antibodies used in this study include biotinylated goat anti-rat antibody (1:400; Cat #, BA-9400; VECTOR, Burlingame, CA) and donkey anti-goat IgG antibody (1:400; Cat #, 705-065-003; Jackson ImmunoResearch Inc., West Grove, PA). The streptavidin-peroxidase conjugate was purchased from Jackson Immuno Research Laboratories (1:400; Cat #, 016-030-084) and AEC substrate kit was from VECTOR (Cat #, SK-4200).

2.6 | Statistical analysis

The measurement data are expressed as the mean ± SEM. For single time point data, one-way ANOVA analysis was conducted. For double time point data, two-way ANOVA followed by multiple comparisons was used to determine the significance between different groups. For nonnormal distribution data, differences were tested using the nonparametric test. All statistical analysis were performed by PRISM 7.0 and $p < .05$ was considered significant.

3 | RESULTS

3.1 | PPE successfully induces aortic dilation in Kunming mice and AAA in C57BL/6J mice

The abdominal aorta surgery was successfully performed in all mice (Figure 1A). Generally, the baseline aorta diameters of Kunming mice were bigger than those of C57BL/6J mice, but the infrarenal abdominal aorta of Kunming mice usually possessed fewer branches arteries needing to be ligated. PPE successfully induced AAA in C57BL/6J mice on day 14 after surgery; the aorta diameter increased from 0.52 to 1.23 mm (Figure 1B–D). Pressurized infusion of both PPE and PBS resulted in aorta dilation, but in all Kunming mice the dilation did not reach the diagnostic criteria for aneurysm, which required aortic dilation more than 50% compared to the baseline level (Figure 1A–D). On day 14 after surgery, the aorta diameter in PBS-infused Kunming mice had increased by 0.22 mm and was similar to the increase seen in PPE-infused mice (0.23 mm) (Figure 1C,D). However, in C57BL/6J mice, the aorta diameter increased by 0.72 mm after PPE infusion, which was significantly higher than that in PBS-infused mice (Figure 1C). These differences in response to PPE infusion between Kunming and C57BL/6J mice may be due to their genetic background.

3.2 | PPE induces slight elastin degradation in Kunming mice and severe elastin destruction in C57BL/6J mice

Elastin degradation is one of the main histological hallmarks of human AAA. As shown in Figure 2A, although the infusion of PPE or PBS resulted in aortic elastin degradation, the integrity of aortic lamina remained unbroken in Kunming mice. Elastin degradation was associated with PPE infusion was a little more severe than in the PBS application group, but the difference was not significant (Figure 2A,B). The H&E, EVG and Masson's trichrome staining results for PBS infusion were similar in Kunming mice and C57BL/6J mice; however, PPE application in C57BL/6J mice led to total elastin destruction and severe collagen digestion compared to the other three groups in this study (Figure 2A). The elastin degradation score was significantly higher in PPE-infused C57BL/6J mice, and less collagen remained in the aortic wall. PPE infusion destroyed the aortic histological integrity in C57BL/6J mice, but not in Kunming mice. This evidence showed that the failure of PPE to induce AAA may be related to its inability to degrade elastin and collagen in Kunming mice.

3.3 | PPE infusion promotes aortic SMC depletion and macrophage infiltration in C57BL/6J mice, but has less effect in Kunming mice

Aortic SMC depletion and macrophage infiltration are two other features of AAA. IHC staining of SMCs showed that pressurized infusion of both PPE and PBS could induce some degree of vascular SMC marker loss, but the morphological integrity remained in Kunming mice (Figure 3A). However, PPE infusion destroyed the entire aortic SMC ring in C57BL/6J mice and the SMC loss score was significantly higher (Figure 3A,B). Analysis of IHC staining of macrophages presented similar results: C57BL/6J mice were more sensitive to PPE-induced macrophage infiltration into the aortic wall than Kunming mice (Figure 3A–C). After pressurized infusion injury, SMC depletion was also aggravated by PPE-induced inflammation. The above data suggested that the weak effects of PPE on AAA formation in Kunming mice may be associated with failure to boost leukocyte infiltration.

