Periodontitis aggravates pulmonary fibrosis by *Porphyromonas gingivalis*-promoted infiltration of neutrophils and Th17 cells

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Introduction: Idiopathic pulmonary fibrosis (IPF) is a fatal interstitial lung disease. However, the pathogeny of IPF is poorly understood, and therapeutic options are very limited. Periodontitis (PD) is a chronic inflammatory disease that leads to dysbiosis of both the oral microbiome and host immune responses. While previous studies have suggested a PD-IPF association, insights into the mechanisms remain limited.

Methods: The PD mouse model was established by the ligation of molars and oral inoculation of subgingival plaques from PD patients and subsequently incorporated with a bleomycin-induced pulmonary fibrosis model. The effect of PD on pulmonary fibrosis was determined. Changes of immune cells were analysed using flow cytometry. Moreover, the microbiome changes of the lungs and oral cavity were assessed by 16S rRNA gene sequencing and fluorescence in situ hybridization. Finally, the effect and mechanism of the specific PD pathogen on pulmonary fibrosis were determined.

Results: PD significantly aggravated pulmonary fibrosis in mice by increasing the infiltration of neutrophils and Th17 cells. Neutrophils and Th17 cells are critical in PD-induced aggravation of pulmonary fibrosis, and Th17 cells regulate neutrophils via IL-17A. The PD pathogen *Porphyromonas gingivalis (Pg)* was detected enriched in both the oral cavity and lungs. *Pg* was further determined to exacerbate pulmonary fibrosis by increasing the expansion of neutrophils and Th17 cells in mice.

Conclusion: PD aggravates pulmonary fibrosis in mice, which is likely induced by *Pg*-promoted infiltration of neutrophils and Th17 cells. Treatment targeting PD or *Pg* might be a promising strategy to clinically ameliorate IPF.

Introduction

Idiopathic pulmonary fibrosis (IPF) is the most common and morbid type of idiopathic interstitial pneumonia (Podolanczuk et al., 2023). IPF is a chronic, progressive and incurable pulmonary disease. The prognosis of IPF patients is poor. Without treatment, the median survival of IPF patients is merely 3–5 years, which is even worse than the prognosis of many cancers (Bonella et al., 2023). The etiology of IPF is very complicated and poorly understood, and risk factors include genetic mutation, environmental exposures, dysbiosis of lung microbiome, and inflammation (Moss et al., 2022; Karampitsakos et al., 2023). Consequently, current treatments for IPF are limited to pirfenidone and nintedanib, which to some extent slow the development of IPF but never improve or stabilize pulmonary function (Spagnolo et al., 2021). Therefore, identifying the pathogenesis of IPF and further developing promising treatments for IPF are urgently needed.

The respiratory system is adjacent to oral cavity and has a close relationship with it. Oral diseases can disrupt the homeostasis of the lungs and even aggravate various lung diseases. Periodontitis (PD) is one of the most common oral diseases and is a complex inflammatory disease (Meyle and Chapple, 2015). PD causes the loss of teeth and periodontal tissues, not only causing oral dysfunction but also endangering overall health as a chronic lesion (Hajishengallis and Chavakis, 2021). PD is accompanied by dysbiosis of both the oral microbiome and host immune responses, which causes systemic dissemination of oral pathogens and related virulence factors as well as immune cells and inflammatory cytokines (Kinane et al., 2017). Consequently, invasion of these oral pathogens and associated inflammatory cascades can promote the development of systemic diseases (Teles et al., 2022). In recent decades, various studies have indicated correlations between periodontitis and at least 43 systemic diseases, such as diabetes, inflammatory bowel disease, hypertension, and respiratory diseases (Slots, 2017).

Recently, studies have indicated a potential correlation between PD and IPF. The previous clinical study revealed that 32.84% of lung microbiome genes are derived from the oral microbiome, and compared with the smoking group (control group), 38% of the differentially enriched genes in the IPF group are from the oral microbiome (Tong et al., 2019). Other studies have illustrated that *Streptococcus* and *Veillonella* are significantly enriched in IPF

patients compared with control subjects (Molyneaux et al., 2014; Invernizzi et al., 2021). *Streptococcus* and *Veillonella* are common oral bacteria and are closely associated with PD (Kumar et al., 2005; Koyanagi et al., 2013). Adult cystic fibrosis patients often have poor oral hygiene habits, more tooth plaques and calculus (Coffey et al., 2024), and exhibit widespread presence of periodontal pathogens in subgingival biofilms and in sputum (Rivas Caldas et al., 2015; Pawlaczyk-Kamieńska et al., 2019). Due to anatomic proximity, the oral microbiota may enter the lungs by microaspiration, and the lung microbiota resembles the oral microbiota more than the nasal microbiota does (Bassis et al., 2015; Gaeckle et al., 2020). Clinical and animal studies have demonstrated that incremental lung bacteria are strongly related to faster development and a greater rate of IPF mortality (O'Dwyer et al., 2019; Amati et al., 2022). PD pathogens such as *Porphyromonas gingivalis* (*Pg*) and *Fusobacterium nucleatum* have been verified to be present in the lungs and to promote lung diseases by increasing inflammation (Li et al., 2021; Feng et al., 2024). However, there is no direct evidence indicating whether PD is correlated with IPF and whether PD exacerbates IPF.

In this study, we aimed to reveal the relationship between PD and IPF and identify the underlying mechanisms. First, we investigated the effects of PD on BLM-induced (bleomycin-induced) pulmonary fibrosis in mice. Next, we analyzed changes of immune cells, and determined critical immune cells and their interactions in the PD-induced aggravation of pulmonary fibrosis. Finally, we identified a key PD pathogen enriched in both the oral and lungs and verified its importance and mechanisms for promoting pulmonary fibrosis in mice.

Materials and methods

Mice and treatments

Eight-week-old C57BL/6 male mice were ordered from Beijing Vital River Laboratories (Beijing, China). The mice were housed in a specific pathogen-free (SPF) facility. Mice aged 10–12 weeks were used in the study. PD was established in the mice as previously described (Bai et al., 2022). The bilateral maxillary second molars of the mice were ligatured with 5–0 silk. Then, mice were orally inoculated with subgingival plaques (1×10^9 colony-forming units

per mouse) from PD patients every other day for 2 weeks. Patients with moderate to severe PD were recruited and those who smoke, suffer systemic diseases, have less than 8 teeth, take antibiotics or probiotics and undergo periodontal treatments within the last 6 months were excluded. Subgingival plaques were then collected from PD patients and mixed well and stored in the -80° C freezer in advance. Subsequently, pulmonary fibrosis was induced in the mice with BLM (HY-17565A, MedChemExpress, Shanghai, China). Briefly, mice were first anaesthetized with isoflurane gas (4%) and then intratracheally administered BLM (0.5 mg/kg body weight, 30 μl per mouse) or saline control by an atomizing needle (Bio Jane Trading Co., Ltd., Shanghai, China). Mice were sacrificed 3 weeks later or at the indicated time points for further analysis.

The *Pg*-induced PD mouse model was established by ligation of bilateral maxillary second molars with 5–0 silk sutures as previously described and then orally inoculated with *Pg* (ATCC 33277, $1x10^9$ colony-forming units per mouse) every other day for 2 weeks. Then BLM was intratracheally administered, and the sutures were maintained until the end of the experiment. The Institute of Animal Care and Use Committee (IACUC) of Cyagen Biosciences, China (Approval No. ICU21-0080), approved the conduct of all the animal experiments. Collecting subgingival plaques from PD patients was approved by the Institutional Review and Ethics Board of Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine (No. SH9H-2021-T115–1). All participants provided informed consent.

To deplete neutrophils, mice were intraperitoneally injected with 200 μg of anti-mouse Ly6G antibody (BE0075-1, BioXCell) every 2 days since BLM administration to the end of the experiment. To neutralize IL-17A, mice were intraperitoneally injected with 200 μg of anti-mouse IL-17A antibody (BE0173, BioXCell) every 3 days since BLM administration until the end of the experiment.

Analysis of alveolar bone loss

To determine PD-induced alveolar bone loss as previously described (Abe and Hajishengallis, 2013), maxillae of mice were acquired, and the gingiva were removed, followed by bleaching and then rinsing. 1% methylene blue was then used to stain the maxillae. The staining maxillae were captured by a Leica digital camera (Leica, Germany). The distance between the cementoenamel junction (CEJ) and alveolar bone crest (ABC) was measured by ImageJ software (National Institutes of Health, Bethesda, USA).

