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Original Article

Modulation of lectin-like oxidized low-density lipoprotein receptor-1 by *Porphyromonas gingivalis* promoting progression of atherosclerosis in apolipoprotein $E^{-/-}$ mice

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KEYWORDS
Porphyromonas gingivalis;
Atherosclerosis;
LOX-1;
Poly(I:C);
Plaque;
Macrophage

Abstract *Background/Purpose: Porphyromonas gingivalis* (*P. gingivalis*), the primary pathogenic bacterium in periodontitis, can infiltrate the cardiovascular system via the bloodstream and actively contribute to various pathological processes associated with atherosclerosis. The scavenger receptor lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) plays a crucial role in atherosclerosis pathogenesis. Previous studies have shown that LOX-1 is involved in endothelial cell activation injury, monocyte migration, and adhesion to endothelial cells induced by *P. gingivalis*. The objective of this study was to further investigate the potential role of LOX-1 in promoting *P. gingivalis*-induced atherosclerosis in mice.
Materials and methods: Using apolipoprotein E $(APOE)^{-/-}$ mice fed with high-fat diet for an established model. Intravenous injection of *P. gingivalis* was performed to create *P. gingivalis* blood model while intraperitoneal injection of Polyinosinic-polycytidylic acid (Poly (I:C)) served as an inhibitor for LOX-1. After 12 weeks, plaques and blood lipids were examined.
Results: Results showed that induction with *P. gingivalis* led to increased expression of LOX-1 in both the aortic root and blood samples, increased plaque area, reduced plaque stability, elevated expression levels of vascular adhesion molecule-1(VCAM-1), Interleukin-6(IL-6) and

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M1 macrophages. However, pretreatment with Poly (I:C) resulted in decreased plaque area improved plaque stability and reduced expression levels of VCAM-1 and IL-6.
Conclusion: These findings suggest that LOX-1 may serve as an intermediary factor promoting atherosclerosis associated with periodontitis.

Introduction

Periodontitis is the sixth most common disease, which affects 11.2 % of the global population.[1] Periodontitis is an inflammatory disease characterized by the disruption of host immune response due to oral pathogens, resulting in the destruction of tooth support tissue and eventual tooth loss. Furthermore, microbial immune disruption not only affects periodontal tissue homeostasis locally but also impairs immune surveillance and balance systemically (e.g., atherosclerotic plaque, placenta, and brain), thereby facilitating or accelerating the pathogenic process.[2,3] The presence of bacteremia caused by periodontal infections is a significant contributing factor to the overall impact of periodontitis on an individual's health. The principal periodontal pathogen is *Porphyromonas gingivalis* (*P.gingivalis*).

Atherosclerosis (AS), a prevalent pathology affecting the large arteries, constitutes a significant etiology of cardiovascular diseases and cerebrovascular accidents. It accounts for approximately 50 % of mortality cases.[4] Substantial evidence supports the importance of immune pathways in the development of atherosclerosis, and infection may affect the course of the disease directly or indirectly, acutely, and chronically.[5] The mechanism of *P. gingivalis* affecting cardiovascular disease has always been worthy of attention. The mechanism underlying the acceleration of AS by *P. gingivalis* has been a focus of research, with particular emphasis on the role of oxidative stress, inflammation, and immune responses induced by these bacteria in disease progression. However, despite extensive clinical data analysis and scientific reports, the precise mechanism through which *P. gingivalis* expedites the onset of AS remains elusive.

The lectin-like oxidized low density lipoprotein receptor 1 (LOX-1) is renowned for its pivotal role as a receptor for oxidized low density lipoproteins (oxLDL), as initially documented by Sawamura et al., in 1999.[6] The LOX-1 receptor recognizes, binds to, and internalizes oxLDL, thereby initiating inflammatory responses and oxidative stress. It has been identified as a crucial receptor in the pathogenesis of atherosclerosis. The overexpression of LOX-1 induces endothelial dysfunction and cellular apoptosis, while also promoting the formation of foam cells in macrophages and vascular smooth muscle cells.[7] The LOX-1 receptor not only interacts with oxLDL, but also exhibits recognition and binding capabilities towards various other ligands, including diverse pathogens.

Polyinosinic-polycytidylic acid (Poly (I:C)) is a synthetic double-stranded RNA that has been utilized in various research studies as an inhibitor of LOX-1 and as a combination therapy for enhancing anti-tumor immunity due to its action as an agonist of Toll-like receptors (TLRs).[8–10] Poly (I:C) preconditioning reduces proinflammatory state in obesity-related insulin resistance and promotes glucose homeostasis by enhancing immune tolerance.[11]

In vitro study has demonstrated that LOX-1 facilitates *P. gingivalis*-mediated endothelial cell injury and monocyte adhesion, thereby playing a pivotal role in the development of periodontitis-induced atherosclerosis.[12] Our study was designed to further investigate the regulation of LOX-1 by *P. gingivalis*-induced atherosclerosis through in vivo.

