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Original Article

Polyinosinic-polycytidylic acid modulates *Porphyromonas gingivalis*-induced cell apoptosis via the janus kinase/ signal transducer and activator of transcription signaling pathway

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KEYWORDS
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Abstract *Background/Purpose: Porphyromonas gingivalis* (*P. gingivalis*) has been shown to induce apoptosis in endothelial cells and contribute to the progression of atherosclerosis. While Polyinosinic-polycytidylic acid (Poly (I:C)) is known to activate the innate immune response against infections, its potential interference with *P. gingivalis*-induced atherosclerosis remains unclear. This study aimed to elucidate the role and underlying mechanisms of Poly (I:C) in mediating human umbilical vein endothelial cells (HUVECs) apoptosis induced by *P. gingivalis*.

Materials and methods: A mice model of atherosclerosis and a model of *P. gingivalis*-induced bacteremia were established to investigate the effects of Poly (I:C) on *P. gingivalis*-induced apoptosis in the aortic root, as well as the expression levels of apoptosis-related proteins including Caspase 3, Caspase 9, Bax, and Bcl-2. Subsequently, HUVECs were cultured in vitro to compare cell apoptosis and the expression of these apoptosis-related proteins under stimulation with *P. gingivalis*, both with and without Poly (I:C) treatment; additionally, the activation status of the JAK/STAT signaling pathway was assessed.

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Results: The administration of Poly (I:C) diminished apoptosis in the aortic root cells of mice, enhanced the expression of the anti-apoptotic protein Bcl-2, and decreased the levels of Bax, Caspase 3 and 9. Furthermore, Poly (I:C) exhibited similar effects on HUVECs cultured in vitro. Additionally, treatment with Poly (I:C) activated the JAK/STAT signaling pathway, while STAT inhibitor was found to attenuate its effects.
Conclusion: Poly (I:C) attenuated *P. gingivalis*-induced cellular apoptosis, with the involvement of the JAK/STAT signaling pathway in this mechanism.

Introduction

The process of apoptosis, a form of programmed cell death, was initially postulated in 1972.[1] The initiation of apoptosis relies on the activation of a cascade of enzymes known as cysteine-aspartate proteases, specifically caspases. The activation of the caspases initiates a sequence of events leading to nucleic acid endonuclease activation, nuclear protein and cytoskeleton degradation, protein cross-linking, phagocytic ligand expression, and apoptotic body formation resulting in DNA fragmentation.[2] There exists a delicate equilibrium of apoptosis, and an excessive level of apoptosis may give rise to various pathological conditions.

Atherosclerosis (AS) is a chronic inflammatory condition that represents a primary contributor to acute cardiovascular and cerebrovascular events. The extent of cellular apoptosis plays a pivotal role in the advancement and rupture of atherosclerotic plaques. The progression of atherosclerosis involves the apoptosis of macrophages,[3] while the apoptosis of vascular smooth muscle cells determines the stability of plaques. Endothelial cells are ubiquitously distributed throughout the entire vascular network and play a pivotal role in the pathogenesis of atherosclerosis. When conditions that induce cellular apoptosis occur, endothelial cell function is compromised, contributing to the development of atherosclerosis.[4]

The principal periodontal pathogen is *Porphyromonas gingivalis* (*P.gingivalis*). Several studies have demonstrated that *P. gingivalis* is capable of inducing cell apoptosis.[5] *P. gingivalis* triggers apoptosis in HUVECs via the NF-κB pathway, disrupting vascular endothelial homeostasis.[6] Outer membrane vesicles derived from *P. gingivalis* induce apoptosis in lung epithelial cells.[7] The gingipains and Lipopolysaccharide of *P. gingivalis* induces apoptosis in osteoblasts.[8,9] The onset of AS is typically accompanied by endothelial cell dysfunction, and the occurrence of endothelial cell apoptosis represents a significant indication of functional impairment.[10]

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).[11–13] Poly (I:C) preconditioning reduces proinflammatory state in obesity-related insulin resistance and promotes glucose homeostasis by enhancing immune tolerance.[14] This study aimed to elucidate the role and underlying mechanisms of Poly (I:C) in mitigating *P. gingivalis*-induced apoptosis, while also investigating the potential of Poly (I:C) as a therapeutic agent for *P. gingivalis*-induced atherosclerosis.

