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# *P. gingivalis* alters lung microbiota and aggravates disease severity of COPD rats by up-regulating Hsp90α/MLKL

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**ABSTRACT**

**Background:** Epidemiological evidence has confirmed that periodontitis is an essential and independent risk factor of chronic obstructive pulmonary disease (COPD). *Porphyromonas gingivalis*, a major pathogen implicated in periodontitis, may make a vital contribution to COPD progression. However, the specific effects and molecular mechanism of the link between *P. gingivalis* and COPD are not clear.

**Methods and Results:** A COPD rat model was constructed by smoke exposure combined intratracheal instillation of E. coli-LPS, then *P. gingivalis* was introduced into the oral cavity of COPD rats. This research observed that lower lung function, more severe alveolar damage and inflammation occurred in COPD rats with *P. gingivalis* group. Meanwhile, P. gingivalis/gingipains could colonize the lung tissues and be enriched in bronchoalveolar lavage fluid (BALF) of COPD rats with *P. gingivalis* group, along with alterations in lung microbiota. Proteomic analysis suggested that Hsp90α/MLKL-meditated necroptosis pathway was up-regulated in *P. gingivalis*-induced COPD aggravation, the detection of Hsp90α and MLKL in serum and lung tissue verified that Hsp90α/MLKL was up-regulated.

**Conclusion:** These results indicate that *P. gingivalis* could emigrate into the lungs, alter lung microbiota and lead to aggravation of COPD, which Hsp90α/MLKL might participate in.

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## Introduction

As the main cause of tooth loss, periodontitis impairs people's quality of life and is an essential and independent risk factor of chronic obstructive pulmonary disease (COPD) [1,2]. COPD is a common and chronic respiratory disease which is characterized by irreversible airflow limitation due to airway and/or alveolar abnormalities [3]. Sometimes COPD patients suffer from severe exacerbations which is associated with significantly lower survival outcomes [4,5]. Poor periodontal health, especially higher plaque scores and fewer teeth, is associated with the increased frequency of exacerbations in COPD patients [6]. Some researches focused on the involvement of periodontal pathogens in the development of COPD [7–9]. *Porphyromonas gingivalis*, a major pathogens in periodontitis development, may play a crucial role in COPD progress [10]. A prospective follow-up study found that COPD patients with high serum *P. gingivalis* IgG titers had significantly lower incidence and frequent of exacerbation than patients with normal IgG titers [11]. Our previous studies also found a high detection rate of *P. gingivalis* in respiratory secretions of patients with COPD exacerbation, which is homologous to *P. gingivalis* in dental plaque, supported the hypothesis that *P. gingivalis* may contribute to the pathology of COPD exacerbation [12]. However, the specific effects and molecular mechanism of the link between *P. gingivalis* and COPD are not clear.

In healthy individuals, the lung microbiota is relatively simple and low in abundance that an ecological balance between lung microbiota and host was maintained through mechanical and immune clearance [13]. However, there is a significant disruption of the lung microbiota, characterized by dysbiosis in microbial composition and quantity in COPD [14,15]. Multi-omics analysis found that the altered lung microbiome in COPD interact with host gene expression by their metabolites to promote inflammation [16]. The changes in airway microbes also shift COPD from neutrophilic to eosinophilic inflammation [17]. *Pseudomonas aeruginosa* and *Streptococcus pneumoniae* are common respiratory condition pathogens that may induce acute exacerbation of COPD [18,19]. *P. gingivalis* was known to enhance the adhesion of *Streptococcus pneumoniae* to alveoli by promoting the expression of Platelet-

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Activating Factor Receptor through gingipain [20]. *P. gingivalis* or its virulence factors can also cooperate with *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* to promote alveolar epithelial cell apoptosis and aggravate respiratory inflammation [21,22]. However, no study has reported the impact of *P. gingivalis* on lung microbiota in COPD progress.

Inflammation is the main mechanism of COPD progression, which can lead to small airway damage, alveolus destruction, and lung function reduced [23]. As a cytosolic molecular chaperone, Heat shocked protein 90 α (Hsp90α) could regulate the phosphorylation, oligomerization and membrane transport of Mixed Lineage Kinase Domain Like Pseudokinase (MLKL) [24,25]. Activated MLKL can translocate to the cell membrane, leading to plasma membrane rupture and cell necroptosis [26]. With the rupture of the plasma membrane, cells swell, chromatin shrinks, and damaged cells release a significant quantity of damage associated molecular patterns (DAMPs), inflammatory factors and cellular contents which directly causing inflammation and indirectly spreading inflammatory responses [27–29]. Early study has found that Hsp90α in serum of patients with moderate-to-severe COPD are significantly higher than those of healthy smokers and non-smoking controls [30]. The expression of MLKL/p-MLKL protein is increased in respiratory epithelial cells and macrophages of patients with severe COPD [31]. Therefore, Hsp90α/MLKL may participate in the occurrence and development of COPD.

In the present study, by constructing COPD rat model and *P. gingivalis* inoculation in COPD rats' oral cavity, we want to investigate the specific effects and molecular mechanism of the link between *P. gingivalis* and COPD. So as to clarify the role of *P. gingivalis* in COPD progression and provide a novel idea on the prevention and treatment of COPD.