Histology and hydroxyproline quantification

Lung lobes were fixed by 4% paraformaldehyde, dehydrated and then embedded in paraffin. Paraffin-embedded sections were subsequently performed with H&E staining or Masson's

trichrome staining. All images were obtained with a Leica DMi8 microscope (Leica, Wetzlar, Germany). The level of interstitial fibrosis was assessed as acknowledged protocols (Hübner et al., 2008). The hydroxyproline level was quantified by a hydroxyproline assay kit (A030-3-1, Nanjing Jiancheng, China).

Quantitative real–time PCR

RNA of lung tissues was isolated by TRIzol reagent (15596018, Invitrogen, USA). RNA was then reverse-transcribed to cDNA by a reverse transcription kit (Takara, Shiga, Japan). A LightCycler 480 II (Roche) was used to perform real-time PCR. Primers used in this study were listed in Supplementary Table S1.

Western blot analysis

Lung tissues were homogenized using RIPA lysis buffer containing phenylmethylsulfonylfluoride (PMSF, Sigma–Aldrich) and protease inhibitor (Med Chem Express). Protein concentration was then measured by the BCA protein assay kit (88512, Thermo Fisher Scientific). The 10% SDS–PAGE was used to separate proteins. Detection was performed with ECL Western Blotting Substrates (Thermo Fisher Scientific). The antibodies used in this study were anti-TGF-β1 (ab215715, Abcam), anti-Fibronectin (ab2413, Abcam) and anti-GAPDH (14c10, Cell Signaling Technology).

Preparation of single-cell suspensions from tissues

Single-cell suspensions from the lungs were prepared according to previous protocol (Jungblut et al., 2009). The lungs were put in a gentleMACS C Tube (130-093-237, Miltenyi Biotec) containing 5 mL of HEPES buffer supplemented with DNase I (80 U/mL, A37780050, applichem) and collagenase IV (300 U/ml, A004186-0001, worthington). The C Tube was attached to the gentleMACS Dissociator (130-093-235, Miltenyi Biotec) to run the program "m_lung_01". Then the lung tissues were digested for 30 minutes with agitation at 37°C and followingly run the program "m_lung_02" to obtain suspensions. PBS was added to terminate the digestion, and the suspensions were filtered by a cell strainer to acquire single lung cells. The cervical lymph nodes (cLNs) of the mice were ground on a cell strainer and rinsed with PBS to obtain single cells. Bone marrow cells were prepared by flushing the femur with a syringe.

Flow cytometry analysis

Single-cell suspensions from the lungs, cLNs and bone marrow were centrifuged and stained with Live/Dead Fixable Viability Stain 510 (564406, BD Biosciences). The cell samples were incubated with

CD16/32 antibodies (101320, Biolegend) to block Fc receptors before surface staining and then stained with antibodies. For cytokine staining, cells were stimulated with the cell activation cocktail (423303, BioLegend), washed with PBS and finally stained with antibodies. Antibodies against the following molecules were used in this study: CD45 (557659, Biosciences), CD11b (101205, Biolegend), Ly6G (127616, Biolegend), Ly6C (128018, Biolegend), CD64 (139321, Biolegend), CD11c (117333, Biolegend), I-A/I-E (562564, Biosciences), SiglecF (155506, Biolegend), CD3 (100203, Biolegend), CD4 (100540, Biolegend), CD8 (100722, Biolegend), B220 (103251, Biolegend), F4/80 (123116, Biolegend), CD115 (135515, Biolegend), IFN-γ (505810, Biolegend) and IL-17A (506903, Biolegend). Samples were run on a BD LSRFortessa X-20 flow cytometer (BD Biosciences).

Analysis of microbiome

Total genomic DNA from lungs and oral silk ligatures was extracted by an OMEGA Soil DNA Kit (M5635-02, Omega Bio-Tek). Qualified DNA samples were performed PCR amplification of the nearly full-length bacterial 16S rRNA genes. PCR amplicons were then sequenced by the PacBio Sequel platform (Shanghai Personal Biotechnology Co., Ltd., China). Microbiome bioinformatics was performed with QIIME software and R packages (v3.2.0). The alpha diversity was analysed by the ASV table in QIIME2, and beta diversity was performed with Bray–Curtis metrics. Differentially abundant taxa between groups were analysed by linear discriminant analysis effect size (LEfSe) with the default parameters. The raw sequences were deposited in the NCBI Sequence Read Archive database with the accession number PRJNA1138471.

Fluorescence *in situ* hybridization

Identifying the presence of *Pg* was by fluorescence *in situ* hybridization (FISH) according to a published protocol (Rudney et al., 2005; Niu et al., 2024). Lung tissue slides were incubated with a specific Alexa Fluor 594-labelled *Pg* probe (5'-CAATACTCGTATCGCCCGTTATTC-3') at 46°C overnight. Samples were washed with solution (100 mM Tris–HCl, 0.9 M NaCl, pH 7.5) for 25 min at 48°C, then distilled water, air-dried and counterstained with DAPI (Invitrogen). Images were obtained with a Leica DMi8 microscope (Leica, Wetzlar, Germany).

Statistical analysis

The experimental data are presented as the mean ± SEM. Unpaired Student's t test was used for two-group comparisons. One-way ANOVA was used for comparisons among more than two groups. All statistical analyses were performed with GraphPad Prism software. Values of $P < 0.05$ were considered to indicate statistical significance. Information about the statistical details and methods is also included in Figure legends.

Results

PD aggravates BLM-induced pulmonary fibrosis

To determine the impact of PD on pulmonary fibrosis, the PD mouse model combined with pulmonary fibrosis was established. The molars of the mice were ligatured with silk sutures, and subgingival plaques from PD patients were inoculated in mouse oral cavity to establish PD with humanized oral microbiota, followed by BLM administration to induce pulmonary fibrosis (Figure 1A). PD caused severe alveolar bone loss (Figure 1B) and significantly increased the distance between the CEJ and ABC in mice (Figure 1C).

Moreover, the results of H&E and Masson's trichrome staining indicated that PD induced more severe destruction of lung architecture and obviously increased collagen accumulation in mouse lungs (Figure 1D). Ashcroft scores, which assess the severity of fibrosis, also indicated that PD aggravated pulmonary fibrosis (Figure 1E). The lungs in the BLM combined with PD group were obviously heavier than those in the BLM group (Figure 1F). The hydroxyproline content, a marker of lung fibrosis severity, was markedly greater in lungs of the BLM combined with PD group than the BLM group (Figure 1G). Consistent with these observations, PD also obviously promoted the expression of fibrotic genes (Figure 1H). The western blot results also demonstrated that PD significantly increased the fibrotic protein levels such as Fibronectin and TGF-β1 (Figures 1I, J). Collectively, these results illustrated that PD exacerbated pulmonary fibrosis in mice.

PD increases the infiltration of neutrophils and Th17 cells in mice with pulmonary fibrosis

Chronic inflammation is generally considered as a risk factor of pulmonary fibrosis, and many studies have verified that various immune cells are important for lung fibrosis (Yang et al., 2019). To explore immune mechanisms underlying PD-induced aggravation of pulmonary fibrosis, we next analyzed changes of immune cells. Myeloid cells and lymphoid cells in the lungs were analysed in an unbiased manner via flow cytometry at 0, 7 and 21 days after BLM treatment. Both the percentage and number of neutrophils were markedly greater in the lungs of the BLM+PD group than the BLM group at 0, 7 and 21 days after BLM administration (Figures 2A, B). Compared with the BLM group, the percentage and number of Th17 cells were also significantly greater in the BLM+PD group at the 3 indicated time points (Figures 2C, D). However, the percentage of other myeloid cells and lymphoid cells in the lungs did not always show the same significant trend as the number at the 3 indicated time points (Supplementary Figure S1). This indicated that neutrophils and Th17 cells were the most important immune cells that mediated the PD-induced aggravation of pulmonary fibrosis.