Materials and methods

Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from ScienCell (San Diego, CA, USA) and cultured in endothelial cell medium (ECM, ScienCell) supplemented with 5 % fetal bovine serum (ScienCell) and 1 % endothelial cell growth supplements (ScienCell) at 37 °C and 5 % CO_2 in a humidified incubator. HUVECs in passages 3–6 were used for all experiments.

Bacterial culture and challenge

The strain *P. gingivalis* W83 was cultured as described.[15] For logarithmic-phase bacteria, the bacterias were harvested via centrifugation (6000 rpm, 4 °C, for 10 min) and subsequently washed three times with PBS before being resuspended. The bacterial suspension was adjusted to an optical density (OD) of 0.5 at 600 nm, which corresponds to a concentration of approximately 10^8 CFU/ml.

Stimulation protocol

HUVECs were seeded in 60-mm dishes in 3 mL of ECM and cultured reached 80–90 % confluence, they were stimulated with 10 μg/mL of Poly (I:C) (Sigma, St. Louis, MO, USA) or PBS for 24 h.[16] Following the multiplicity of infection (MOI) of 100:1, the bacterial suspension was added to HUVECs for a culture period of 24 h. The co-culture of *P. gingivalis* and HUVECs has been previously detailed.[15] Expression of Caspase 3, Caspase 9, Bax, Bcl-2 and JAK/STATs was then determined. In the STAT inhibition experiment, Fludarabine (STAT1 Activation Inhibitor) (Selleck, Shanghai, China) or Dimethyl sulfoxide (DMSO, Solarbio, Beijing, China) was pre-treated for 2 h prior to the addition of Poly (I:C), followed by stimulation with *P. gingivalis*. The expression of these proteins was then observed.

Animals and diets

18 8-week-old male APOE$^{-/-}$ mice were purchased from Beijing Vital River Laboratory Animal Technology Company (Beijing, China). In a specific pathogen-free (SPF) laboratory, mice were fed with a high-fat diet (D12109C, FBSH, Shanghai, China).[17] The mice were 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) 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.[18] The experiment lasted 12 weeks. Mice were euthanized at indicated time points, and hearts were collected. 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).

TUNEL straining

After fixation, dehydration, and embedding of the mouse heart, continuous 5-micron-thick sections were prepared from the aortic root, starting at the location where the first three aortic valves were initially observed. Cell apoptosis in the aortic root tissue was assessed using a TUNEL staining kit (Beyotime, Shanghai, China) in accordance with the manufacturer's instruction.

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 was then used to induce the formation of a colored precipitate at the tissue antigen-binding sites. Restaining the nucleus with hematoxylin. Primary antibodies against the following proteins were used: Caspase 3 (abcepta, Suzhou, China), B-Cell Lymphoma-2 (Bcl-2, Zenbio, Chengdu, China) and Bcl2-associated x (Bax, Abclonal, Wuhan, China). The sections were acquired using a light microscope (Olympus, Tokyo, Japan).

Flow cytometry

Utilize the Annexin-V-FITC Apoptosis Detection Kit (Elabscience Biotechnology, Wuhan, China) for quantitative assessment of HUVECs apoptosis. Following the kit instructions, harvest cells through trypsin digestion without EDTA and rinse with pre-chilled PBS (Solarbio). Subsequently, incubate FITC-Annexin V and PI at room temperature for 20 min before analyzing apoptotic cells, encompassing both early and late apoptotic populations, using flow cytometry.

Western blot analysis

Conduct the Western Blot experiment utilizing the protocol previously outlined. The protein bands were imaged by chemiluminescence and quantified by densitometry using ImageJ 9.0 (National Institutes of Health, Bethesda, MD, USA). The relative expression of proteins was normalized to β- Tubulin levels. Primary antibodies against the following were used: Caspase 9 (Abclonal), Bax (Abclonal), phospho-janus kinase 1 (p-JAK1, Tyr1022, Zenbio), phospho-JAK2 (p-JAK2, Tyr1007/1008, Zenbio), JAK1 (Zenbio), JAK2 (Affinity Bioscience, Jiangsu, China), Bcl-2 (Zenbio), Caspase 3 (abcepta) and β- Tubulin (ZSGB-Bio, Beijing, China), phospho-signal transducer and activator of transcription 1 (p-STAT1, Tyr701, Abclonal, Fig.4C), p-STAT1 (Tyr701, Affinity Bioscience, Fig. 4A), STAT1 (Affinity Bioscience).