Materials and methods

Bacterial culture

The strain *P. gingivalis* W83 was kindly provided by Professor Lai Chen-hsiung from Kaohsiung University. The *P. gingivalis* was grown for 4–6 days on brain heart infusion (BHI) blood agar plates (OXOID, Waltham, MA, USA) which contained 5 % defibrinated sheep blood (Baiaolaibo, Beijing, China), 5 μg/mL hemin (Hopebiol, Qingdao, China), and 1 μg/mL vitamin K1 (Hopebiol) in an anaerobic system (10 % H2, 85 % N2, and 5 % CO2) at 37 °C. Bacterial colonies were then inoculated into BHI broth medium supplemented with 5 μg/mL hemin, and 1 μg/mL vitamin K1, and cultured for 24 h. The bacteria were subsequently harvested through centrifugation (6000 rpm, 4 °C, 10 min), washed and resuspended with phosphate buffered salt solution (PBS, PH = 7.2). The bacterial resuspension should be adjusted to an optical density (OD) of 0.5 at a wavelength of 600 nm, which corresponds to a concentration of 10^8 CFU/mL.

Animals and diets

18 8-week-old male apolipoprotein E $(APOE)^{-/-}$ mice (purchased from Beijing Vital River Laboratory Animal Technology Company, Beijing, China) were reared in a SPF laboratory and fed a high-fat diet (HFD, 40 % kcal fat, 40 % kcal carbohydrate, 20 % protein; D12109C, FBSH, Shanghai, China).[13] Feeding under 12:12 light/dark cycle conditions. The mice were equally randomly divided into three groups: PBS + PBS, PBS + *P. gingivalis*, Poly (I:C) + *P. gingivalis*. For *P. gingivalis*/PBS infection, mice were injected with *P. gingivalis* (10^7 CFU in 0.1 mL PBS)/PBS by tail-vein injection twice a week. Two hours before *P. gingivalis* infection in the tail vein, 200 μL of Poly (I:C) (Sigma, St. Louis, MO, USA)

was intraperitoneally injected into the Poly (I:C) group according to 5 μg/g body weight, and 200 μL of PBS was injected into the control group.[14] The experiment lasted 12 weeks. Mice were euthanized at indicated time points, and tissues and blood were collected. The aorta and heart are frozen in liquid nitrogen or fixed fluid. Blood serum was collected, sorted and frozen in −80 °C refrigerator after centrifugation. All animal experiments were carried out with the consent of the Institutional Animal Care and Use Committee of Peking University Health Science Center (BeiJing, China) (approval number LA2018087).

HE staining

In order to assess the atherosclerotic lesions in aortic root, mouse hearts were dehydrated and embedded with paraffin. A continuous cross-sections (5 μm thick) of the aortic root were prepared from the location where the three aortic valves first appeared. Mice aorta paraffinized sections were deparaffinized with xylene and hydrated with gradient ethanol. Hematoxylin and eosin stain the nucleus and cytoplasm respectively. The sections were photographed using an optical microscope (Olympus, Tokyo, Japan). The Image J software analyzed and compared the average plague area of five consecutive sections.

Masson staining

Mice aorta paraffinized sections were deparaffinized with xylene and hydrated with gradient ethanol. The subsequent steps should be carried out in accordance with the guidelines provided by Masson's dyeing kit for dyeing (Solarbio, Beijing, China).

Oil red O staining

The aorta was meticulously dissected from the ascending aorta to the iliac branch, and subsequently longitudinally incised using ophthalmic scissors. Following staining with oil red O, the tissue was immersed in saturated oil red O solution (Sigma, St. Louis, MO, USA) and incubated at 37 °C in a water bath for 30 min, resulting in differentiation into an orange-red hue upon exposure to 60 % isopropanol. After capturing images using a Canon camera, proceed with their analysis utilizing the Image J software.

The enzyme-linked immunosorbent assay (ELISA)

Analyzed samples were the serum of mice. Cytokine responses [vascular adhesion molecule-1(VCAM-1) and Interleukin-6(IL-6)] were measured using an ELISA kit (MEIMIAN, Jiangsu, China) in accordance with the manufacturer's instruction. Cytokine responses (oxLDL) were measured using an ELISA kit (CUSABIO, Wuhan, China).

Immunohistochemistry (IHC) and imaging

Mice aorta paraffinized sections were deparaffinized with xylene and hydrated with gradient ethanol. The sections were blocked for 1 h at room temperature and then stained with the primary antibodies overnight at 4 °C followed by incubation with horseradish peroxidase-conjugated secondary antibody. The DAB substrate solution (ZSGB-Bio, Beijing, China) was then used to induce the formation of a colored precipitate at the tissue antigen-binding sites. Restaining the nucleus with hematoxylin (Solarbio, Beijing, China). Primary antibodies against the following proteins were used: VCAM1 (Cell Signaling Technology, St. Danvers, MA, USA), IL-6 (Abclonal, Wuhan, China). The sections were acquired using a light microscope (Olympus, Tokyo, Japan).