## Materials and methods

### Bacterial cultures

*P. gingivalis* W83 (Provided by the Department of Periodontology, School of Stomatology, China Medical University) were cultured in fresh brain heart infusion (BHI) agar plate with 0.5% hemin, 0.1% menadione (vitamin K1),0.05% yeast extract and 5% sterile defibrinated sheep blood at 37°C in an anaerobic jar for 3–5 days. Monoclonal colonies in good growth condition (dark black and shiny) were selected to BHI liquid medium without sheep blood. After 16–18 h culturation, bacteria were collected by centrifugation at 3500 rpm for 5 min, and resuspended in sterile PBS. OD was measured using a UV spectrophotometer at 600 nm, and $1 \times 10$ [9] CFU/mL was used as the bacterial concentration at an absorbance of one.

### Animal model

The Sprague-Dawley (SD) rats (180–200 g, 6–7 weeks-old) were purchased from Changsheng Biotechnology Co., Ltd. (Liaoning, China). After being fed adaptively in SPF laboratory animal room for 1 week, the rats were randomly divided into three groups ($n = 6$ per group): Control, COPD, COPD with *P. gingivalis* (COPD+P.g). The method of COPD rat modeling was minor modified according to some references [32,33]. Briefly, COPD group and COPD+P.g group rats were exposed to cigarette smoke in an automatic smoke exposure device (C-100, Shanghai Yuyan Instruments Co. Ltd. $50 \times 40 \times 29$ cm) at a rate of 30 minutes of 12 cigarettes (Hong He, Yunnan, China; tar: 18 mg, nicotine content: 1.1 mg) twice a day for successive 4 weeks, and intratracheal instillation of 200 μg of Escherichia coli lipopolysaccharide (*E. coli*-LPS) (O55:B5, Sigma, America) under anesthetize condition on day 1 and day 14. The Control group was feed in fresh air and received intratracheal instillation of 200 μg of sterile PBS under anesthetize condition on day 1 and day 14. (The detail of intratracheal instillation is in the supplementary material) At the end of week 4, lung function tests were performed to estimate if the COPD model was successfully made on the basis of pulmonary functional impairment. At the begin of week 5, COPD+P.g group rats were anesthetized with sodium pentobarbital, a 0.2-mm wire was placed in the dentogingival area of both maxillary first molars, and received$1 \times 10^9$ CFU/mL(1 mL) of *P. gingivalis* W83 with 5% carboxymethylcellulose sodium (CMC-Na) in the gingival sulcus and oral cavity once every other day for 2 weeks. Other groups received normal breeding for 2 weeks (Figure 1a). At the end of week 6, lung function tests were carried out to demonstrate the effects of *P. gingivalis* on COPD rats. Then all rats were sacrificed for following experiments. All procedures were approved by the Animal Research Committee of China Medical University (Permit number: K2020031).

### Lung function test

At the end of week 4 and 6, lung function was measured using the EMKA pulmonary system (GYD-003). After calibration of the parameters, the rats were placed in a closed plethysmograph box, and the data were recorded when the rats were quiet and breathing smoothly. The detection time for each rat was 4 minutes, and the parameters included tidal volume (TV), peak inspiration flow (PIF) and peak expiration flow (PEF).

### Histological analysis

For histological analysis, the right-lung tissue of rat was formalin-fixed and paraffin-embedded. The