Additionally, myeloid cells and lymphoid cells in the cLNs and bone marrow of mice were also analysed at 0, 7 and 21 days after BLM administration. PD markedly increased neutrophils and Th17 cells in

the cLNs (Figures 2E–H). PD also led to obvious changes in other myeloid cells and lymphoid cells in the cLNs (Supplementary Figures S2A–L). A significant increase in B cells was also detected among the changes caused by PD in the cLNs (Supplementary Figures S2A–F). PD also affected myeloid cells and lymphoid cells in the bone marrow

of mice at different time points (Supplementary Figures S2M–R). Compared with the BLM group, the percentage and number of neutrophils in the bone marrow were markedly greater in the BLM +PD group (Figures 2I, J). Together, these results demonstrated that for mice with pulmonary fibrosis, PD increased the expansion of
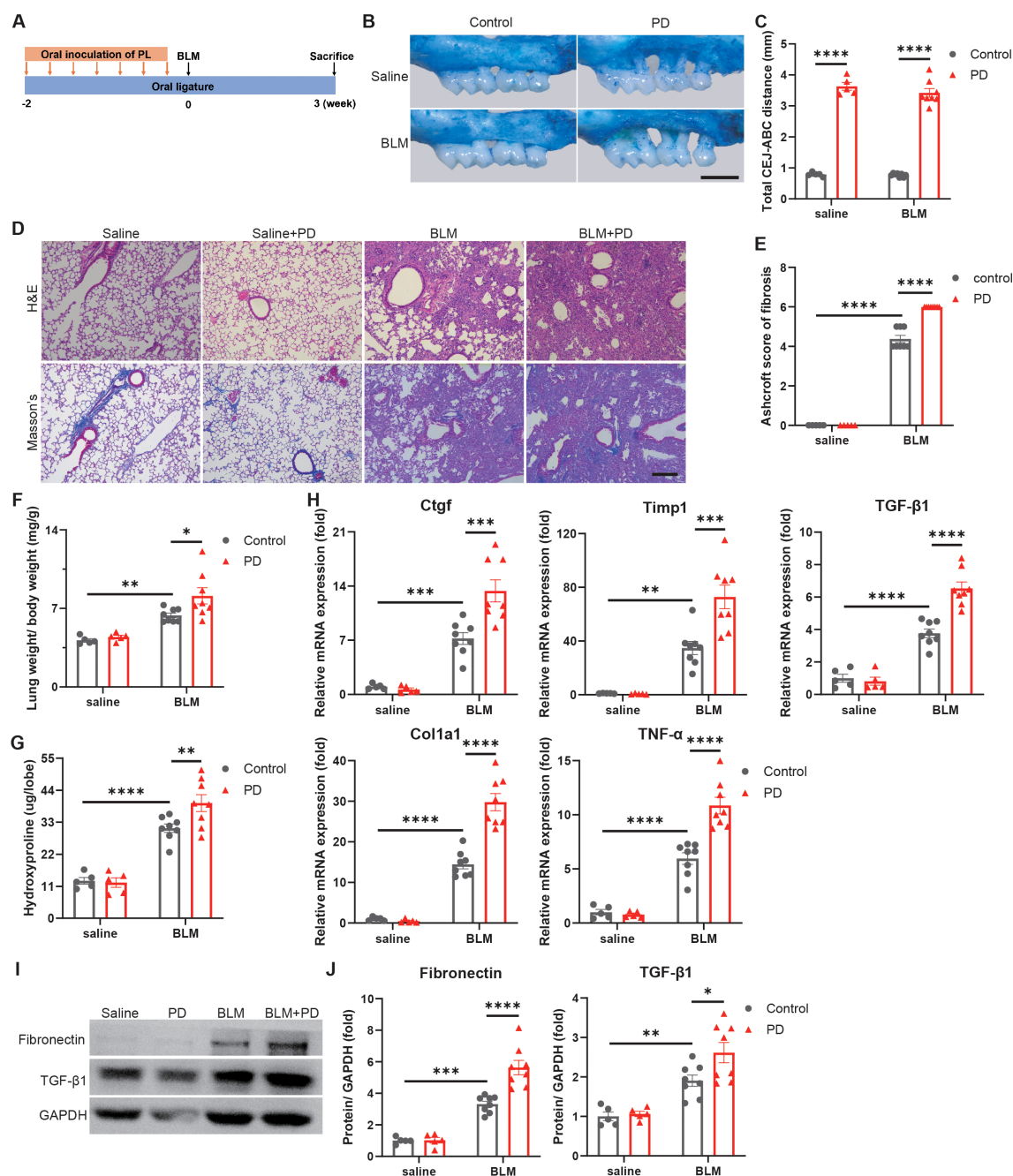

FIGURE 1

Periodontitis (PD) aggravates bleomycin-induced pulmonary fibrosis in mice. **(A)** Schematic illustration of the experimental design. PD was induced in mice by silk ligatures tied around molars and oral inoculation of subgingival plaques (PL) from patients with PD. Pulmonary fibrosis was induced by intratracheally administration of bleomycin (BLM). **(B)** Representative methylene blue staining of alveolar bones. Scale bar: 1 mm. **(C)** Quantification of the distance from the buccal cementoenamel junction (CEJ) to alveolar bone crest (ABC). **(D)** Representative images of H&E and Masson's trichrome staining of mouse lung sections. Scale bar: 200 μm. **(E)** Quantitative analysis of pulmonary fibrosis as Ashcroft score based on Masson's trichrome staining. **(F)** Lung weight to body weight ratio of mice. **(G)** Quantification of hydroxyproline content in mouse lungs. **(H)** qRT−PCR analysis of fibrotic genes in mouse lungs. **(I–J)** Representative Western blotting analysis **(I)** and quantifications **(J)** of Fibronectin and TGF-β1 in mouse lungs. n=5:5:8:8. Data are presented as mean ± SEM. One-way ANOVA was used for statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.
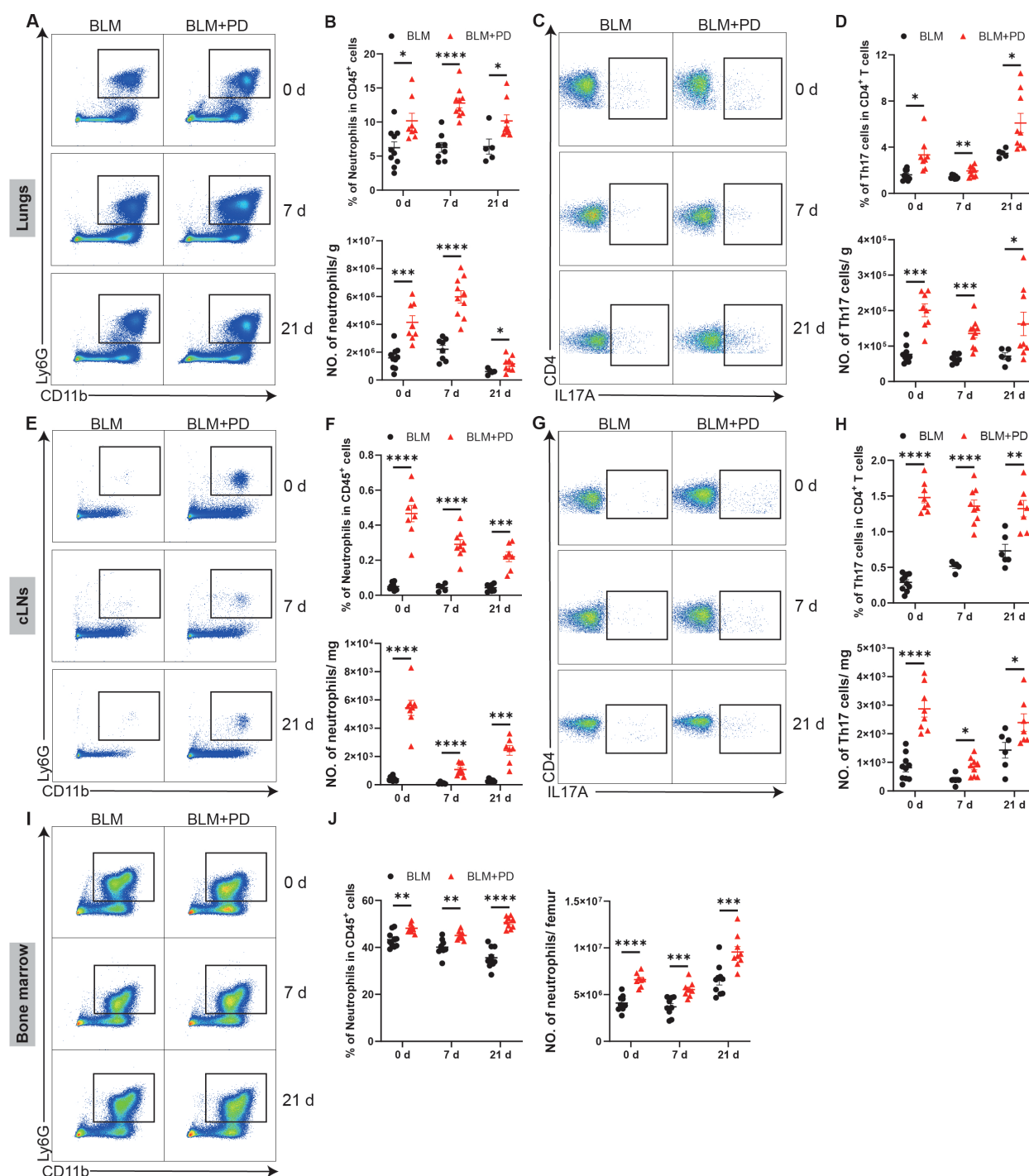
FIGURE 2
PD increases the infiltration of neutrophils and Th17 cells in mice with pulmonary fibrosis. **(A)** Representative flow cytometry plots of neutrophils in mouse lungs at 0 day, 7 days and 21 days after BLM administration. **(B)** Quantifications of neutrophils in mouse lungs as percentages and numbers. NO., number. **(C)** Representative flow cytometry plots of Th17 cells in mouse lungs at 0 day, 7 days and 21 days after BLM administration. **(D)** Quantifications of Th17 cells in mouse lungs as percentages and numbers. **(E)** Representative flow cytometry plots of neutrophils in cervical lymph nodes (cLNs) at 0 day, 7 days and 21 days after BLM administration. **(F)** Quantifications of neutrophils in mouse cLNs as percentages and numbers. **(G)** Representative flow cytometry plots of Th17 cells in mouse cLNs at 0 day, 7 days and 21 days after BLM administration. **(H)** Quantifications of Th17 cells in mouse cLNs as percentages and numbers. **(I)** Representative flow cytometry plots of neutrophils in mouse bone marrow at 0 day, 7 days and 21 days after BLM administration. **(J)** Quantifications of neutrophils in mouse bone marrow as percentages and numbers. n=5-10. Data are presented as mean ± SEM. Student's t-test was used for statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