Statistical analysis

Each experiment was performed in triplicate with the results expressed as mean ± SD. Data analysis was performed using SPSS (version 22.0). Statistical analyses were performed utilizing unpaired t-tests and one-way ANOVA followed by Kruskal–Wallis test. Statistical significance was set at $P < 0.05$ ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

Results

Poly (I:C) mitigated aortic plaque-induced cell apoptosis triggered by *P. gingivalis* in APOE$^{-/-}$ mice

The results of TUNEL staining for the aortic root in different experimental groups are depicted in Fig. 1. The red

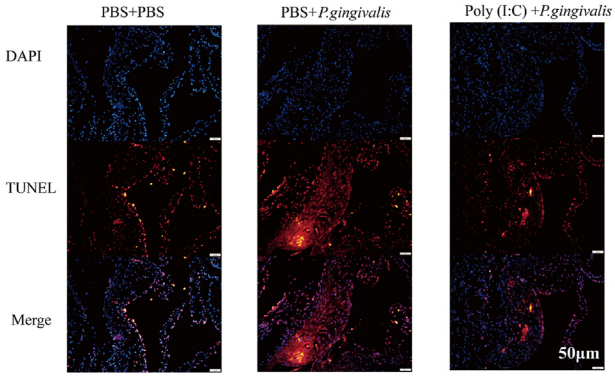

Figure 1 Poly(I:C) mitigated aortic plaque-induced cell apoptosis triggered by *P. gingivalis* in APOE$^{-/-}$ mice. TUNEL staining was performed on the aortic root across various experimental groups (White scale:50 μm). The red fluorescence observed corresponds to cells undergoing apoptosis. DAPI, 40,6- diamidino-2-phenylindole; Poly (I:C), Polyinosinic-polycytidylic acid; *P. gingivalis*, *P. gingivalis*; PBS, Phosphate buffered saline; TUNEL, Terminal dUTP nick end labelling.

fluorescence observed corresponds to cells undergoing apoptosis. After intravenous injection of *P. gingivalis*, the number of red apoptotic cells in the aortic plaque increased; however, in mice pretreated with Poly (I:C), the number of apoptotic cells in the aortic plaque was significantly reduced compared to that in the group injected with *P. gingivalis*.

Poly (I:C) attenuated the expression of apoptosis-related proteins in the aortic plaques induced by *P. gingivalis* in APOE-/ mice.

The effect of *P. gingivalis* infection on apoptosis-related proteins expression in the aortic plaques was attenuated by Poly (I:C) (Fig. 2). Fig. 2 shows that after *P. gingivalis* stimulation, the levels of Caspase 3 and Bax increased, while the level of Bcl-2 decreased. In mice treated with Poly (I:C), the downregulation of Caspase 3 and Bax expression induced by *P. gingivalis*, along with the reduction in Bcl-2 expression, was effectively reversed.

Poly (I:C) counteracted the alterations in expression of apoptosis-related proteins caused by *P. gingivalis* in HUVECs.

The effect of *P. gingivalis* infection on apoptosis in HUVECs was attenuated by the Poly (I:C) (Fig. 3). As shown in Fig. 3A and B, the levels of apoptosis-related proteins were attenuated after 10 μg/mL Poly (I:C) pretreated compared with the *P. gingivalis* control. Poly (I:C) pretreated HUVECs exhibited decreased protein expression of Bax ($P < 0.01$), Caspase 9 and Caspase 3 ($P < 0.01$). Meanwhile, Poly (I:C) pretreated increased the protein level of Bcl-2 ($P < 0.001$). Flow cytometry analysis revealed a decrease in the number of apoptotic ($P < 0.001$), early apoptotic ($P < 0.1$) and total apoptotic ($P < 0.001$) HUVECs following Poly (I:C) pretreatment (Fig. 3C). Dual staining with FITC and PI identifies apoptotic cells, whereas single staining with FITC specifically marks early apoptotic cells. The cumulative count of both reflects the total population of apoptotic cells. Fig. 3D illustrates the statistical analysis corresponding to the results obtained from flow cytometry.