Immunocytofluorescence (IF) and imaging

The expression of CD86 and CD206 in mouse aorta was detdemined by Double-Fluorescence immunohistochemical mouse/rabbit kit (ImmunoWay Biotechnology, Plano, TX, USA), according to the manufacturer's instructions. The sections were acquired using a light microscope (Olympus, Tokyo, Japan). Primary antibodies against the following proteins were used: CD86(ImmunoWay Biotechnology) and CD206(Cell Signaling Technology).

Statistical analysis

Data analysis was performed with SPSS 22.0 (SPSS Inc). Results were expressed as means ± SD. Data were analyzed by two-sample *t* test or one-way analysis of variance (ANOVA). Values of $P < 0.05$ were considered statistically significant (*: $P < 0.05$, **: $P < 0.01$ and ***:$P < 0.001$).

Results

The stimulation of *P. gingivalis* enhances the expression of LOX-1 in the aortic root of mice

The expression of LOX-1 is upregulated upon infection with *P. gingivalis* (Fig. 1). The level of LOX-1 was observed to significantly increase in the mouse aortic root after 12 weeks of *P. gingivalis* stimulation, as depicted in Fig. 1A. The expression of LOX-1 in mouse serum exhibited an upward trend following *P. gingivalis* infection ($P < 0.05$, Fig. 1B).

The administration of Poly (I:C) can attenuate the *P. gingivalis*-induced increase in plaque area

The HE staining revealed an increase in the proportion of core necrotic area within aortic root plaques following *P. gingivalis* stimulation ($P < 0.01$, Fig. 2A–C), which was mitigated by Poly (I:C) application resulting in reduced plaque area ($P < 0.05$, Fig. 2A and B) and core necrotic proportion ($P < 0.05$, Fig. 2A–C). Oil red O staining revealed an increase in the percentage of plaques throughout the entire aorta following infection with *P. gingivalis* ($P < 0.01$, Fig. 2D, E), and a decrease in the percentage of plaques after inhibition of LOX-1 ($P < 0.001$, Fig. 2D and E).

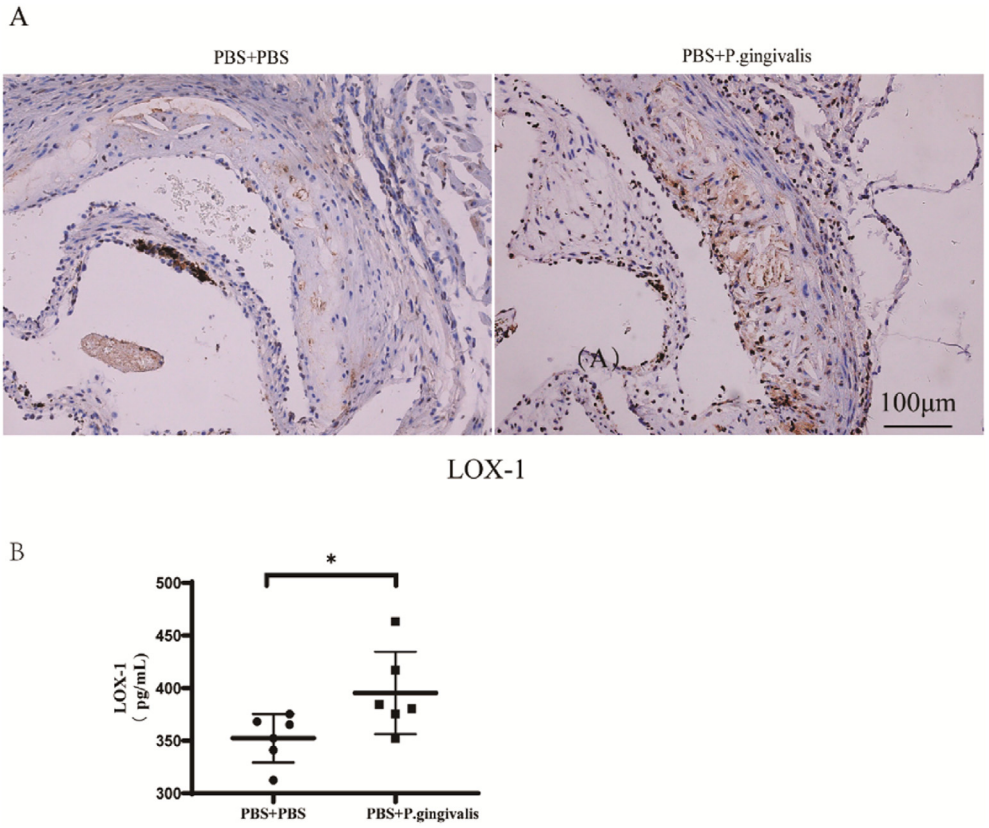

Figure 1 LOX-1 expression in the aortic root and serum in mice. The expression of LOX-1 in the aortic root pre- and post-infection with *P. gingivalis* (A). The expression of LOX-1 in the serum pre- and post-infection with *P. gingivalis* (B). (Black scale:100 μm). *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$. n = 6. Poly (I:C), Polyinosinic-polycytidylic acid; *P. gingivalis*, *Porphyromonas gingivalis*; PBS, Phosphate buffered saline; LOX-1: lectin-like oxidized low-density lipoprotein receptor-1.