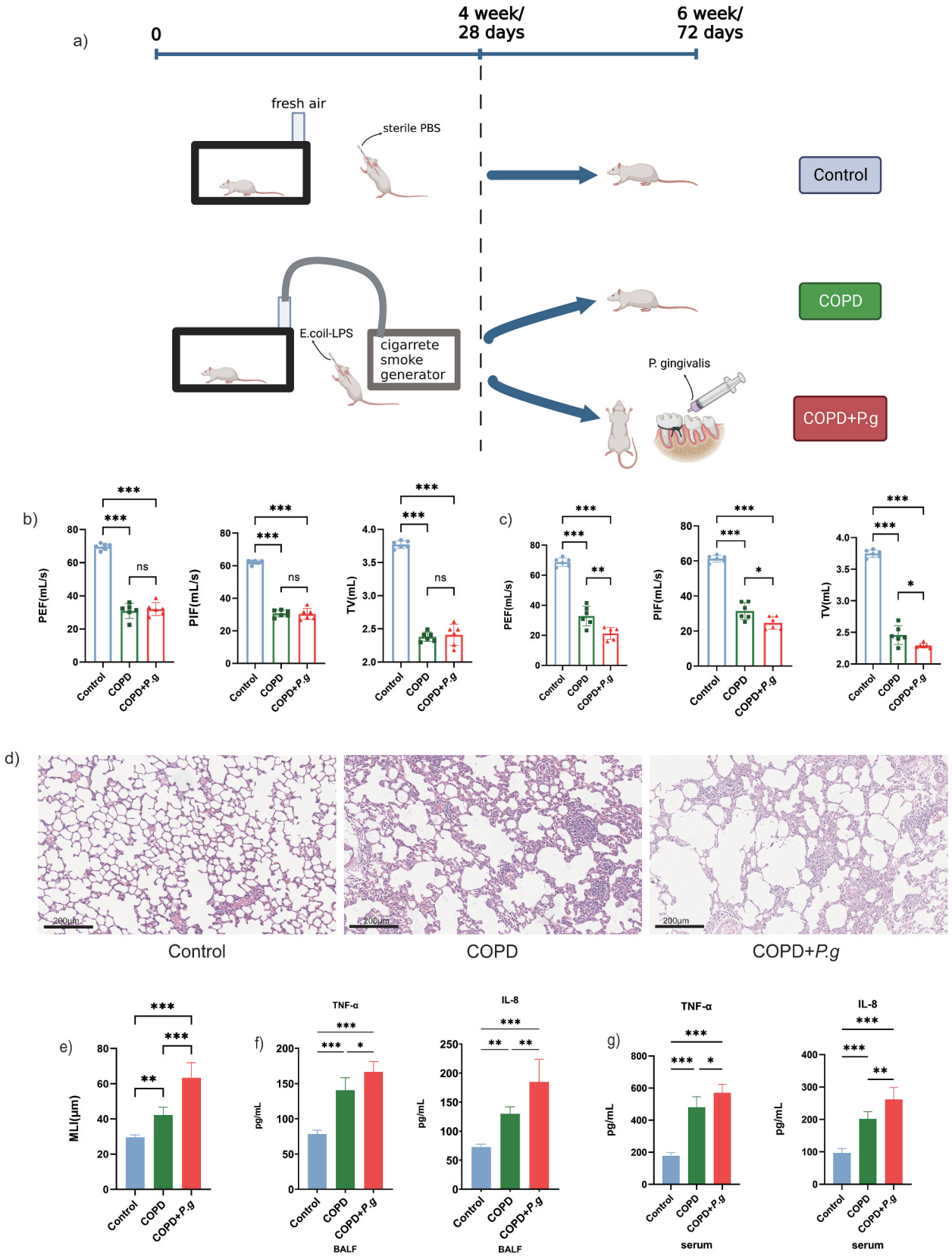


**Figure 1.** *P. gingivalis* exacerbates alveolus destruction, impairs lung function and promotes inflammation in COPD rats. (a) Flowchart of animal experiments including COPD modeling and *P. gingivalis* inoculation in COPD rats' oral cavity. (b) Lung function test at the end of week 4($n = 6$). (c) Lung function test at the end of week 6($n = 6$). (d) Representative HE images of lung tissue. Scale bar:200 μm. (e) Quantification of MLI (mean linear intercept) in the lung tissue ($n = 6$). (f-g) the content of IL-8 and TNF-α in BALF and serum ($n = 6$). ns: no significant difference; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. PEF: peak expiratory flow; PIF: peak inspiratory flow; TV: tidal volume.

deparaffinized and rehydrated lung tissue slices (3 μm) of rat were stained with hematoxylin and eosin (H&E), we calculated the mean linear intercept (MLI) to reflect the mean alveolar diameter [34]. Briefly, we captured five histological fields (upper, middle, lower, left, and right) per rat at 200× magnification from lung section, Using Image J software to measure the total length of the diagonal line (L) and count the number of alveolar septa (Ns) across the diagonal line of the visual field, and MLI=L/Ns.

### Immunofluorescence staining

Immunofluorescence staining was performed to detect the colonization of *P. gingivalis*/gingipain in lung tissue. First, antigen retrieval was performed by using citrate buffer for 2 minutes at 100°C on deparaffinized and rehydrated lung sections (3 μm) of rat. Then incubated by 3% hydrogen peroxide to remove endogenous peroxidase and blocked with 5% goat serum for 15 minutes. Finally, lung sections were incubated with monoclonal mouse anti-rgpA/kgp/hagA antibodies (61BG1.3, DSHB, USA) at a 1:100 dilution overnight at 4°C, followed by incubation with goat anti-mouse IgG Dylight 800 secondary antibody (1:100, Abbkine, USA) and DAPI in the dark. Images were captured by a fluorescence microscope (Nikon).

### 16S rRNA sequencing and data analysis

To clarify the effects of *P. gingivalis* on lung microbiota, we collected bronchoalveolar lavage fluid (BALF) by lavaging the left lung three times with 3 mL of cold sterile PBS and remove the cellular debris from lavage fluid. Four BALF samples were randomly picked out from each COPD and COPD+P.g group to investigate the lung microbiota difference between the two groups. The 16S rRNA sequencing of BALF was performed by BGI Genomics Co. Ltd (Shanghai, China). After DNA extraction and amplification of the V3-V4 region of bacterial 16S rRNA in BALF, the validated libraries qualified by the Agilent 2100 bioanalyzer were sequenced using the Illumina HiSeq2000 platform. The tags were clustered into OTUs (Operational Taxonomic Units) and compared with the RDP database to annotate the taxa. Alpha diversity and beta diversity analysis were conducted to compare the diversity difference of bacteria between COPD and COPD +P.g group. Linear discriminant analysis effect size (LEfSe) method was used for identifying differentially abundant taxa between two groups with LDA threshold >4.