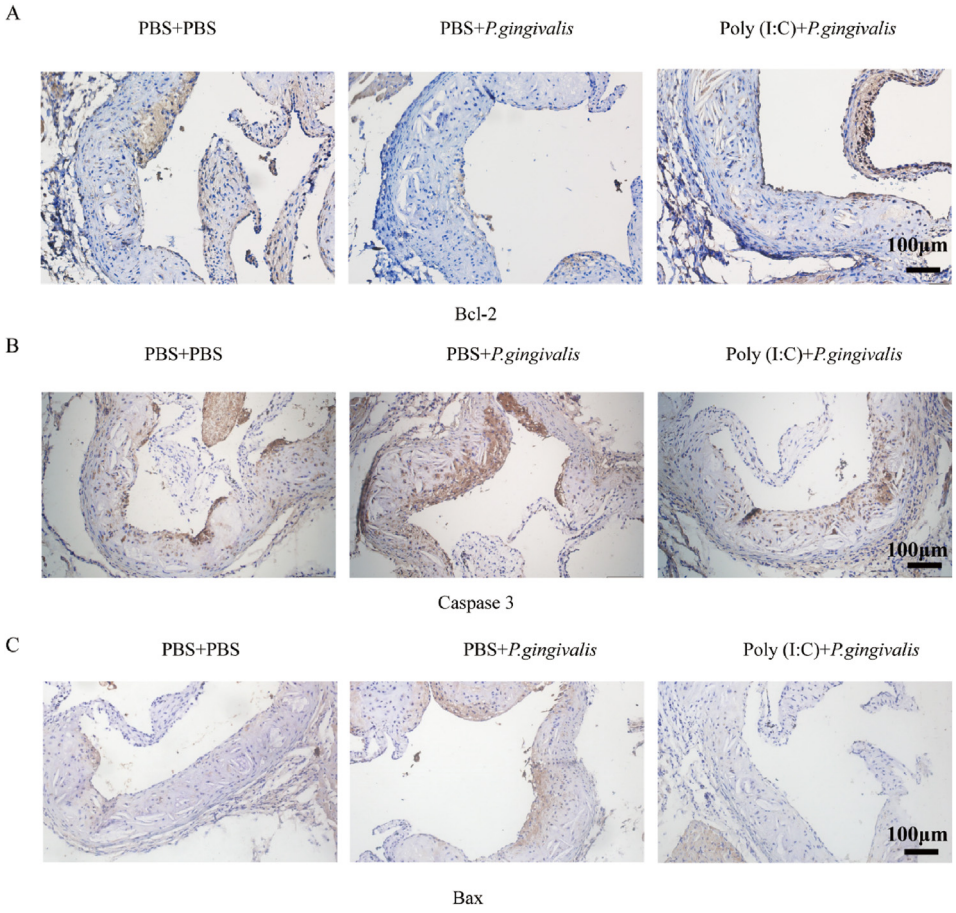

Figure 2 Poly(I:C) attenuated the expression of apoptosis-related proteins in the aortic plaques induced by *P. gingivalis* in APOE$^{-/-}$ mice. (Black scale:100 μm). *P. gingivalis* increases the expression of pro-apoptotic proteins Bax and Caspase 3 in plaques, while concurrently reducing the levels of Bcl-2. Notably, pre-treatment with Poly (I:C) counteracts this effect induced by *P. gingivalis*, resulting in decreased expression of Bax and Caspase 3 alongside an elevation in Bcl-2 levels compared to the *P. gingivalis* group. Poly (I:C), Polyinosinic-polycytidylic acid; *P. gingivalis*, *P. gingivalis*; PBS, Phosphate buffered saline; Bcl-2, B-Cell Lymphoma-2; Bax, Bcl2-associated x.