3.4 | PPE is more effective in inducing aortic leukocyte infiltration in C57BL/6J mice than Kunming mice

Abnormal infiltrated leukocytes can release excessive inflammatory cytokines and promote aortic SMC dysfunction and elastin or collagen melting. In this study, $CD4^+$ T cells, $CD8^+$ T cells and B cells were found to have infiltrated the aortic wall and hyperplastic adventitia (Figures 3–5). Infiltration of all the above leukocyte subtypes was significantly more severe in PPE-infused C57BL/6J mice than that in Kunming mice. Though there was a trend for PPE infusion to induce more aortic leukocyte infiltration than PBS controls in the Kunming strain, the difference was not significant (Figures 4B,C and 5A,B). The above leukocyte analysis provides evidence that the general effects of PPE are weaker at boosting aortic inflammation in Kunming mice than in C57BL/6J mice.

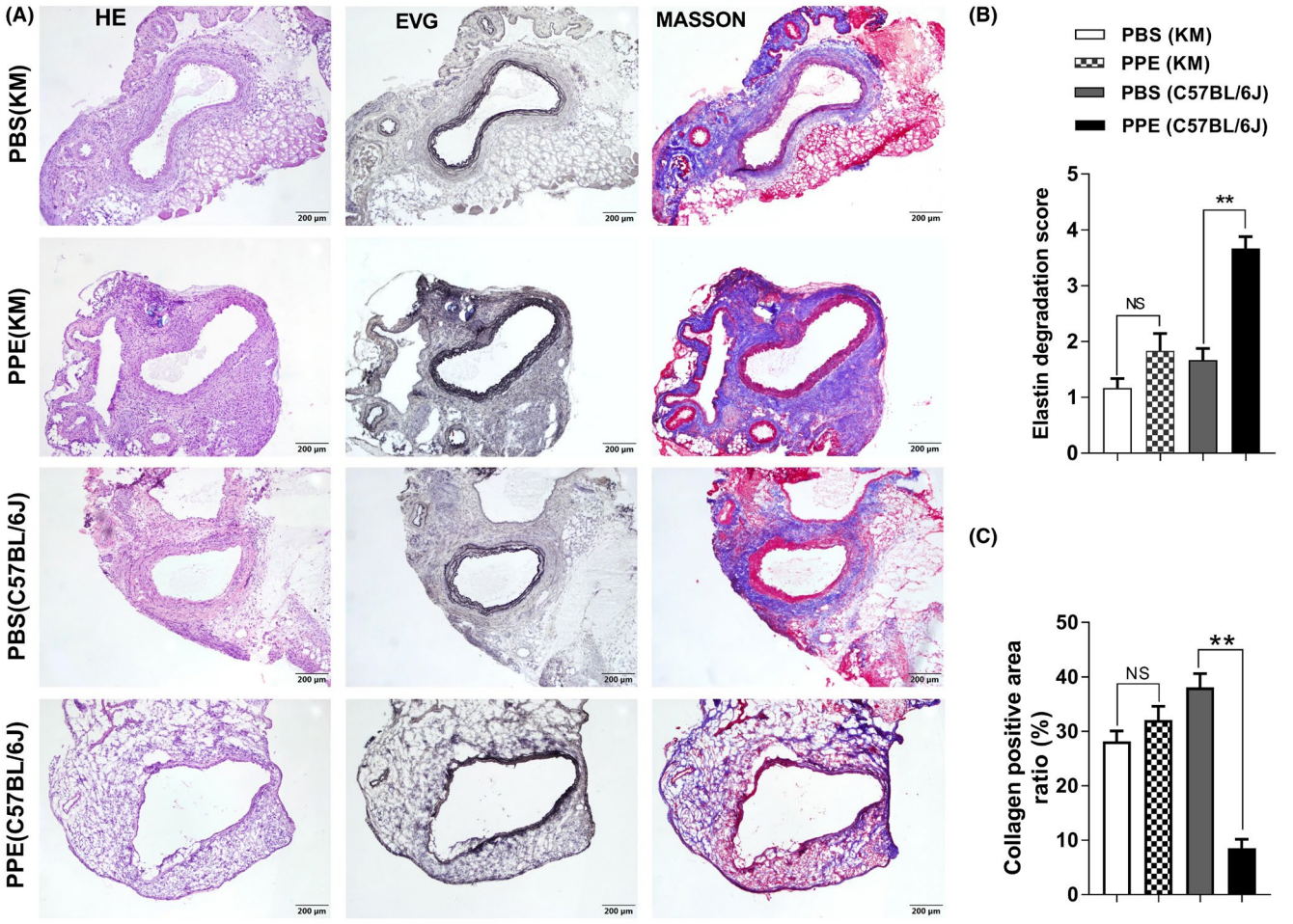

FIGURE 2 Representative H&E, EVG, and Masson's trichrome staining micrographs of PPE- or PBS-infused mice. (A) Representative histological micrographs of PPE- or PBS-infused mice. (B) Quantitative analysis of elastin degradation at 14 days after PPE/PBS infusion; (C) Quantitative analysis of aortic collagen digestion after PPE/PBS infusion in mice. The collagen positive stained area ratio was calculated as blue stained area/the whole aortic wall area. One-way ANOVA followed by nonparametric Kruskal-Wallis test, $^{**}p < .01$ between two groups. $n = 6$ mice in each group; NS, not significant

3.5 | PPE induced weak inflammation but failed to induce obvious mural angiogenesis and upregulate MMP2 and 9 expression in Kunming mice