neutrophils and Th17 cells in lungs, and that cLNs and bone marrow were likely the sources of increased neutrophils and Th17 cells.

Neutrophils and Th17 cells are critical to PD-induced aggravation of pulmonary fibrosis, and Th17 cells regulate neutrophils via IL-17A

To further investigate the importance of neutrophils and Th17 cells in PD-induced aggravation of pulmonary fibrosis, we depleted

neutrophils or IL-17A (mainly generated by Th17 cells) to assess the severity of lung fibrosis in mice. The lung damage and collagen accumulation were notably attenuated in the BLM+PD+Anti-Ly6G group and BLM+PD+Anti-IL17A group than those in the BLM+PD group (Figures 3A, B). The weights of the lungs and the hydroxyproline level were significantly reduced in PD mice after depletion of neutrophils or IL-17A (Figures 3C, D). Consistent with above results, the expression of fibrosis-associated genes and proteins also markedly reduced in PD mice after depletion of neutrophils or IL-17A (Figures 3E–G).

We next evaluated the relationship between neutrophils and Th17 cells in PD-induced aggravation of pulmonary fibrosis.
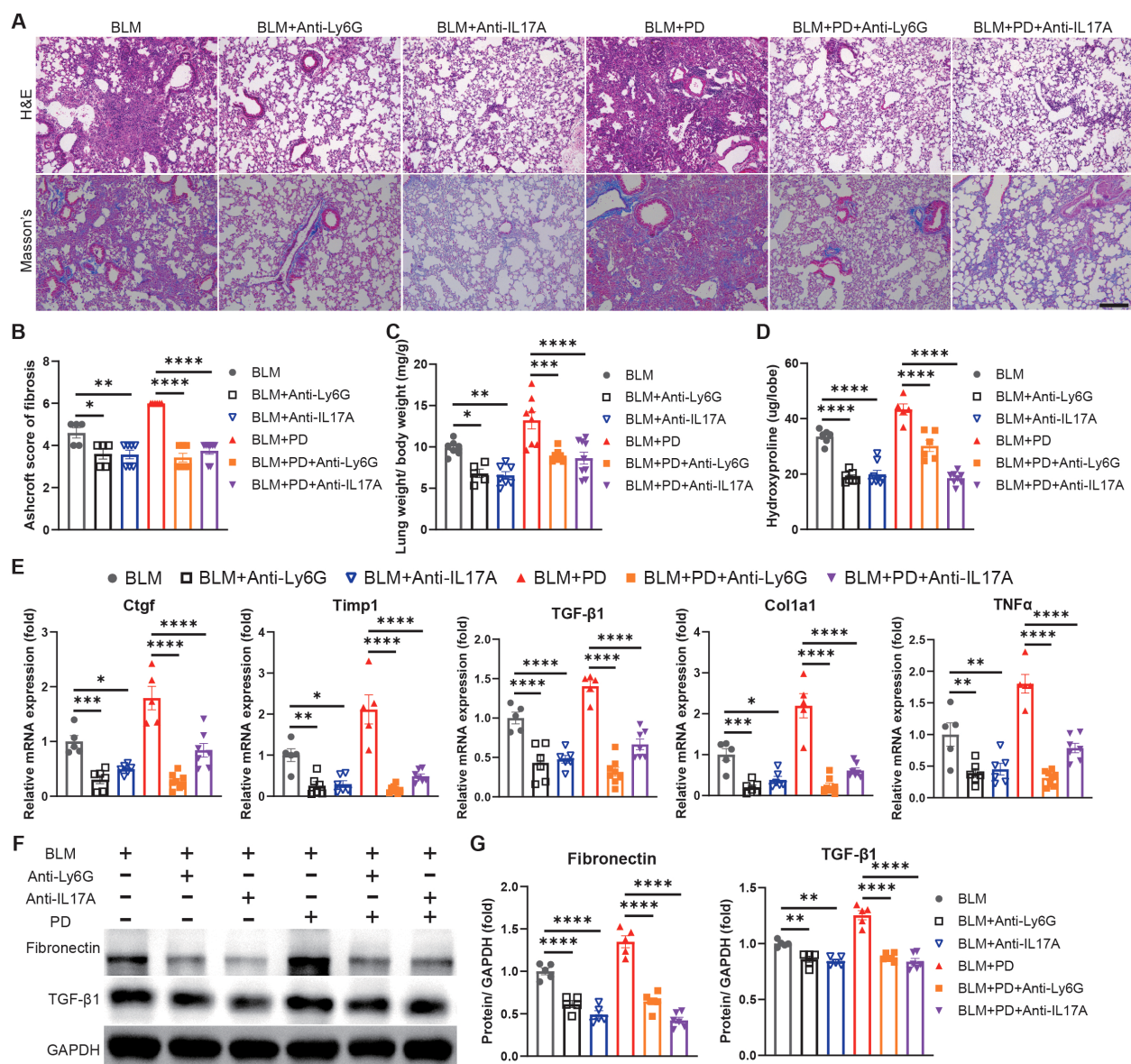

FIGURE 3

Neutrophils and Th17 cells mediate PD-induced aggravation of pulmonary fibrosis. **(A)** Representative images of H&E and Masson's trichrome staining of mouse lung sections. Anti-Ly6G, anti-Ly6G antibody; Anti-IL17A, anti-IL17A antibody. Scale bar: 200 μm. **(B)** Quantitative analysis of pulmonary fibrosis as Ashcroft score based on Masson's trichrome staining. **(C)** Lung weight to body weight ratio of mice. **(D)** Quantification of hydroxyproline content in mouse lungs. **(E)** qRT−PCR analysis of fibrotic genes in mouse lungs. **(F-G)** Representative Western blotting analysis **(F)** and quantifications **(G)** of Fibronectin and TGF-β1 in mouse lungs. n=5-9. Data are presented as mean ± SEM. One-way ANOVA was used for statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