### Label-free LC-MS/MS proteomics analysis

To further explore the potential molecular mechanism in the effects of *P. gingivalis* on COPD rats, we collected serum samples from all rats and randomly picked out three serum samples from each COPD and COPD+P.g group for Label-free proteomics analysis, that was performed by PTM Bio (Hangzhou, Zhejiang, China). The raw mass spectral data was processed by MaxQuant software (v.1.5.2.8) based on the standard protocol [35]. Tandem mass spectra were searched against the protein sequence of rat in the Uniport database (54,764 entries), and the LFQ (label-free quantification) intensity of each protein was calculated based on the database search results [36]. These quantitative values were then log2-transformed, and statistical tests (three-sample, two-tail t-test) were applied to test for differential expression between COPD and COPD+P.g group. A fold change cutoff of >1.5 or <1/1.5 and a p-value cutoff of <0.05 were used to identify significant differentially expressed proteins and visualized via Volcano plot. KEGG pathway enrichment analysis was performed using the ClusterProfiler package in RStudio to identify enriched biological pathways associated with upregulated differential expressed proteins. Principal Component Analysis (PCA) was also conducted using the factoextra and factoMineR packages in RStudio to visualize the differences between COPD and COPD+P.g groups.

### Immunohistochemistry staining

We employed Immunohistochemistry staining to evaluate the expression of Hsp90α and MLKL in the lung tissue of each group. The deparaffinized and rehydrated lung sections (3 μm) of rat were prepared. After antigen retrieval and blocking, the lung sections were incubated with anti-MLKL dilution (1:1,000, Proteintech Group, China) or anti-Hsp90α dilution (1:800, Proteintech Group, China) overnight at 4°C, followed by incubation with enzyme-labeled sheep anti-mouse/rabbit IgG-polymer (Gene Tech, China). Finally, DAB solution was added to visualize the antibody complexes and sections were counterstained with hematoxylin dye. IHC images were obtained using an optical microscope (Nikon). The mean optical density of the positive staining area was evaluated by ImageJ software.

### ELISA

BALF and serum samples were collected from each group as described above. According to the manufacturer's protocols, using ELISA kit to detect the content of IL-8 (ABIN2535650, 4A Biotech, Germany), TNF-α (SEA133Ra, Cloud-Clone, China) in BALF and serum of rats. We also detect the content of Hsp90α (SED523Mi, Cloud-Clone, China) and MLKL (SER645Ra, Cloud-Clone, China) in serum of rats.

### Statistical analysis

Statistical analysis of 16S rRNA sequencing and proteomics has been described above. The other results were presented as mean ± standard deviation (*SD*) and analyzed by statistical software GraphPad prism 9.0. The normality of the data was assessed using the S–W test. In case of normal distribution, One-way ANOVA analysis was employed to compare the differences among groups followed by the Tukey multiple comparison test. *p*-Values <0.05 were considered significant.

## Results

### *P. gingivalis* exacerbates alveolar destruction, impairs lung function, and promotes inflammation in COPD rats

To investigate the effect of *P. gingivalis* on COPD progression, we constructed a COPD rat model by smoke exposure combined with endotracheal instillation of *E. coli*-LPS (Figure 1a), and measured lung function at the end of week 4 to verify the PEF, PIF and TV were significantly lower in the COPD+P.g and COPD groups than that in the Control group ($p<0.001$) (Figure 1b). After *P. gingivalis* was inoculated into the oral cavity of the COPD rats for 2 weeks (Figure 1a), we observed an increase in the severity of COPD. Compared with these in the COPD group, the PEF, PIF and TV in COPD+P.g group were significantly lower ($p<0.01$, $p<0.05$, $p<0.05$) (Figure 1c). For the histopathological changes in the lungs, HE staining images revealed that the Control group rats had relatively intact alveolar structures with orderly cells. However, the alveoli of the COPD and COPD+P.g group were enlarged, and some of the alveolar walls had ruptured, leading to the formation of pulmonary bullae with inflammatory cell infiltration in the alveolar septa (Figure 1d). Quantitative analysis of the alveolar mean linear interval (MLI) showed that the mean alveolar diameter in the COPD+P.g group was significantly greater than that in the COPD and Control group ($p<0.001$) (Figure 1e). Moreover, increased levels of TNF-α and IL-8 were found in BALF and serum of the COPD+P.g group versus COPD group (Figure 1f,g) ($p < 0.05$, $p < 0.01$). These data indicated that *P. gingivalis* exacerbates alveolar destruction, impairs lung function and promotes inflammation in COPD rats.