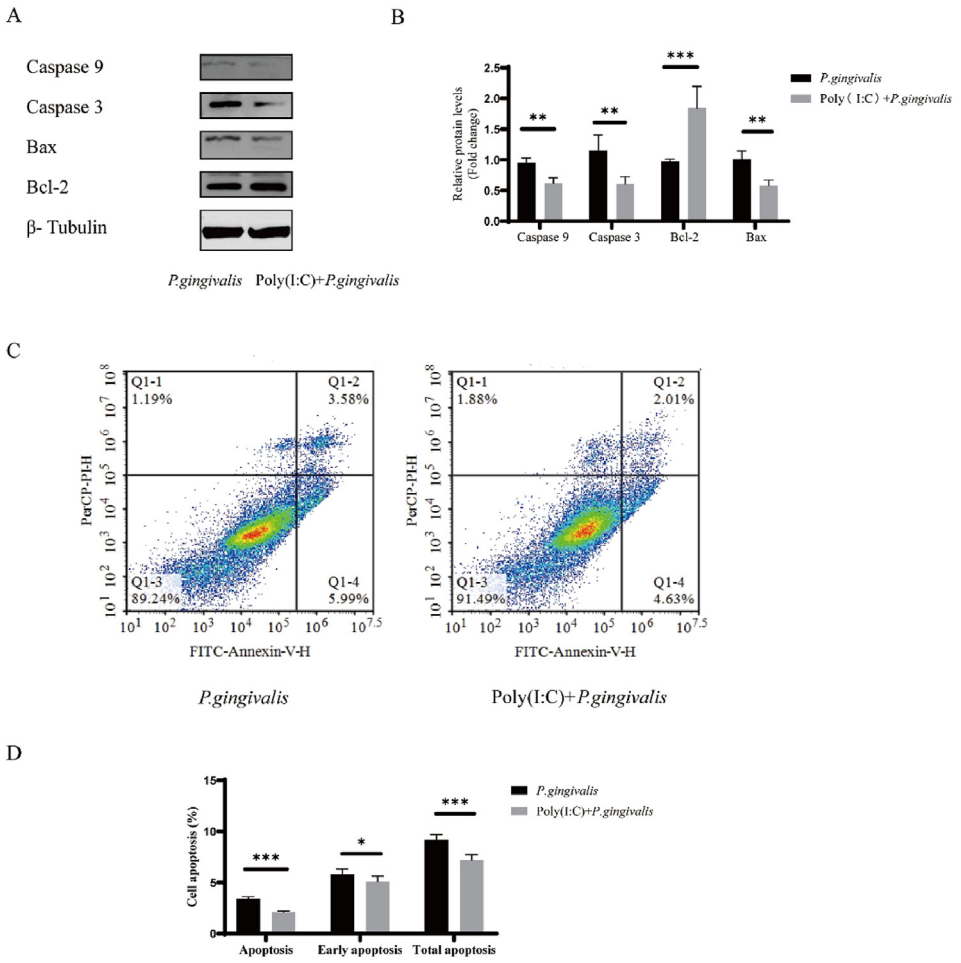

Figure 3 Pretreatment of Poly (I:C) reduced apoptosis induced by *P. gingivalis* in HUVECs. (A, B) Following pretreatment with Poly (I:C) or its absence, *P. gingivalis* stimulation was assessed, and protein expression levels of Caspase 3, Caspase 9, Bcl-2 and Bax were evaluated using Western blot analysis. (C) Following pretreatment with Poly (I:C) or without, *P. gingivalis* was stimulated, and HUVECs apoptosis was assessed using flow cytometry. (D) Statistical analysis of Flow Cytometry. Dual staining with FITC and PI identifies apoptotic cells, whereas single staining with FITC specifically marks early apoptotic cells. The cumulative count of both reflects the total population of apoptotic cells. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. Poly (I:C), Polyinosinic-polycytidylic acid; *P. gingivalis*, *P. gingivalis*; PBS, Phosphate buffered saline; Bcl-2, B-Cell Lymphoma-2; Bax, Bcl2-associated x; FITC, Fluorescein isothiocyanate; PI, Propidium iodide.

JAK/STAT are involved in the regulation of Poly (I:C)-mediated apoptosis suppression

HUVECs with or without Poly (I:C)-pretreated were treated with *P. gingivalis* for 24 h (Fig. 4). The result of Western blot showed that Poly (I:C)-pretreated led to an increased expression of p-STAT1 ($P < 0.05$), p-JAK1 ($P < 0.01$) and p-JAK2 ($P < 0.05$) which indicated the activation of JAK/STAT signaling pathway by Poly (I:C) (Fig. 4A and B). Subsequently, Fludarabine (100 μM, 2 h) was incorporated for inhibiting the STAT1 activation, and the p-STAT1 expression in HUVECs was considerably decreased by Fludarabine (100 μM, 2 h) treatment (Fig. 4C, D, $P < 0.001$). When Fludarabine was administered as a pretreatment, the expression levels of Caspase 9, 3($P < 0.01$), and Bax ($P < 0.1$) were significantly elevated compared to those observed with Poly (I:C) treatment alone, while Bcl-2 expression was notably reduced ($P < 0.001$). This indicates that the anti-apoptotic effect of Poly (I:C) was diminished under Fludarabine pretreated, furthermore, it implies that Poly (I:C)'s protective role against *P. gingivalis* -induced cell apoptosis may be mediated through the JAK/STAT signaling pathway.