Neutrophils in the lungs were comparable between BLM-administrated mice and BLM+Anti-IL17A treated mice, while the percentage and number of neutrophils were significantly lower in BLM+PD+anti-IL17A mice than in BLM+PD mice (Figures 4A, B). These results indicated that IL-17A was the upstream signal triggering neutrophils to mediate PD-induced aggravation of pulmonary fibrosis. However, Th17 cells in lungs were comparable between the BLM+PD group and BLM+PD+Anti-Ly6G group (Figures 4C, D). This result indicated that neutrophils did not influence the number of Th17 cells in mice with pulmonary fibrosis. Hence, these results revealed that neutrophils and Th17 cells are critical for the PD-induced
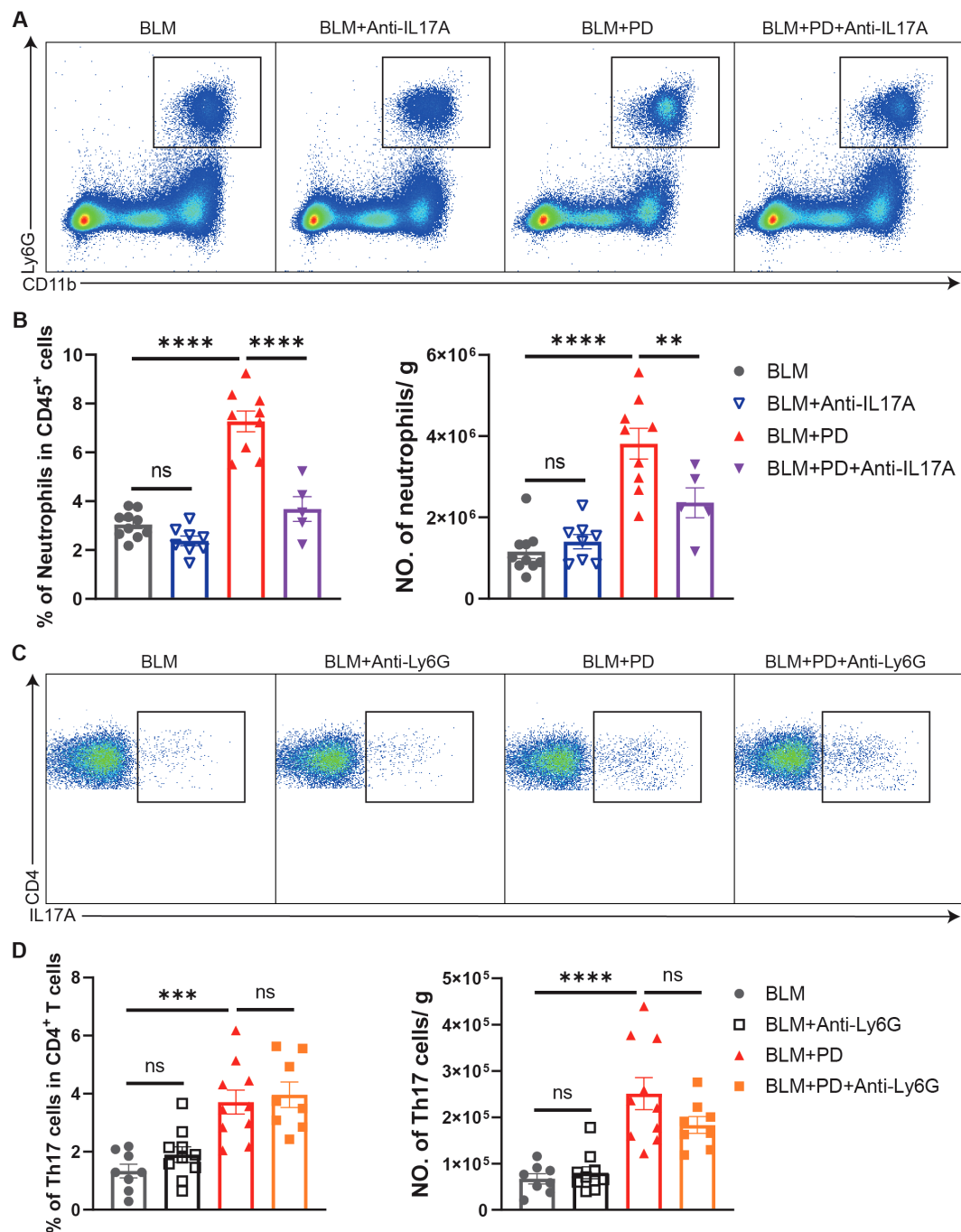

FIGURE 4
Th17 cells regulate neutrophils via IL-17A in PD-induced aggravation of pulmonary fibrosis. **(A)** Representative flow cytometry plots of neutrophils in mouse lungs at 7 days after BLM administration. **(B)** Quantifications of neutrophils in mouse lungs as percentages and numbers. NO., number. **(C)** Representative flow cytometry plots of Th17 cells in mouse lungs at 7 days after BLM administration. **(D)** Quantifications of Th17 cells in mouse lungs as percentages and numbers. NO., number. n=5-10. Data are presented as mean $\pm$ SEM. One-way ANOVA was used for statistical analysis. ns, not significant; *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

aggravation of pulmonary fibrosis and that Th17 cells regulate neutrophils via IL-17A.

PD enriches *Pg* in the lung and *Pg* aggravates BLM-induced pulmonary fibrosis in mice

Oral microbes were detected in the BALF microbiome of IPF patients, which possibly promoted the occurrence or progression of IPF (Tong et al., 2019). Therefore, we next assessed whether PD-related pathogenic bacteria transferred from the oral cavity to the lung and consequently impacted pulmonary fibrosis. We analysed both the lung microbiome and oral microbiome in the BLM group and BLM+PD group by 16S rRNA gene sequencing. For the lung microbiome, the alpha diversity and beta diversity were not notably different between the BLM+PD group and BLM group (Supplementary Figure S3A, B). In contrast, the alpha diversity and beta diversity of the oral microbiome were significantly different between the two groups (Supplementary Figure S3C, D). The LEfSe results of the enriched bacterial species in lungs and oral ligatures demonstrated that *Porphyromonas gingivalis* was among the top-ranked PD-related species in the BLM+PD group (Figures 5A, B).

To determinate the effect of *Pg* on pulmonary fibrosis in mice, we established a *Pg*-induced PD model by ligation of bilateral maxillary second molars and oral inoculation of *Pg* in the PG group and subsequently administrated BLM (Figure 5C). *Pg* was detected in lung sections of mice from both the BLM+PD group and BLM+PG group, which confirmed *Pg* translocating from the oral to the lungs (Figure 5D). According to the results of H&E and Masson's trichrome staining, *Pg* caused more severe injury and significantly increased collagen accumulation in lungs of the mice (Figures 5E, F). *Pg* also notably promoted the expression of fibrotic genes (Figure 5G) and fibrotic proteins (Figures 5J, K). Compared with the BLM group, mice in the BLM+PG group presented markedly heavier lungs and higher levels of hydroxyproline in the lungs (Figures 5H, I). Overall, *Pg* translocated from the oral to the lung and aggravated BLM-induced pulmonary fibrosis in mice.

Pg increases the infiltration of neutrophils and Th17 cells in mice with pulmonary fibrosis

To explore immune mechanisms underlying *Pg*-driven exacerbation of pulmonary fibrosis, we performed flow cytometry analysis of the lungs, cLNs and bone marrow at 7 days after BLM administration, which was an early inflammation stage that determines the following development of pulmonary fibrosis. Consistent with the PD-induced infiltration of neutrophils and Th17 cells (Figure 2), *Pg* also significantly increased neutrophils and Th17 cells in lungs of mice (Figures 6A–D). The percentages and numbers of other myeloid cells and lymphoid cells did not exhibit the same significant changes between the two groups

(Supplementary Figure S4A–C). We further analysed immune cells in cLNs and the bone marrow in an unbiased manner. In line with subgingival plaque-induced PD, *Pg* also caused notable increases of neutrophils and Th17 cells in cLNs (Figures 6E–H). Additionally, *Pg* notably increased the percentage and number of B cells in cLNs (Supplementary Figures S4D–F). Compared with the BLM group, the percentage and number of neutrophils in the bone marrow of the BLM+PG group were greater (Figures 6I, J). To some extent, *Pg* also affected other myeloid cells and lymphoid cells in the bone marrow (Supplementary Figures S4G, H). Taken together, these results illustrated that *Pg* promoted the expansion of neutrophils and Th17 cells in lungs, cLNs and bone marrow of mice with BLM-induced pulmonary fibrosis.