### *P. gingivalis* colonizes the lungs and alters the lung microbiota of COPD rats

To elucidate the impact of *P. gingivalis* on the lung microbiota in COPD rats, we first observed by immunofluorescence staining that *P. gingivalis*/gingipains could colonize the lungs, not only in the bronchus but also the alveolus (Figure 2a). Then we conducted 16S rRNA sequencing of the BALF from the COPD and COPD+P.g groups. A total of 21.5% of OUTs were distinct from those in the COPD+P.g group according to the Venn diagram of bacteria (Figure 2b). Principal coordinates analysis (PCoA) based on weighted UniFrac revealed that the differences of the beta diversity in lung microbiota composition between the COPD and COPD+P.g groups were significant (ANOSIM, $R = 0.4479$, $p = 0.027$) (Figure 2c). There was also a noticeable increase in the Shannon index of the alpha diversity of the lung microbiota in the COPD+P.g group compared with that in the COPD group ($p = 0.029$) (Figure 2d). At the phylum level, the abundances of *Veillonella* and *Streptococcus* were greater in the COPD+P.g group than in the COPD group (Figure 2f). Consistent with immunofluorescence staining results, *P. gingivalis* abundance increased in COPD+P.g group at the species level (Figure 2e). Notably, *Porphyromonas* was the remarkable differential microbiota constituent between the COPD and COPD+P.g groups, according to the LEfSe results (Figure 2g). These data indicated that *P. gingivalis* can colonize the lungs and change the lung microbiota of COPD+P.g rats.

### Hsp90α and MLKL are up-regulated during *P. gingivalis*-induced COPD aggravation

To explore the underlying molecular mechanism involved in *P. gingivalis*-induced COPD aggravation. we identified differentially expressed proteins in the serum of the COPD group and COPD+P.g group by proteomics analysis with a threshold of FC > 1.5 or <0.667 and a P. value <0.05. There were 79 up-regulated proteins and 77 down-regulated proteins in the COPD+P.g group (Figure 3b). PCA revealed a distinct difference between the COPD and COPD+P.g group, which can be divided into two groups (Figure 3a). However, KEGG enrichment analysis of the up-regulated proteins suggested that necroptosis pathway participate in *P. gingivalis*-induced COPD aggravation (Figure 3c). Furthermore, Hsp90α and MLKL were involved in the necroptosis pathway (Figure 3d). Consistent with the proteomics results, the serum levels of Hsp90α and MLKL in COPD+P.g group were significantly greater than those in the COPD group ($p < 0.001$) (Figure 4a,b). Spearman's correlation analysis revealed that the serum levels of Hsp90α and MLKL were correlated positively with the MLI ($p < 0.01$) (Figure 4c). Accordingly, the expression of Hsp90α and MLKL in lung tissue was measured by immunohistochemistry staining (Figure 4d,f), and the average optical density of positive cells of Hsp90α and MLKL in the lung tissue of the COPD +P.g group was significantly greater ($p < 0.01$, $p < 0.05$) (Figure 4e,g). However, the changes in Hsp90α in both