The presence of Poly (I:C) alters the plaque instability induced by *P. gingivalis*.

The MASSON staining results revealed a reduction in the proportion of blue collagen fibers within the aortic root plaques following *P. gingivalis* stimulation ($P < 0.01$, Fig. 3A and B), leading to decreased plaque stability. However, application of Poly (I:C) demonstrated an increase in collagen fiber quantity, a decrease in muscle fiber proportion, and enhanced plaque stability ($P < 0.05$, Fig. 3A and B).

Poly (I:C) mitigated the upregulation of VCAM-1 and IL-6 expression induced by *P. gingivalis*

The effect of *P. gingivalis* infection on VCAM-1 and IL-6 expression was attenuated by the Poly (I:C) (Fig. 4). As shown in Fig. 4A and B, the levels of VCAM-1 and IL-6 were increased in the aortic root plaques after challenge of *P. gingivalis* stimulation. Poly (I:C) downregulated the expression of VCAM-1 and IL-6 levels. The same pattern was observed in serum VCAM-1 ($P < 0.01$, Fig. 4C) and IL-6 ($P < 0.05$, Fig. 4D) levels after Poly (I:C) preprocessing.

Poly (I:C) induces alterations in the lipid profile of mice infected with *P. gingivalis*

The changes in blood lipids of mice were depicted in Fig. 5. Following *P. gingivalis* stimulation, there was a decrease observed in the expression of high-density lipoprotein (HDL-C) (Fig. 5A, $P < 0.05$), and the application of Poly (I:C) exhibited a reversal trend; Subsequent to *P. gingivalis* stimulation, an increase was noted in the expression of low-density lipoprotein (LDL-C) (Fig. 5B, $P < 0.001$) and total cholesterol (TC) (Fig. 5C, $P < 0.05$) levels, which were mitigated by Poly (I:C) application to counteract *P. gingivalis* -induced elevation in LDL-C (Fig. 5B, $P < 0.001$) and TC (Fig. 5C, $P < 0.01$) ratio. Moreover, Poly (I:C) application demonstrated potential for reducing triglyceride (TG) levels (Fig. 5D, $P < 0.05$).

The polarization of mouse macrophages induced by *P. gingivalis* can be modulated through the inhibition of LOX-1

The immunofluorescence results demonstrated an upregulation of M1 macrophages (CD86 positive) during the active phase in the aortic root of mice infected with *P. gingivalis*,