Discussion

Several clinical studies have reported that oral microbiota was possibly a risk factor for pulmonary fibrosis (Molyneaux et al., 2014; Tong et al., 2019; Invernizzi et al., 2021). As a very prevalent oral infectious disease, PD causes oral microbiota dysbiosis and notably increases oral pathogens (Kinane et al., 2017). However, there is a lack of identification and mechanistic exploration of the relationships among PD, oral microbiota and pulmonary fibrosis. Here, our results demonstrated that PD aggravated BLM-induced pulmonary fibrosis in mice and that the ectopically colonized PD pathogen *Pg* promoted the infiltration of neutrophils and Th17 cells, and Th17 cells regulated neutrophils via IL-17A to exacerbate pulmonary fibrosis (Figure 7).

Our results revealed an association between PD and IPF, and verified that PD exacerbates BLM-induced pulmonary fibrosis in mice. PD caused more severe lung damage and obviously increased collagen accumulation in lungs, heavier lungs as well as higher levels of hydroxyproline. PD also promoted the mRNA and protein levels of fibrotic markers. Our models used in this study mimicked those in clinical practice to some extent and provided more insights into the causal relationship between PD and the progression of pulmonary fibrosis. Subgingival plaques from PD patients were orally inoculated into mice to simulate the oral microbiome of human patients (Zhou et al., 2023b). The BLM model is acknowledged as the most extensively applied experimental model in IPF research (Tashiro et al., 2017). The administration of BLM first led to excessive inflammatory infiltrates and subsequent extracellular matrix accumulation, ultimately resulting in pulmonary fibrosis (Della Latta et al., 2015). PD leads to systemic chronic inflammation, which may partially explain why PD exacerbated pulmonary fibrosis in our study.

To reveal the mechanisms by which PD aggravated pulmonary fibrosis, we next performed flow cytometry analysis and found that PD increased the infiltration of neutrophils and Th17 cells. PD expanded neutrophils and Th17 cells before BLM administration (0 day), during the inflammation period (7 days) and fibrosis period (21 days). These results were same as previous studies revealing that PD leads to chronically deregulated inflammatory conditions and impacts the systemic immune response (Albuquerque-Souza and
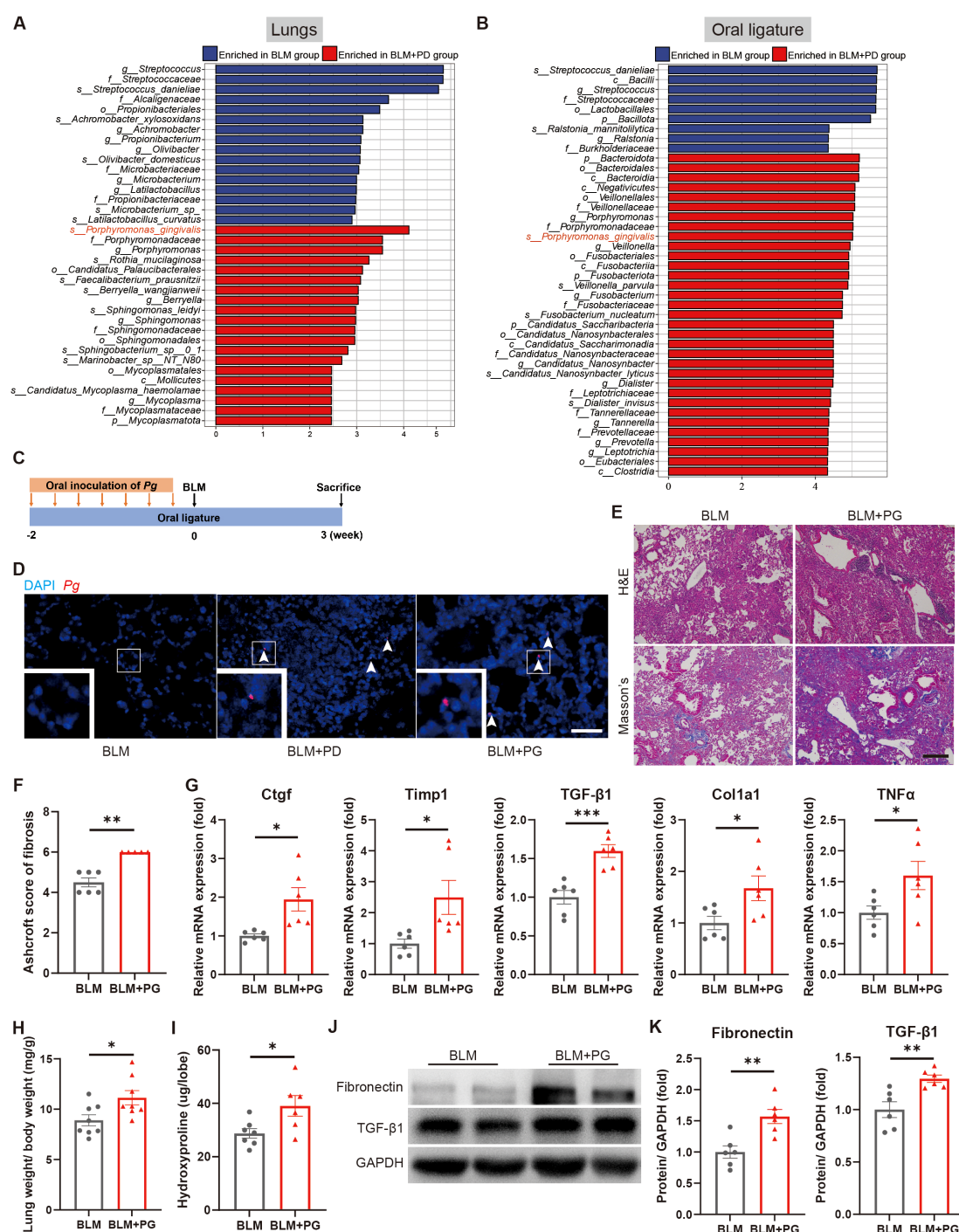
FIGURE 5

Porphyromonas gingivalis (*Pg*) aggravates BLM-induced pulmonary fibrosis in mice. **(A, B)** Linear discriminant analysis effect size (LEfSe) of enriched bacterial species in lungs **(A)** and oral ligatures **(B)** of mice with pulmonary fibrosis and with or without PD by 16S rRNA gene sequencing. n=4:5. **(C)** Schematic illustration of the experimental design. Periodontitis was induced in mice of the PG group by ligation of molars and oral inoculation of *Pg*. **(D)** Representative fluorescence *in situ* hybridization (FISH) for *Pg* in mouse lungs using Alexa Fluor 594-conjugated *Pg* probe. Boxed areas are shown in higher magnification by inset images. Scale bar: 100 µm. **(E)** Representative images of H&E and Masson's trichrome staining of lung sections of mice with pulmonary fibrosis and with or without *Pg* inoculation. Scale bar: 200 µm. **(F)** Quantitative analysis of pulmonary fibrosis based on Masson's trichrome staining. **(G)** qRT−PCR analysis of fibrotic genes in mouse lungs. **(H)** Lung weight to body weight ratio of mice. **(I)** Quantification of hydroxyproline content in mouse lungs. **(J−K)** Representative Western blotting analysis **(J)** and quantifications **(K)** of Fibronectin and TGF-β1 in mouse lungs. n=5-8. Data are presented as mean ± SEM. Student's t-test was used for statistical analysis. *P < 0.05, **P < 0.01, and ***P < 0.001.
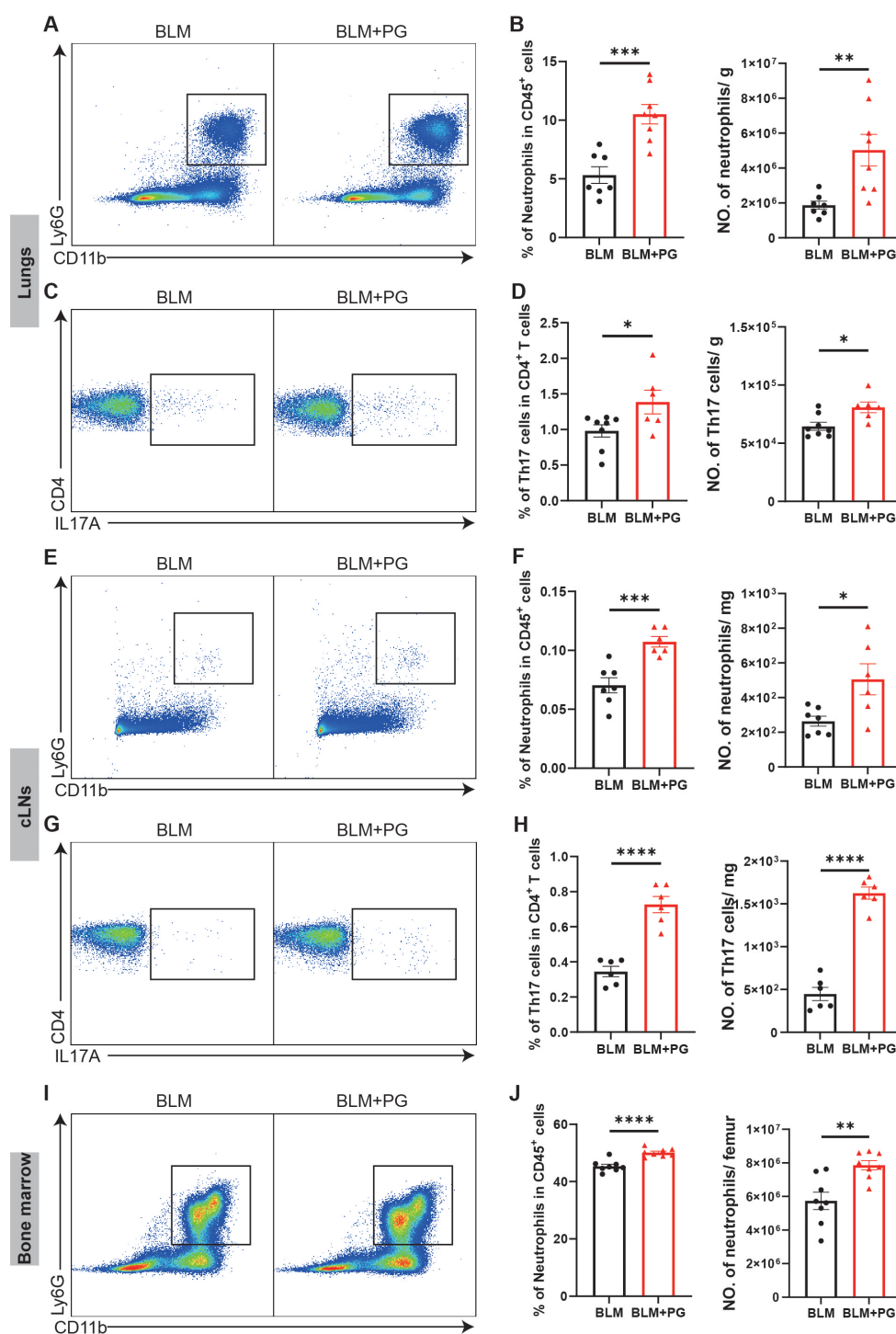
FIGURE 6