Excessive leukocyte infiltration usually upregulates expression of MMP2 and 9 and promotes mural angiogenesis. Abnormal mural angiogenesis may exacerbate aortic inflammation and promote the progression of AAA. As shown in Figure 5, PPE infusion significantly increased the mural angiogenesis in C57BL/6J mice, but the effect was weak in Kunming mice. In C57BL/6J mice, the number of neovessels in the PPE-treated group was more than twofold that in the PBS-infused group (Figure 5C). However, PBS and PPE application induced similar levels of angiogenesis in Kunming mice (Figure 5A,C). PPE also did not play an important role in regulating MMP2 and 9 expression in Kunming mice (Figure 6A–C). MMP2 and 9 were significantly upregulated in PPE-infused C57BL/6J mice, and the increase was significantly higher than that of PBS-treated C57BL/6J mice and the other two groups of Kunming mice (Figure 6A–C). Mural infiltration of leucocytes was identified as the main source of MMPs. The failure of PPE to induce inflammation in Kunming mice may explain the inconsistent MMP expression between C57BL/6J and Kunming mice.

4 | DISCUSSION

Several methods are used to create AAAs in mice and PPE infusion remains one of the most widely used methods in this field.[10] PPE infusion can induce elastin breakdown and vascular SMC loss and disrupt media structure.[22] Exogenous PPE can also trigger inflammation by promoting leukocyte infiltration to clean degraded extracellular matrix, efferocytosis of dying SMCs and injured endothelium, and all the subsequent biological events following PPE infusion contribute the progression of AAA.[10,22] As one of the most widely accepted AAA models, the PPE-induced model shows typical elastin degradation/break, SMC depletion and mural leukocyte infiltration, which are the main histological features of human AAA.[10,12,13] Several strains of mice had been tested to create AAA by infusion of PPE, but no data exist for Kunming mouse, which is a most widely used strain in China.