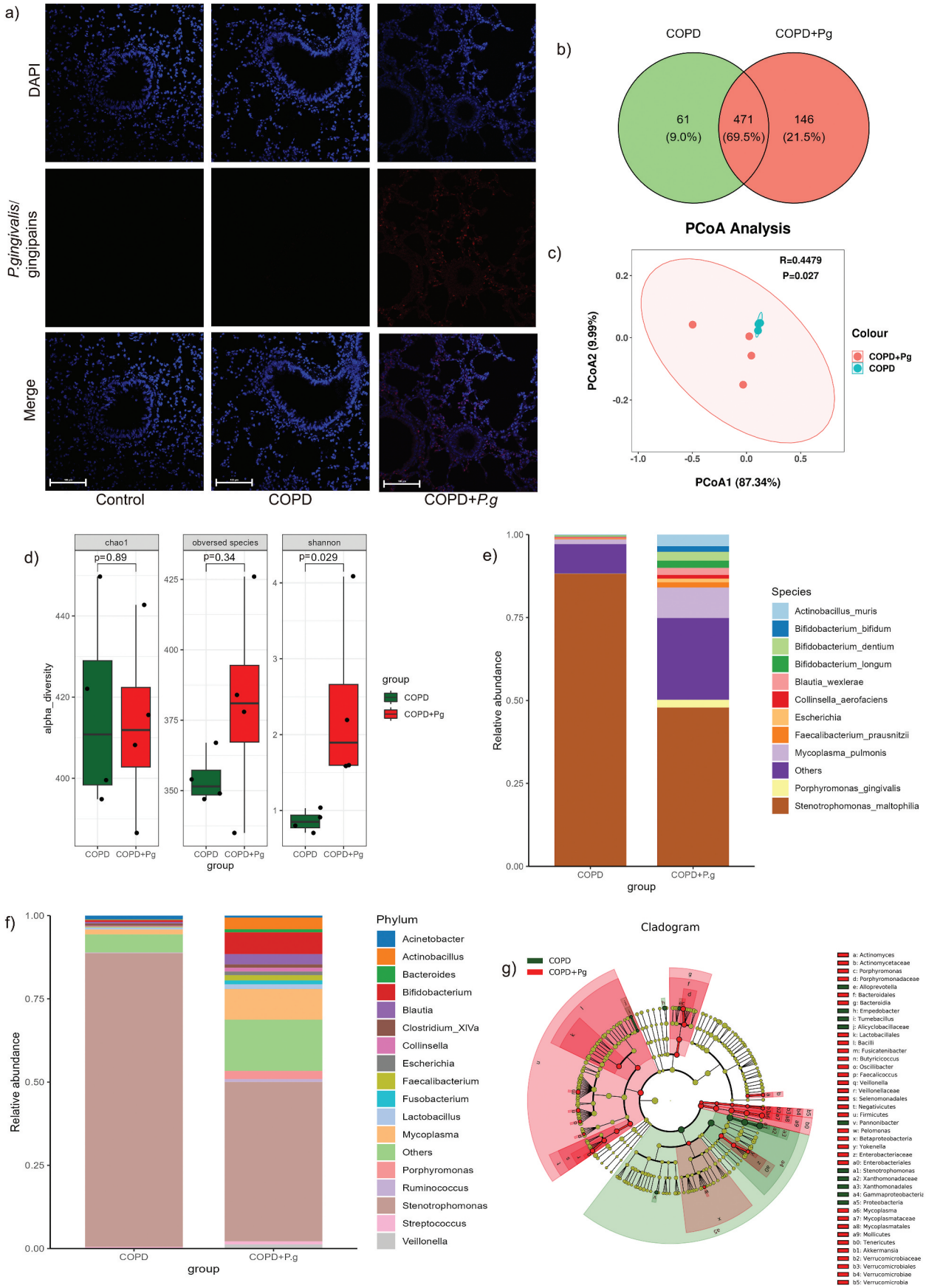


**Figure 2.** *P. gingivalis* colonizes the lungs and alters lung microbiota of COPD rats. (a) Representative immunofluorescence images of *P. gingivalis*/gingipains in lung tissues. Scale bar:100 μm. (b) Venn diagram of shared and unique OTUs between COPD and COPD+P.G group. (c) PCoA plot of beta diversity of lung microbiota based on weighted unifrac. (ANOSIM, $r = 0.4479$ $p = 0.027$). (d)Boxplot of alpha diversity (Chao1, observed species, and Shannon index) of lung microbiota in BALF between COPD and COPD+P.G group ($n = 4$). (e-f) relative abundance of lung microbiota at the species (e) and phylum (f) levels between COPD and COPD+P.G group. (g) Cladograms of LEfSe showing differences in the bacterial taxa between COPD and COPD+P.G group (LDA score > 4).