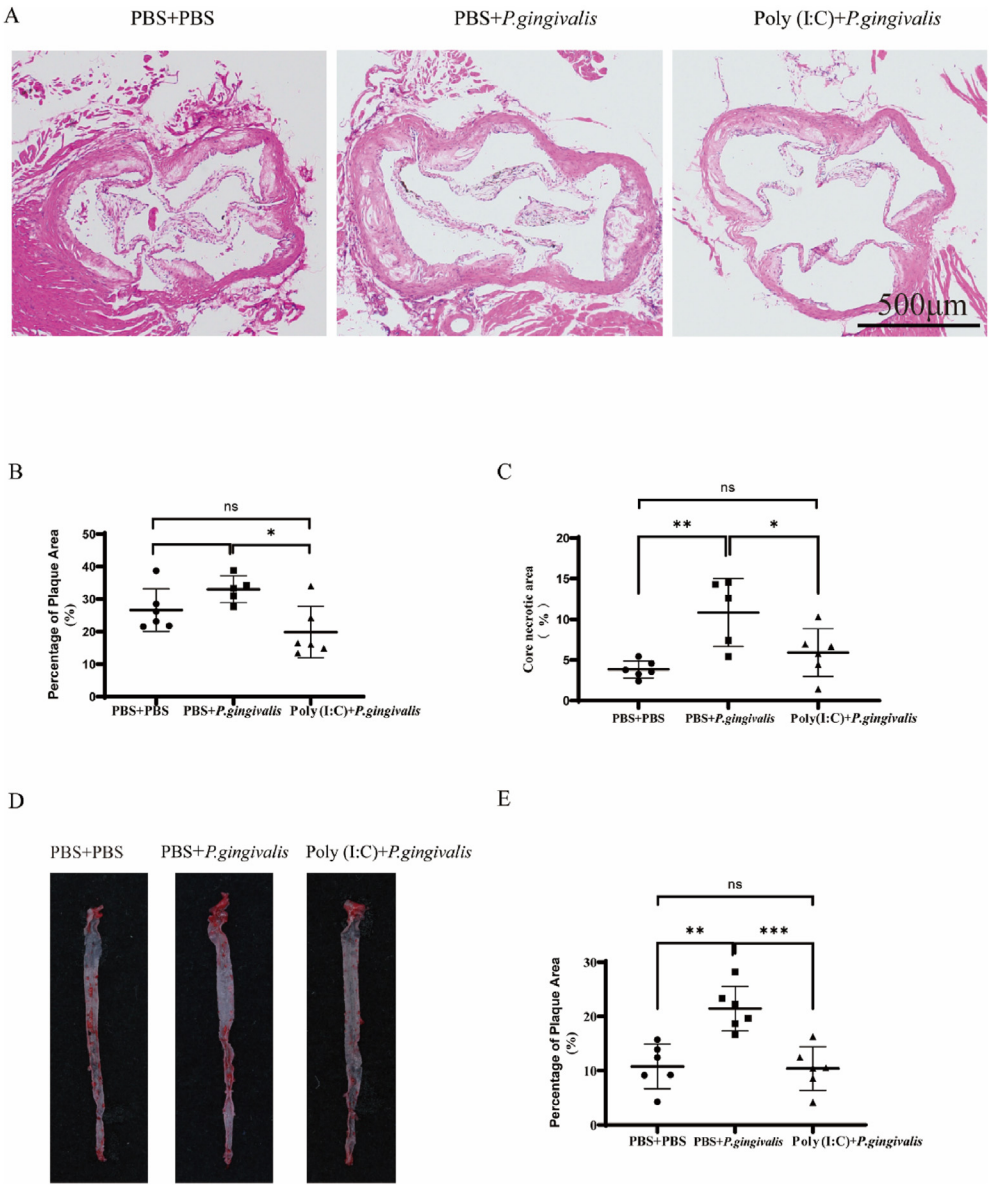

Figure 2 The inhibition of LOX-1 by Poly (I:C) reversed the plaque formation induced by *P. gingivalis*. HE staining (A) and analysis (B, C) of the aortic root. Plaque area, (A, B); core necrotic proportion, (A, C). Oil red O staining (D) and analysis (E) of the aortic. (Black scale:500 μm). *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$. Poly (I:C), Polyinosinic-polycytidylic acid; *P. gingivalis*, *Porphyromonas gingivalis*; PBS, Phosphate buffered saline.

whereas an increase in M2 macrophages (CD206 positive) was observed following inhibition of LOX-1's function (Fig. 6).

Discussion

The significant finding of this study reveals an upregulation in the expression of LOX-1 within the aortic root of mice infected with *P. gingivalis*, while inhibition of LOX-1 function mitigates *P. gingivalis*-induced mouse atherosclerosis. The in vivo experiments further provide compelling evidence supporting the role of LOX-1 as a crucial link between periodontitis and atherosclerosis, building upon previous studies on cellular mechanisms.

The presence of bacteremia caused by periodontal infections is a significant contributing factor to the overall impact of periodontitis on an individual's health. The construction of a bacteremia model involved the intravenous injection of *P. gingivalis*, a well-established method extensively utilized in studies on periodontal disease and systemic diseases.[15–18]

There are multiple factors that induce the expression of LOX-1, including oxLDL, advanced glycation end products (AGEs), angiotensin II, and cytokines, among others. These metabolites and stressors upregulate LOX-1 expression,