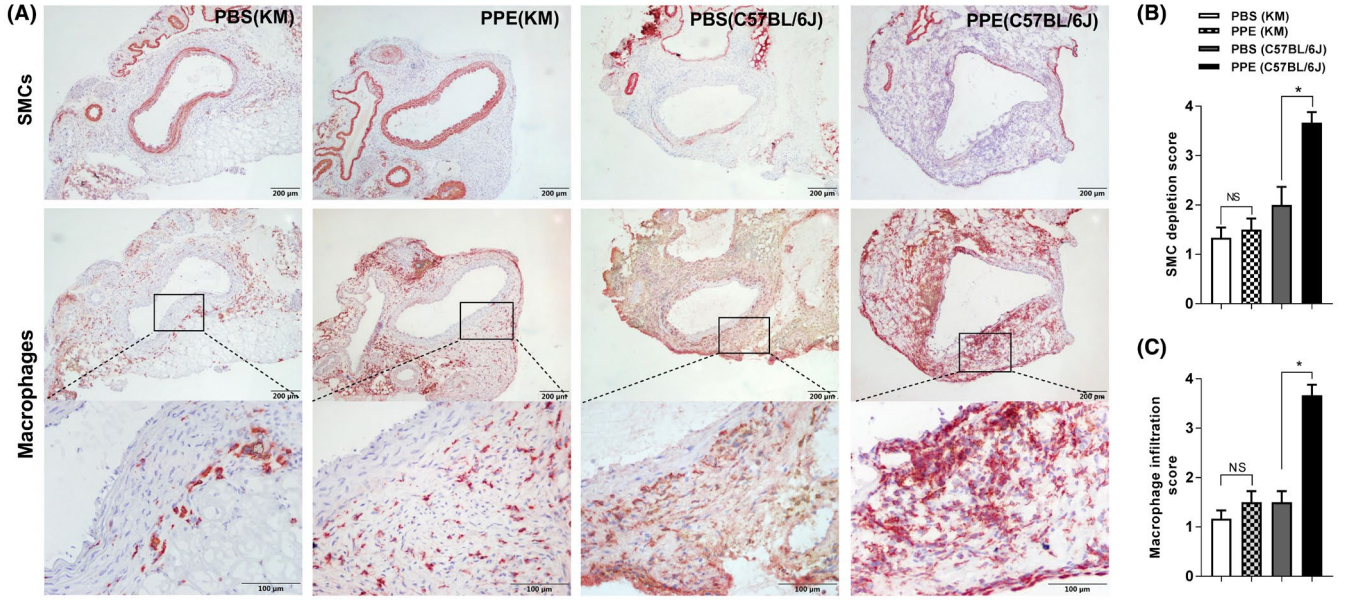

FIGURE 3 Representative aortic IHC micrographs of SMC depletion and macrophage infiltration of aortae of PPE- or PBS-infused mice. Vascular SMC depletion and leucocyte infiltration, two hallmarks of AAA, were detected by IHC staining of aortic aneurysmal sections. (A) Representative micrographs of aortic wall SMCs and mural macrophage infiltration after infusion; (B) Quantitative analysis of vascular SMC loss; (C) Scores for mural macrophage infiltration in the four groups. Nonparametric Kruskal-Wallis test followed by two group comparison, $n = 6$ for each group; $^{*}p < .05$. NS, not significant