Discussion

In this study, the aortic root of mice challenged with *P. gingivalis* exhibited increased apoptosis and upregulation of pro-apoptotic proteins, whereas the administration of Poly (I:C) attenuated the pro-apoptotic effect induced by *P. gingivalis*. Meanwhile, Poly (I:C)'s pretreatment also demonstrated the potential to attenuate *P. gingivalis* -induced apoptosis of HUVECs in vitro, with potential involvement of the JAK/STAT pathway.

Multiple studies have demonstrated that *P. gingivalis* and its constituent components induce apoptosis in

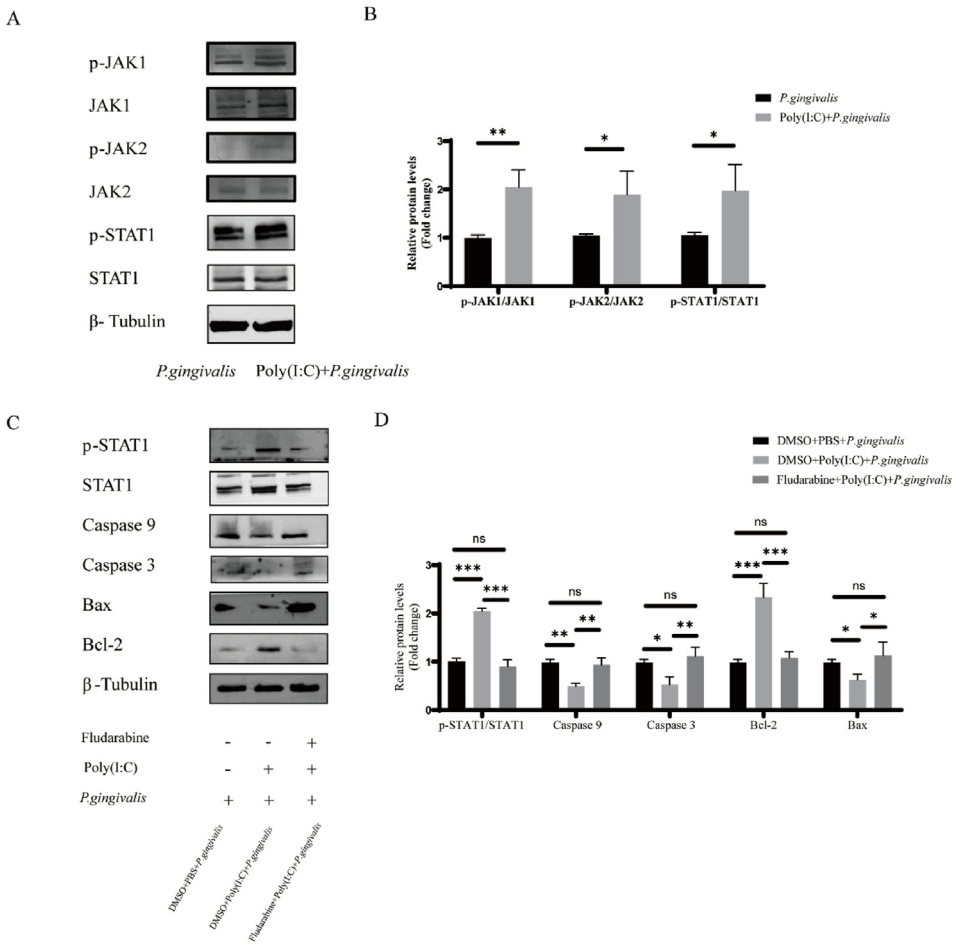

Figure 4 The pretreatment with Poly(I:C) affects the expression of JAK/STAT1 in HUVECs. (A, B) The expression levels of p—STAT1 and p-JAK1/2 in Poly (I:C) pretreaded-HUVECS were detected by Western blot. (C, D) Regardless of Fludarabine pretreatment, subsequent Poly (I:C) stimulation was employed to assess the expression levels of Caspase 3, Caspase 9, Bcl-2 and Bax proteins using Western blot analysis. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. Poly (I:C), Polyinosinic-polycytidylic acid; *P. gingivalis*, *P. gingivalis*; PBS, Phosphate buffered saline; Bcl-2, B-Cell Lymphoma-2; Bax, Bcl2-associated x; STAT, signal transducer and activator of transcription; JAK, janus kinase; p- STAT, phosphor-STAT; p-JAK, phosphor-JAK; DMSO, Dimethyl sulfoxide.