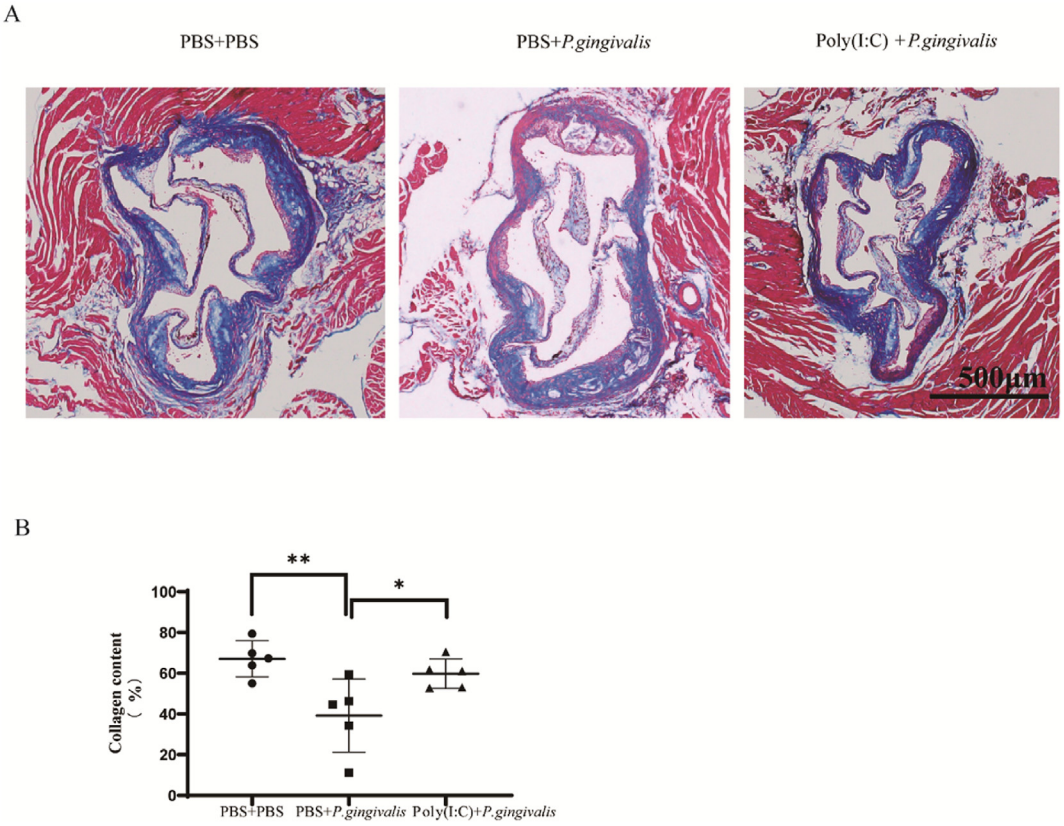

Figure 3 The inhibition of LOX-1 by Poly (I:C) reversed the plaque instability induced by *P. gingivalis*. Masson staining (A) and analysis(B) of the aortic root. Blue, collagen fiber. Red, myofiber. (Black scale:500 μm). *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$. Poly (I:C), Polyinosinic-polycytidylic acid; *P. gingivalis*, *Porphyromonas gingivalis*; PBS, Phosphate buffered saline.

leading to the production of various pro-inflammatory mediators, which subsequently contribute to platelet aggregation, angiogenesis, fibrosis, and endothelial dysfunction. This effect plays a pivotal role in the pathogenesis of cardiovascular diseases.[19] Due to its involvement in initiating pro-inflammatory immune responses, LOX-1 is increasingly recognized as a crucial receptor for pathogens. Notably, LOX-1 exhibits recognition capabilities towards various pathogenic bacteria including *S. pneumoniae*,[20] *S. aureus*, *E. coli*,[21] *Chlamydia pneumoniae*,[22,23] and *Aspergillus fumigatus*.[24] Several studies have demonstrated the impact of *P. gingivalis* infection on LOX-1 expression, which mediates MMP2 expression in human macrophages following *P. gingivalis* infection and thereby regulates periodontitis progression.[25,26] In the context of cardiovascular diseases, our previous study revealed that *P. gingivalis* infection upregulates LOX-1 expression in HUVEC and THP-1 cells, additionally, Huang reported that GroEL from *P. gingivalis* enhances ICAM-1, VCAM-1, and LOX-1 expression in mouse blood vessels.[27] Consistent with these findings, Xuan observed an increase in LOX-1 RNA expression in mouse blood vessels after *P. gingivalis* infection.[17] This study, based on our previous in vitro research, observed the content of LOX-1 in the aortic root of mice infected with *P. gingivalis* and the circulating levels of soluble LOX-1 (sLOX-1), which corroborated findings from prior studies. Meanwhile, sLOX-1 can serve as a diagnostic marker for cardiovascular diseases,[28,29] reflecting the development and instability of AS lesions.[30,31] The increase in sLOX-1 after *P. gingivalis* infection indirectly proves the promoting role of *P. gingivalis* in AS.

The morphological features of atherosclerotic plaques are the most important risk factors for vulnerability to cardiovascular events and may represent characteristics of vulnerability, including the thickness of the fibrous cap, the number of inflammatory macrophages, and the size of the necrotic core.[32] The histological characteristics of vulnerable atherosclerotic plaques encompass delicate fibrous caps, necrotic cores abundant in lipids, and occurrences of intraplaque hemorrhage. The studies revealed that *P. gingivalis* not only facilitates the expansion of plaque area,[33,34] but also induces systemic inflammatory responses and enhances local expression of VCAM-1 and ICAM-1.[35] Furthermore, it may contribute to atherosclerosis by modulating the Th17/Treg imbalance in atherosclerosis, thereby increasing plaque instability.[36] The secretion of gingival protease by *P. gingivalis* ex vivo can enhance macrophage infiltration, while also promoting the polarization of macrophages into the M1 subtype and inducing IL-6 expression through the complement 5a pathway, C5a is present in AS plaques and acts as a pro-AS molecule.[37,38] Among them, macrophages not only play a role in