Pg increases the infiltration of neutrophils and Th17 cells in mice with pulmonary fibrosis. **(A)** Representative flow cytometry plots of neutrophils in mouse lungs at 7 days after BLM administration. Periodontitis was induced in mice of the PG group by ligation of molars and oral inoculation of *Pg*. **(B)** Quantifications of neutrophils in mouse lungs as percentages and numbers. **(C)** Representative flow cytometry plots of Th17 cells in mouse lungs at 7 days after BLM administration. **(D)** Quantifications of Th17 cells in mouse lungs as percentages and numbers. **(E)** Representative flow cytometry plots of neutrophils in mouse cLNs at 7 days after BLM administration. **(F)** Quantifications of neutrophils in mouse cLNs as percentages and numbers. **(G)** Representative flow cytometry plots of Th17 cells in mouse cLNs at 7 days after BLM administration. **(H)** Quantifications of Th17 cells in mouse cLNs as percentages and numbers. **(I)** Representative flow cytometry plots of neutrophils in mouse bone marrow at 7 days after BLM administration. **(J)** Quantifications of neutrophils in mouse bone marrow as percentages and numbers. n=6-8. Data are presented as mean $\pm$ SEM. Student's t-test was used for statistical analysis. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.
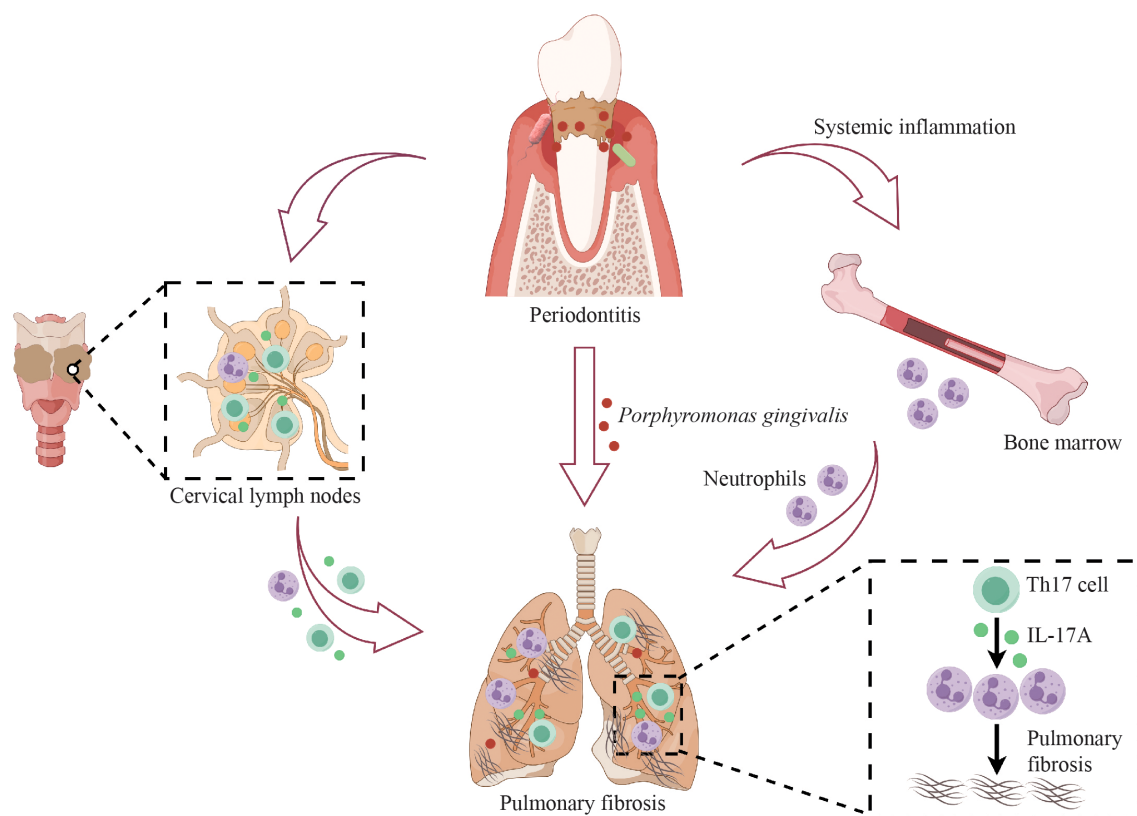
FIGURE 7
Schematic illustration of the mechanisms by which periodontitis (PD) aggravates pulmonary fibrosis. PD aggravates BLM-induced pulmonary fibrosis by promoting the infiltration of neutrophils and Th17 cells in the lungs, and Th17 cells regulate neutrophils via IL-17A in PD-induced aggravation of pulmonary fibrosis. PD facilitates the translocation of the oral pathogen, *Porphyromonas gingivalis*, to the lung and *Porphyromonas gingivalis* also induces the expansion of Th17 cells and neutrophils and aggravates pulmonary fibrosis. This figure was created with the assistance of Figdraw.

Sahingur, 2022). In our study, PD promoted the expansion of neutrophils and Th17 cells in lungs even before BLM administration. Both neutrophils and Th17 cells were determined vital for exacerbating pulmonary fibrosis (Aoki et al., 2005; Yang et al., 2019). Our results showed that PD markedly increased neutrophils and Th17 cells in the cLNs and neutrophils in the bone marrow. Consistent with previous studies, PD significantly enriched neutrophils and Th17 cells in draining cLNs (Papadakou et al., 2017; Zhou et al., 2023a). PD induces a sustained increase in myelopoiesis and the production of neutrophils in the bone marrow (Li et al., 2022). The bone marrow generates neutrophils and then releases to peripheral organs, including lungs (Summers et al., 2010). The lymphatic circulatory system enables leukocyte trafficking among draining lymph nodes in response to inflammation (Au et al., 2002). Hence, these local and systemic immune disorders caused by PD further promote the progression of pulmonary fibrosis.