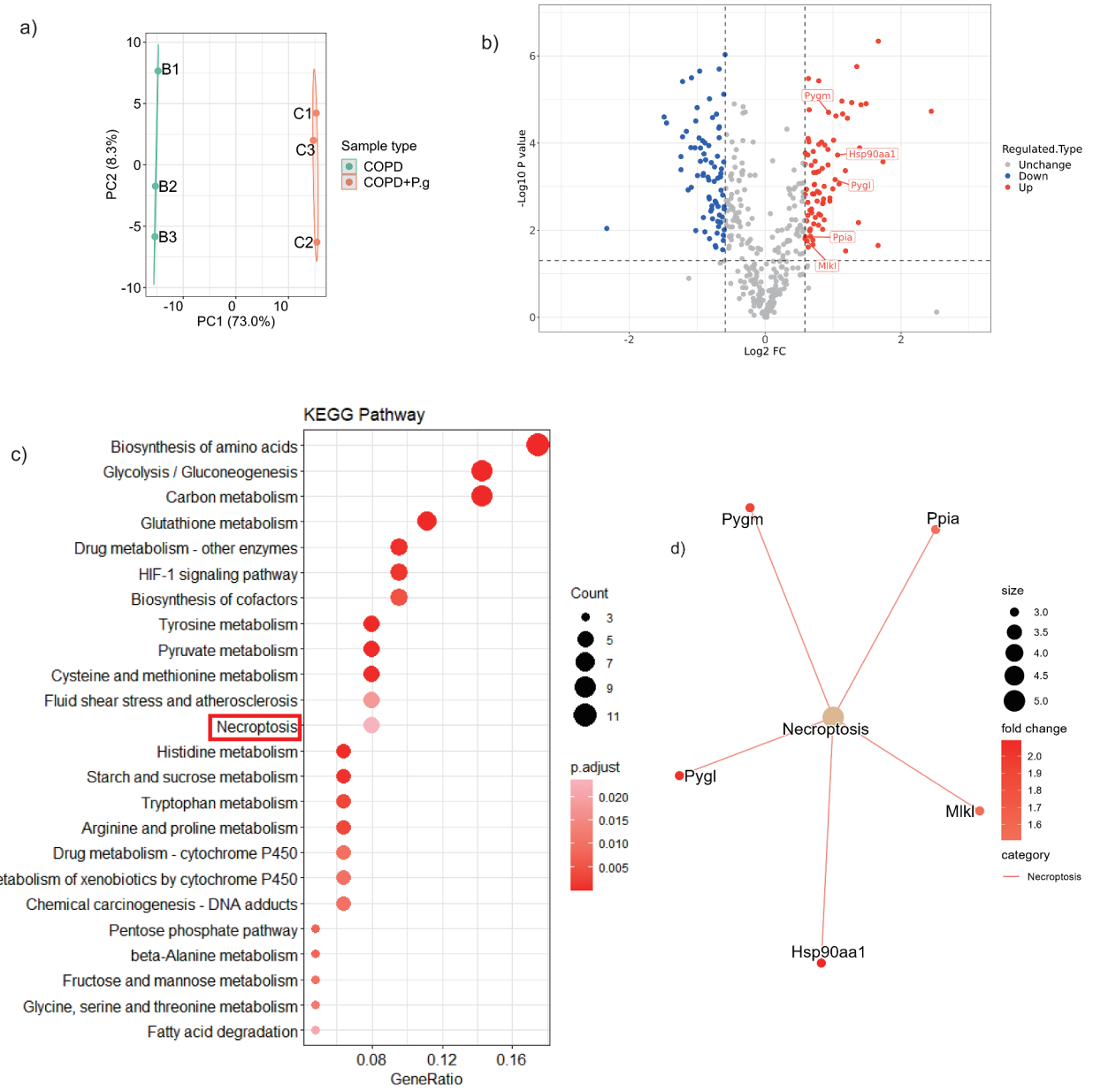


**Figure 3.** Hsp90α and MLKL are up-regulated in *P. gingivalis*-induced COPD aggravation revealed by proteomics. (a) PCA results between COPD and COPD+P.G group($n = 3$). (b) Volcano plot for the differentially expressed proteins (gene names). The X-axis shows the Log2 FC, and the Y-axis shows the -Log10 P. value. The red and blue circles represent upregulated proteins and down-regulated proteins in COPD+P.G group, respectively. (c) The KEGG pathways analysis of all the upregulated differentially expressed protein in COPD+P.G group. (d) Visualization of the relationship between necroptosis pathways and up-regulated proteins (gene names).

the serum and lung tissue were not significantly different between the COPD group and the Control group (Figure 4a,e). On the other hand, heat-inactive *P. gingivalis* promotes the expression of Hsp90α/MLKL in A549 cell under COPD condition (see in supplementary materials). These data revealed that Hsp90α and MLKL are up-regulated during *P. gingivalis*-induced COPD aggravation.

## Discussion

*P. gingivalis*, as one of major pathogens implicated in the development of periodontitis, can adhere to and colonize the periodontal tissues, expressing various of virulence factors to cause inflammatory response and destroy the periodontal tissues [10,37]. What was more, *P. gingivalis* was found to be closely related to COPD. They can be detected in BALF and lower respiratory tract secretions of COPD [8,12]. The level of *P. gingivalis* IgG titers in serum of COPD patients is positively correlated with the frequency of COPD exacerbation [11]. Importantly, the detection rate of *P. gingivalis* in subgingival plaque was negatively correlated with lung function [38]. Therefore, it is particularly important to investigate the impact of *P. gingivalis* on the progress of COPD.