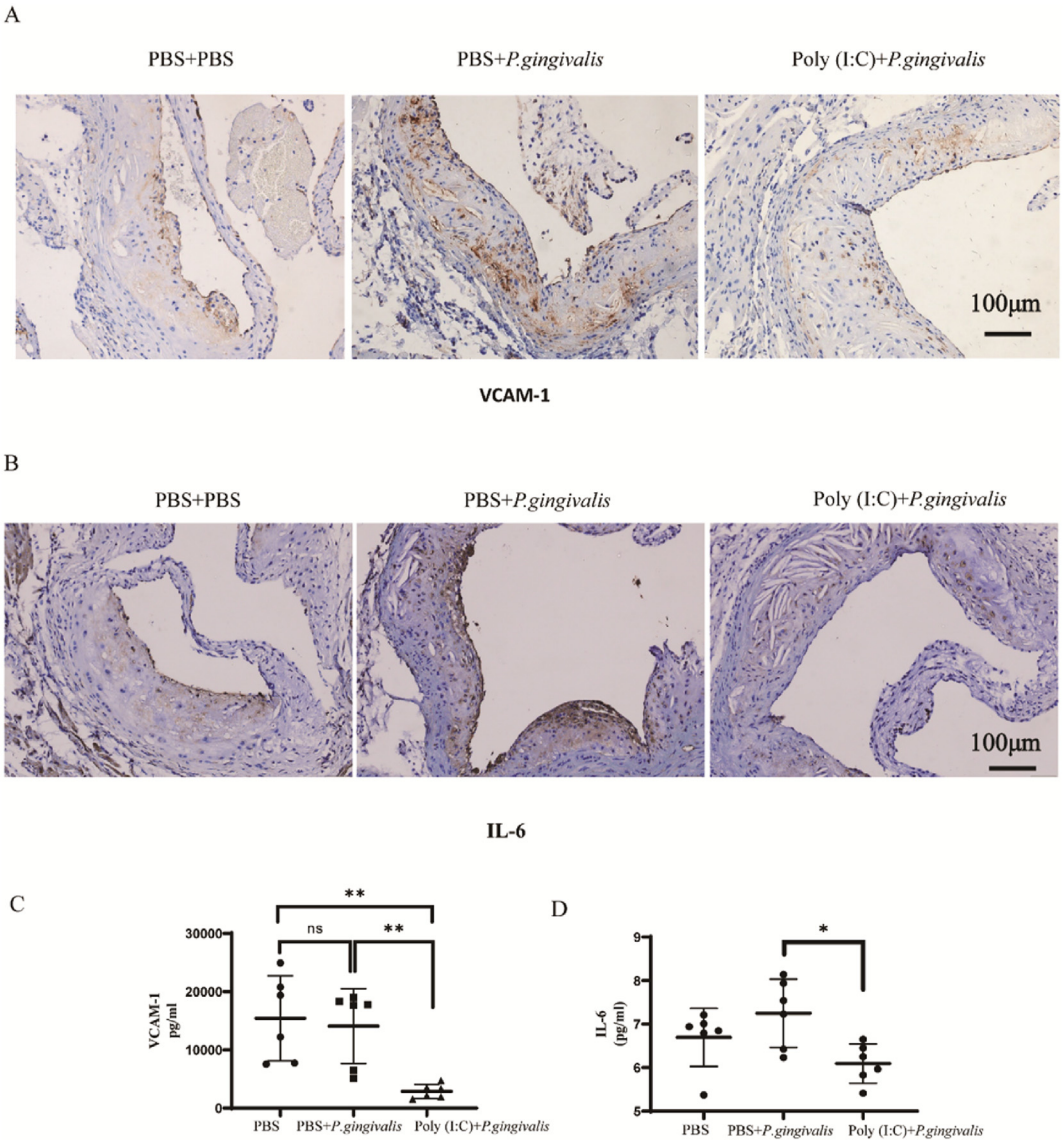

Figure 4 The inhibition of LOX-1 by Poly (I:C) reversed the alterations of VCAM-1 and IL-6 induced by *P. gingivalis*. Immunohistochemical staining was performed to detect the expression of VCAM-1(A) and IL-6(B) in the aortic root. The levels of VCAM-1(C) and IL-6(D) in mouse serum. (Black scale:100 μm). *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$. Poly (I:C), Polyinosinic-polycytidylic acid; *P. gingivalis*, *Porphyromonas gingivalis*; PBS, Phosphate buffered saline; IL-6, Interleukin-6; VCAM-1, vascular adhesion molecule-1.

antibacterial action, but also serve as the main participants in the formation of foam cells and mediating the stability of plaques.[39] The survival of *P. gingivalis* in macrophages induces inflammation activation and M1 polarization, promoting the release of inflammatory factors and accelerating the progression of AS.[40—42] The findings from this study are consistent with the results, as *P. gingivalis* was found to increase the size of necrotic plaque, decrease plaque stability, enhance expression of VCAM-1 and IL-6 in tissue, elevate levels of total cholesterol and low-density lipoprotein (LDL) in blood lipids, reduce high-density lipoprotein (HDL) levels, and promote an increased proportion of M1 macrophages. The impact of *P. gingivalis* on this phenomenon, however, was attenuated following the inhibition of LOX-1.

In conclusion, the findings of this study indicate that *P. gingivalis* stimulation may attenuate the expression of LOX-1 in plaque and serum. LOX-1 is implicated in atherosclerosis promotion by *P. gingivalis*, serving as a crucial link between periodontitis and atherosclerosis. The use of LOX-1 inhibitors will also emerge as a novel approach for managing periodontitis-related atherosclerosis.