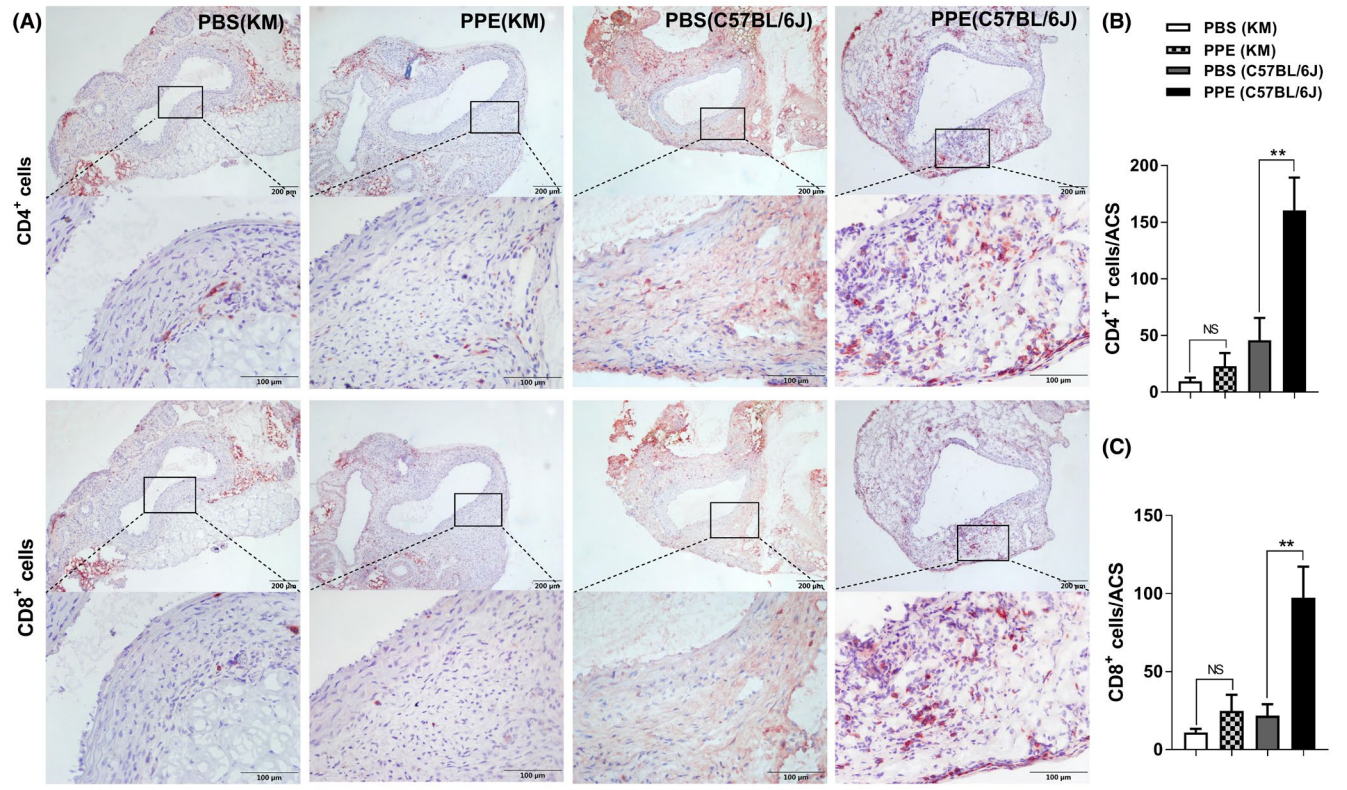

FIGURE 4 Representative aortic IHC micrographs of $CD4^+$ and $CD8^+$ T cells infiltration in aortae of PPE or PBS-infused mice. $CD4^+$ and $CD8^+$ T cell staining was performed on frozen aortic sections and infiltrated cells were counted according as described in the Methods section. (A) Representative aortic mural $CD4^+$ and $CD8^+$ T cell infiltration images; (B) Quantitative analysis of $CD4^+$ infiltration; (C) Quantification of $CD8^+$ T cells in the four groups. One-way ANOVA followed by two group comparison, $^{**}p < .01$ between two groups. $n = 6$ mice in each group. NS, not significant