various oral cells, including periodontal ligament cells, osteoblasts, epithelial cells, and others.[9,19,20] However, some research has also indicated that these components inhibit apoptosis in gingival epithelial cells.[21] In investigations of the impact of *P. gingivalis* on systemic diseases, it has been observed to promote apoptosis in various cell types such as retinal microvascular endothelial cells, brain endothelial cells, and umbilical vein endothelial cells.[22—24] These findings align with the results depicted in Figs. 1 and 2 of this study where *P. gingivalis* facilitated cell apoptosis thus exacerbating atherosclerosis in mice. This may represent one potential mechanism through which *P. gingivalis* induces atherosclerosis.

Poly (I:C) is a double-stranded RNA analogue that activates the innate immune response against infections and exerts both antibacterial and antiviral effects. It is extensively utilized as a vaccine adjuvant to enhance immune responses and efficacy of vaccines.[25] Beyond its antiviral properties, Poly (I:C) serves as a stimulant for innate immune cells, bolstering the resistance of both immunocompetent and immunosuppressed mice to *Escherichia coli* meningitis.[26] Additionally, study have indicated that it can amplify the innate immune response to vibrio alginolyticus.[27] In terms of cell apoptosis, Poly (I:C) primarily demonstrates anti-tumor effects across various cancer cell types and has been shown to cooperate with apoptotic induction in renal cell carcinoma and cervical cancer cells (with activation of Caspases 8 and 9), contrasting with its inhibitory effect on HUVEC apoptosis induced by *P. gingivalis* observed in this study.[28,29] However, other research has reported that Poly (I:C) inhibits virus-induced apoptosis in duck epithelial cells.[30] The variability in Poly (I:C)'s role regarding cell apoptosis may stem from methodological differences, certain studies employ liposome transfection for direct cellular entry of Poly (I:C), whereas this investigation utilized it as a pre-treatment stimulant. Furthermore, differing molecular weights of Poly (I:C) have been found to similarly affect dendritic cell maturation, suggesting that molecular weight could also be an influencing factor.[31]

Numerous theories and experimental findings have demonstrated that the JAK/STAT signaling pathway can be modulated to enhance therapeutic strategies for atherosclerosis. Beyond the inflammatory factors downstream of

JAK/STAT that directly influence atherosclerosis,[32] this signaling pathway also mediates certain molecules associated with cell apoptosis and their implications for atherosclerotic processes.[33] Bcl-2 and Bax are transcriptional regulators downstream of STAT. Bcl-2 exerts an anti-apoptotic effect, inhibiting cell death induced by various cytotoxic agents, while Bax, as a member of the Bcl-2 family, is pivotal in promoting apoptosis. The overexpression of Bax can negate the protective effects of Bcl-2, ultimately leading to cellular demise. The expression levels of STAT can modulate the ratio between Bcl-2 and Bax, thereby influencing apoptotic outcomes.[34] Research has indicated that Poly (I:C) activates STAT1 phosphorylation in murine macrophages and similarly induces STAT1 phosphorylation in fish cells.[35,36] Furthermore, Poly (I:C) regulated the JAK/STAT signaling pathway while mediating antiviral immune responses in grass carp (*Hypophthalmichthys molitrix*).[37] This aligns with our study's observations regarding Poly (I:C)'s regulatory role on the JAK/STAT pathway.

In conclusion, the research findings demonstrate that Poly (I:C) effectively mitigates *P. gingivalis*-induced cell apoptosis both in vivo and in vitro, while also attenuating the expression of apoptosis-related proteins associated with *P. gingivalis* exposure. Furthermore, the JAK/STAT signaling pathway plays a crucial role in this mechanism. Consequently, Poly (I:C) may represent a promising therapeutic candidate for interventions targeting *P. gingivalis*-induced atherosclerosis.

Declaration of competing interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

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