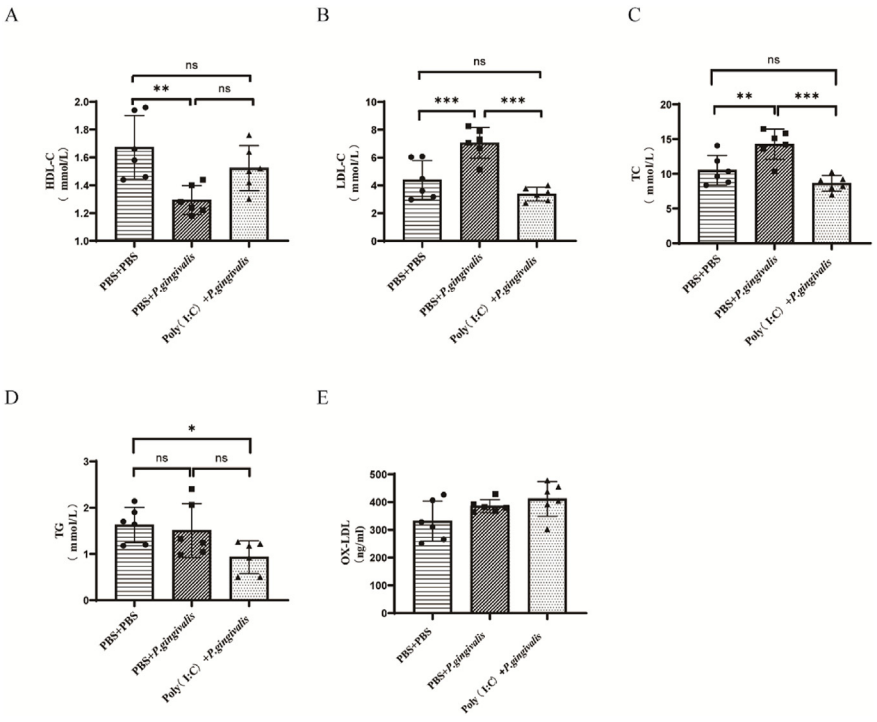

Figure 5 The inhibition of LOX-1 by Poly (I:C) reversed the alterations in mouse blood lipids induced by *P. gingivalis*. HDL-C, (A); LDL-C, (B); TC, (C); TG, (D); oxLDL, (E). Poly (I:C), Polyinosinic-polycytidylic acid; *P. gingivalis*, *Porphyromonas gingivalis*; PBS, Phosphate buffered saline; HDL-C, high-density lipoprotein; LDL-C, low-density lipoprotein; TC, total cholesterol; TG, triglyceride. oxLDL, oxidized low density lipoproteins.

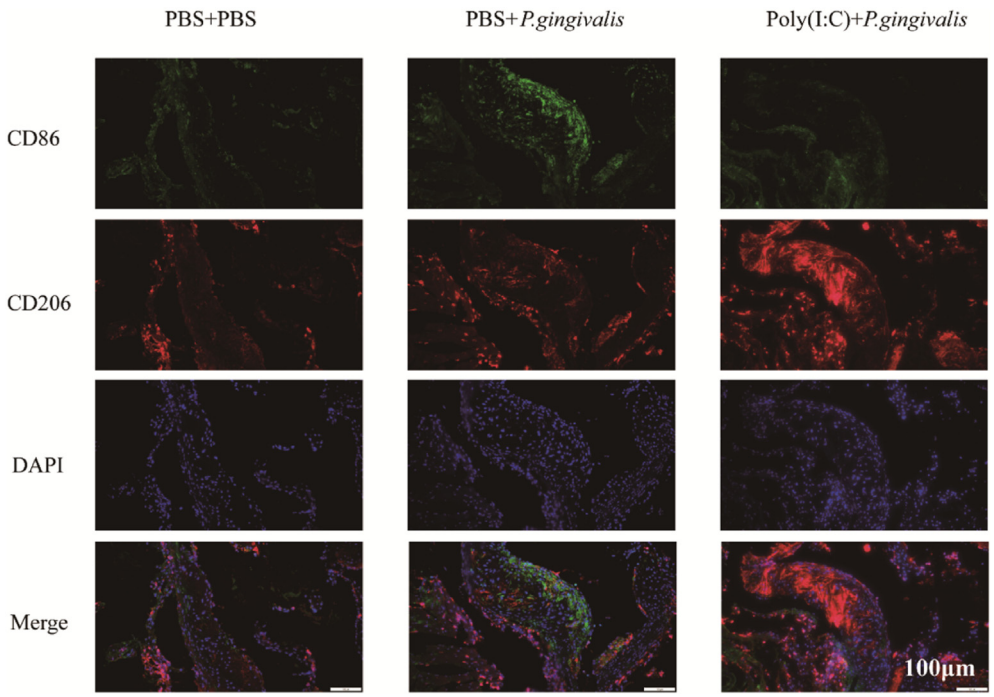

Figure 6 The inhibition of LOX-1 by Poly (I:C) altered the polarization of macrophages induced by *P. gingivalis* in aortic plaques in mice. Green, M1 macrophages (CD86 positive); Red, M2 macrophages (CD206 positive). (White scale:100 μm). DAPI, 40,6- diamidino-2-phenylindole; Poly (I:C), Polyinosinic-polycytidylic acid; *P. gingivalis*, *Porphyromonas gingivalis*; PBS, Phosphate buffered saline; CD, Cludter of Differentiation.

Declaration of competing interest

The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgments

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