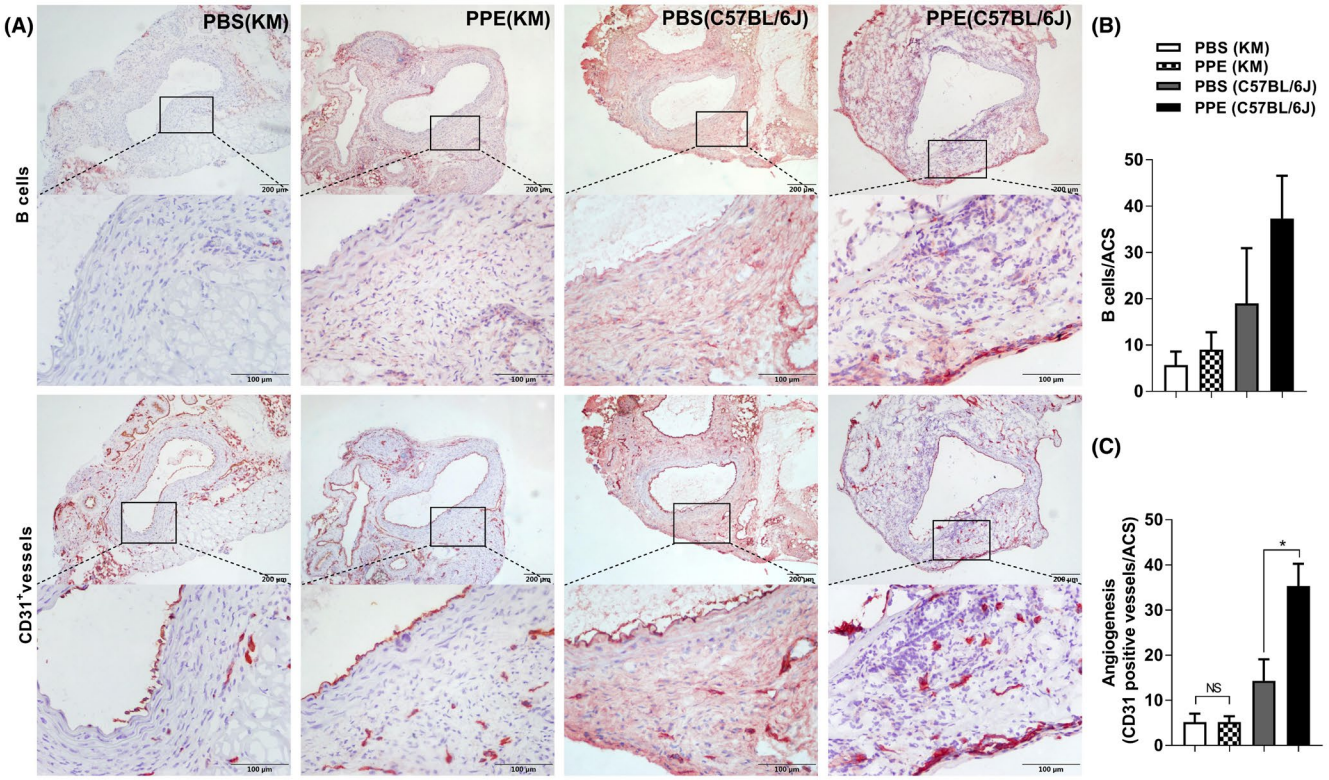

FIGURE 5 Representative aortic IHC micrographs of B cell infiltration and angiogenesis. (A) Representative micrographs of aortic mural B cell infiltration and neovessels after infusion; (B) Quantitative analysis of B cell infiltration; (C) Numbers of aortic mural angiogenesis in the four groups. One-way ANOVA followed by two group comparison, $^{**}p < .01$ between two groups. $n = 6$ mice in each group. NS, not significant