In our study, neutrophils and Th17 cells were demonstrated indispensable for PD-induced exacerbation of pulmonary fibrosis, and Th17 cells regulated neutrophils via IL-17A to aggravate pulmonary fibrosis. Various studies have confirmed the importance of neutrophils, Th17 cells and IL-17A in pulmonary fibrosis. These studies verified that neutrophil or IL-17A depletion attenuated BLM-induced pulmonary inflammation and pulmonary fibrosis (Lei et al.,

2016; Leslie et al., 2020; Chen et al., 2022). Reasonably, our study showed that PD did not aggravate pulmonary fibrosis after neutrophil depletion or IL-17A depletion. IL-17A is generated mainly by Th17 cells (Mills, 2023) and has been demonstrated to promote immune cells like macrophages and neutrophils to aggravate airway inflammation (Sergejeva et al., 2005; Cheung et al., 2008; Vanaudenaerde et al., 2011). Th17 cells reportedly play important roles in neutrophil recruitment and aggregation in the lungs by secreting IL-17A and increasing the expression of chemokines (Liu et al., 2015; Yang et al., 2022). IL-17A acted on many nonimmune cells and prompted them to release chemokines, which were sensed by neutrophils in blood vessels and neutrophils then migrated to the focus (Knochelmann et al., 2018). Additionally, by upregulating G-CSF expression, IL-17A accelerated proliferation and differentiation of neutrophil precursors, and the release of mature neutrophils from bone marrow (Fan et al., 2023). Consistent with these studies, our results also verified that Th17 cells assembled neutrophils via IL-17A in the lung to promote PD-induced aggravation of pulmonary fibrosis.

Healthy lungs are traditionally thought to be sterile, but more and more studies have detected the microbiota in the lungs via advanced sequencing methods in recent years (Whiteside et al., 2021). Many pulmonary diseases are strongly associated with lung dysbiosis or specific microbiota (Durack et al., 2017; Wang et al., 2021). Pathological changes in the lung structure and damaged

mucus clearance because of lung diseases probably contribute to microbial dysbiosis and microbial colonization, which in turn might promote the development of lung diseases by upregulating or downregulating inflammatory signals (Natalini et al., 2023). The oral microbiota is acknowledged as the primary source of the lung microbiota, and the lung microbiota resembles the oral microbiota more than microbiota of other body sites (Venkataraman et al., 2015; Dickson et al., 2016). Greater oral microbial diversity was reported to be related to more severe conditions and a greater risk of death in IPF patients (O'Dwyer et al., 2024). A previous study showed that most translocations (32.84%) of the lung microbiome in IPF patients were from the oral microbiome (Tong et al., 2019). As an entrance of the respiratory tract, oral cavity is anatomically connected with the respiratory system. Provided with the proximity, oral microbiota including periodontal pathogens have more chance to enter the trachea and lung. Oral microbiota is easily translocated to the lung by daily inhalation, coughing and swallowing (Soussan et al., 2019). For those patients who need mechanical ventilation, oral microbiota migrates rapidly from the oral cavity and upper respiratory tract into the lung when performing orotracheal intubation (de Carvalho Baptista et al., 2018). Moreover, the oral cavity turns dry when the patient is bedridden for extended periods. This dry oral environment promotes oropharyngeal bacterial colonization and impaired swallowing function of patients further leads to bacterial stagnation in the pharyngeal cavity, which contributes to the aspiration of oral microbiota to the lung (Rehm et al., 2010). Periodontal pockets are prone to ulceration and bleeding, and therefore periodontal pathogens can also disseminate to the lung by blood (Forner et al., 2006). Besides, conditions like long-term smoking, diabetes and nasal intubation impairs the body immune system, which facilitate the translocation of oral microbiota to the lung (Pathak et al., 2021).

Dysregulated lung bacteria are reported to motivate the expression of neutrophil-recruiting genes and Th17 cell-promoting genes via their outer membrane vesicles to aggravate lung fibrosis in BLM-administrated mice (Yang et al., 2019). Furthermore, it has been reported that subjects with the four periodontal pathogens, *Tannerella forsythia*, *Pg*, *Aggregatibacter actinomycetemcomitans* and *Treponema denticola* in the lung suffered from PD more frequently than those subjects without these periodontal pathogens in the lung. The level of inflammatory aMMP8 tended to be higher in the bronchial fluid in subjects with the four periodontal pathogens. This indicates that PD possibly increases the risk of pulmonary colonization of periodontal pathogens, and such colonization seems to be related with increased lung inflammation (Schmidlin et al., 2015). As a classic pathogen of PD, *Pg* is broadly acknowledged to be related to pneumonia, asthma, chronic obstructive pulmonary disease and other respiratory diseases and is found in the lungs or BALF from patients (Shi et al., 2023). *Pg*-induced PD resulted in more obvious pulmonary inflammation and increased neutrophils in the peripheral blood and lungs, as well as the presence of *Pg* gingipains in lung tissues (Tian et al., 2022). Outer membrane vesicles of *Pg* were reported to induce cell death by disrupting the barrier system in lung epithelial cells (He et al., 2020). Intratracheal

injection of *Pg* culture supernatant significantly exacerbated pneumonia by increasing the production of TNF-α and IL-17, which indicates the importance of virulence factors produced by *Pg* (Okabe et al., 2021). Additionally, *Pg* also led to a significant increase of Th17 cells in cLNs (Sandal et al., 2016). Consistent with these previous results, our study verified that PD contributed to ectopic colonization of *Pg* in the lung, and *Pg* increased the infiltration of neutrophils and Th17 cells in mice.

We acknowledge several limitations in this study. Although we verified that PD aggravated BLM-induced pulmonary fibrosis in mice, we do not know the causal relationship between PD and IPF in humans. In the future, researchers can investigate the effect of PD on IPF and collect clinical samples to explore changes of lung microbiota by clinical studies. Besides, we revealed that *Pg*-induced PD exacerbated pulmonary fibrosis by promoting the infiltration of neutrophils and Th17 cells in mice, but the direct effects of *Pg* and *Pg* related virulence factors on the lung remained unknown. These underlying mechanisms can be explored in future experiments.

Conclusion

The present study demonstrated that PD aggravated BLM-induced pulmonary fibrosis in mice. Mechanistically, PD promoted ectopic colonization of *Pg* in lungs and markedly increased the infiltration of neutrophils and Th17 cells in mice, thereby exacerbating inflammation and subsequent pulmonary fibrosis. Furthermore, our data verified that Th17 cells recruited neutrophils via IL-17A to aggravate pulmonary fibrosis. Together, these data provide insights into the correlation between PD and IPF, reveal novel mechanisms underlying this correlation, and suggest treatment targeting PD or *Pg* as a promising strategy for clinically ameliorating IPF.

Data availability statement

The data that support the findings of this study are available in the NCBI Sequence Read Archive database with the accession number PRJNA1138471.

Ethics statement

The studies involving humans were approved by The Institutional Review and Ethics Board of Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine (No. SH9H-2021-T115–1). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. The animal study was approved by The Institute of Animal Care and Use Committee (IACUC) of Cyagen Biosciences, China (Approval No. ICU21-0080). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

H-LY: Conceptualization, Data curation, Methodology, Validation, Writing – review & editing, Formal Analysis, Investigation, Visualization, Writing – original draft. X-QM: Conceptualization, Data curation, Methodology, Validation, Writing – review & editing, Investigation. HL: Conceptualization, Investigation, Methodology, Writing – review & editing. XS: Conceptualization, Methodology, Writing – review & editing, Resources. W-ZL: Conceptualization, Investigation, Methodology, Writing – review & editing. L-JZ: Conceptualization, Investigation, Methodology, Writing – review & editing, Data curation, Validation. JZ: Data curation, Investigation, Writing – review & editing. CH: Writing – review & editing, Conceptualization, Methodology. SX: Writing – review & editing, Data curation, Investigation. B-YC: Data curation, Writing – review & editing, Methodology. CQ: Methodology, Writing – review & editing, Conceptualization. Y-LL: Writing – review & editing, Data curation, Investigation. Y-LW: Writing – review & editing, Methodology. L-FY: Methodology, Writing – review & editing, Conceptualization. S-ZD: Conceptualization, Methodology, Writing – review & editing, Data curation, Funding acquisition, Project administration, Resources, Supervision, Validation.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2025.1595500/full#supplementary-material

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