In this study, we aimed to test the susceptibility of Kunming mice to PPE-induced AAA formation. To our knowledge, this study is the first attempt to compare the aorta histological features of C57BL/6J mice and Kunming mice after PPE infusion. Only dilation, not the full AAA phenotype, was found in PPE-infused Kunming mice, while the C57BL/6J mice developed typical AAA after PPE application. These results may resemble the human racial/ethnic differences seen in aneurysmal disease.[6–9] The contribution of genetic background to the different susceptibilities to PPE application among mouse strains has also been reported previously.[14,15] Recently, the C57BL/6 substrains C57BL/6J and C57BL/6N were reported to show different responses to Ang II infusion, which is another commonly used method of creating AAA in mice*.[17] Both PPE and PBS infusion can induce significant aortic dilation in Kunming mice, but the failure of PPE to induce typical AAA in Kunming mice, as it does in C57BL/6J mice, may reflect genetic variations. Due to a deficiency of nicotinamide-nucleotide-transhydrogenase, aortic SMCs of C57BL/6J mice display increased oxidative stress, oxidative DNA damage and stronger inflammatory responses after Ang II infusion.[17] Whether this genetic deficiency also contributes to PPE-induced AAA formation remains to be clarified. In Kunming mice, another reason for failure to induce AAA following PPE infusion may be the failure of PPE to boost mural infiltration of leukocytes to bring about secondary injure to the elastin and SMCs.

*[Correction added on 18 January 2022 after the first publication: In this sentence, C57BL/6J has been changed to C57BL/6].

Unlike in C57BL/6J mice, histological results for Kunming mice revealed that infusion of both PBS and PPE led to slight SMC injury, with no obvious difference between PPE and PBS. Similar histological results were also found for elastin staining. PPE infusion also failed to induce significant elastin break or degradation when compared to PBS. Subsequent IHC staining of leukocytes in Kunming mice also showed nearly consistent results between PBS and PPE. Compared with PBS, PPE infusion failed to induce more leukocyte migration and mural infiltration of macrophages, $CD4^+$ T cells, $CD8^+$ T cells and B cells. In addition to aortic dilation induced by pressurized infusion, PPE-related inflammation plays an important role in expansion of the aortic leading to AAA.[10] All data suggest that the failure of PPE to boost inflammation may result in lack of continuously leukocyte-driven aortic expansion in Kunming mice. The weak infiltration of leukocytes in these mice also resulted in no significant promotion of angiogenesis and upregulation of MMP2 and 9 expression.

In this study, the PPE-infusion dose that proved to be effective in C57BL/6J mice did not successfully promote AAA formation in Kunming mice. The differences in susceptibility to forming AAAs between mice strains may be inherited, i.e. genetically determined.[13] PPE infusion initially induces local expansion and acute inflammation of aorta through its effects on the extra cellular matrix, SMCs, and recruitment of inflammatory cells. To identify whether higher doses of PPE can induce inflammatory cell infiltration and AAA progression, we infused 3.0 and 6.0 units/ml PPE into Kunming mice. The results showed that the higher

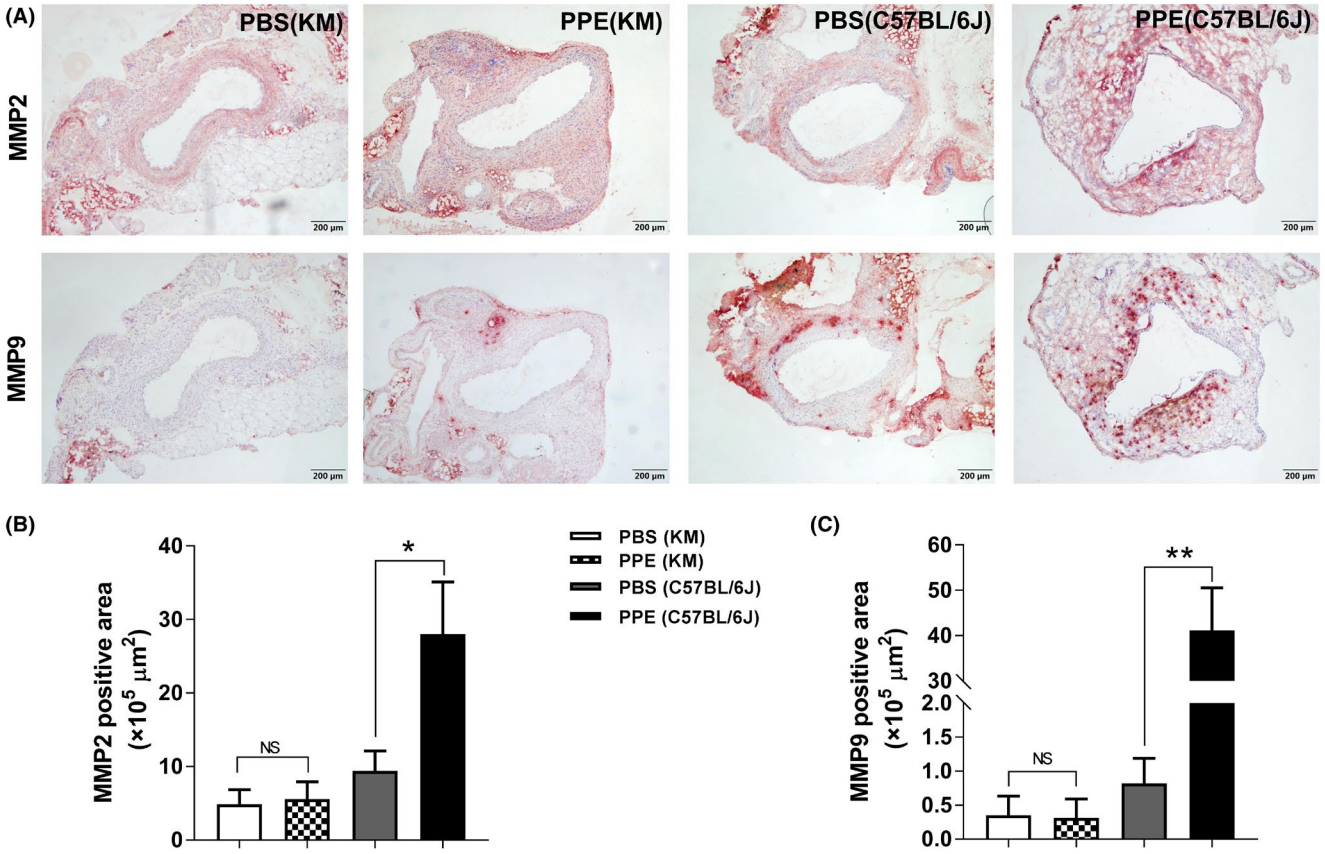

FIGURE 6 The effect of PPE/PBS infusion on MMP2 and 9 expression. (A) Representative micrographs of aortic MMP2 and 9 expression after infusion; (B) Quantitative analysis of MMP2 expression; (C) Quantitative analysis of MMP9 expression. One-way ANOVA followed by two group comparison, $n = 6$ mice in each group. NS, not significant; $^{*}p < .05$ and $^{**}p < .01$

doses tended to promote more severe inflammation and formation of AAA. However, the higher mortality associated with these doses precludes their use in mice studies. Only 55% (6/11) and 10% (1/10) of the mice survived in 3.0 and 6.0 units/ml groups, respectively. Though the detailed genetic and molecular mechanisms that explain why Kunming mice are resistant to PPE-induced AAA remain to be clarified, the lack of effect of PPE at a feasible dose may limit its application in this field.

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CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTIONS

S.Z., B.X. and E.L. designed and supervised this experiment. H.L., K.T., C.X., P.W., B.X., W.F. and Y.L. performed animal experiments. H.L., K.T., Y.L., R.W. and L.B. conducted the histological analysis. W.W., H.L. and S.Z. analyzed and interpreted the data. S.Z. and K.T. wrote the manuscript. All authors read and approved the final manuscript.

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