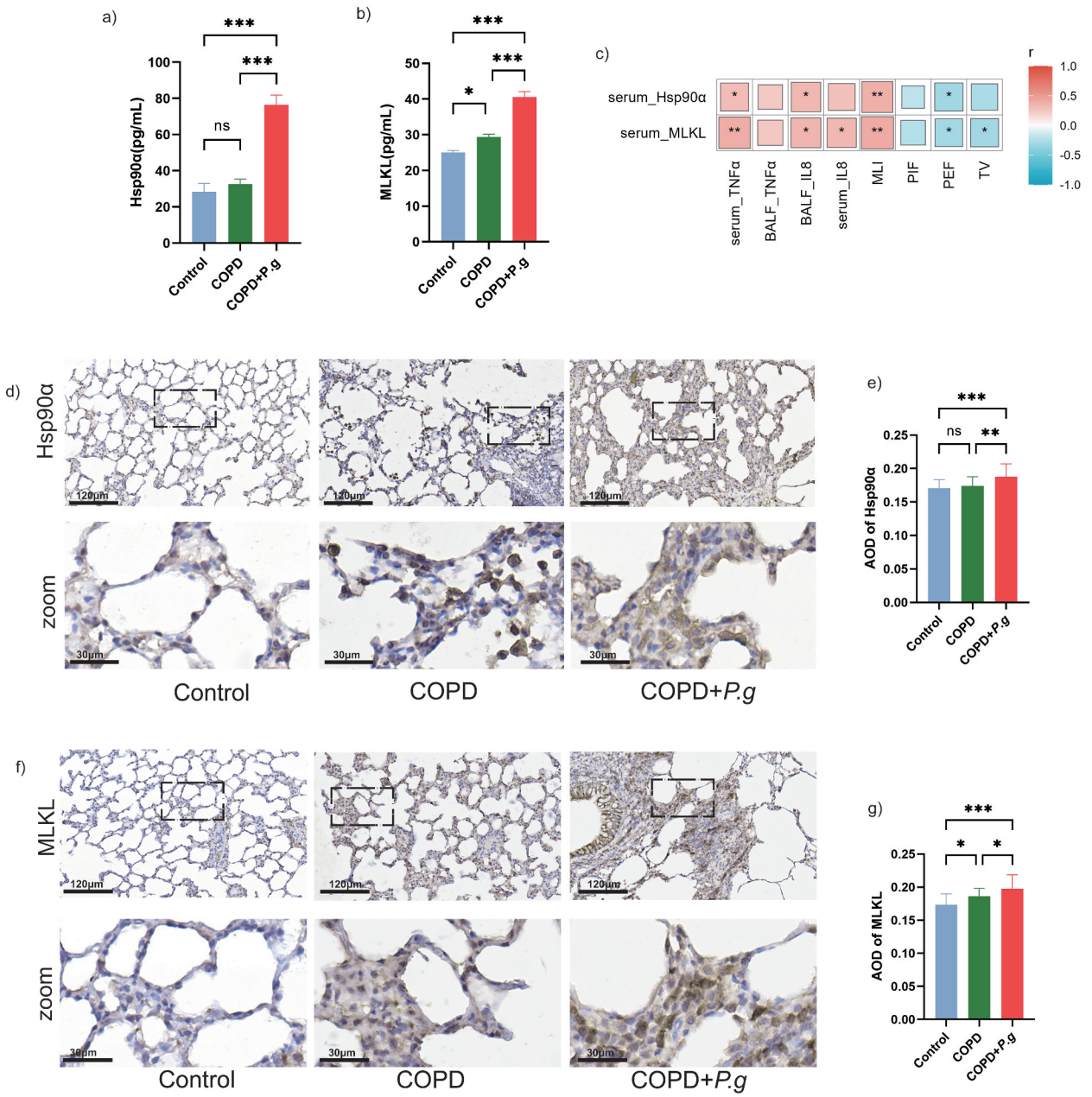


**Figure 4.** Hsp90α and MLKL are up-regulated in lung tissue and serum of COPD+P.G rats. (a,b) The content of Hsp90α and MLKL in serum ($n = 6$). (c) Spearman's correlation analysis of Hsp90α, MLKL, IL-8, TNF-α, PIF, PEF and TV. Color from blue to red showing the negative to positive correlations. (d,f) Representative immunohistochemical staining of Hsp90α and MLKL in lung tissues. Scale bar:120 μm,30 μm. (e,g) Quantification of average absorbance of Hsp90α and MLKL positive cells in the lung tissue ($n = 6$). ns: no significant difference; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Animal experiments found that periodontitis promoted the inflammation of COPD, but it is mainly considered the cause of periodontitis-related inflammatory factors [39]. Another study established an animal model of COPD by elastase induced emphysema, and found that *Fusobacterium nucleatum* aggravates COPD inflammatory response after it is injected into the trachea [40]. Considering the pathogenesis of COPD is complex, it is associated primarily with smoking, early-life respiratory infections and genetics [41], moreover the location of *P. gingivalis* is mainly in the periodontal pocket. We constructed the rat model of COPD by cigarette smoke exposure combined with endotracheal instillation of *E. coli*-LPS, and made *P. gingivalis* inoculated in COPD rats' oral cavity by orthodontic wire ligation and local application of *P. gingivalis* suspension. After *P. gingivalis* inoculating in oral cavity of COPD rats for 2 weeks, we found the lung function of COPD+P.g group was significantly impaired comparing to COPD group. Accordingly, we measured the MLI of lung tissue to reflect the alveolar size, and the results showed that *P. gingivalis* worsened alveolar damage in COPD+P.g group. Importantly, the levels of TNF-α and IL-8 in BALF and serum of COPD+P.g group were significantly higher than those of COPD and Control group, which means *P. gingivalis* aggravated local and systemic inflammation of COPD rats. On

the other hand, the inflammatory endotype of COPD will be observed in our subsequent studies.

Although inflammation is the main mechanism of COPD progression, alterations in the lung microbiome are correlated with decreased lung function and enhanced airway inflammation of COPD [17,42]. The 'vicious circle hypothesis' explains how the lung microbiome play a role in COPD pathogenesis [43]. Inhalation of toxic gases or acute changes in the lung microbiota disrupt innate defenses, allow dysbiosis of lung microbiota from diversity and abundance, and lead to adverse inflammatory response. The continuous inflammatory response further causes serious imbalance of microbial composition and quantity, and finally forms a vicious cycle of continuous destruction of lung tissue. In our study, we observed *P. gingivalis* colonized and were enriched in lung of COPD rats after *P. gingivalis* inoculating in oral cavity for 2 weeks, which indicated *P. gingivalis* could emigrate and flourish in lungs of COPD rats. From 16S rRNA gene sequencing of lung microbiome in the BALF, we found a significant discriminate of beta diversity between COPD and COPD+P.g according to PCoA, and an increased Shannon index of alpha diversity in COPD+P.g. Although a decreased Shannon index was observed with increasing COPD severity in some clinical researches [44,45]. A multicenter cohort study found great differences in microbial alpha and beta diversity variability of COPD exacerbation across study centers compared to baseline [46], and the lung microbiome diversity was increased in COPD of eosinophilic inflammation [13]. In addition, we observed that the relative abundance of *Veillonella* and *Streptococcus* was increased in the COPD+P.g group at phylum level. Multi-omics study found that lower lung function and more severe symptoms were positively associated with *Streptococcus* and *Veillonella* in moderate state of COPD [47]. More importantly, *Porphyromonas* was the remarkable differential microbiota between the COPD and COPD+P.g groups according to LEfSe, which means *P. gingivalis* is the biomarker in altered lung microbiome of COPD+P.g rats. Therefore, we conduct that *P. gingivalis* could alter lung microbiota and aggravate disease severity of COPD rats.

To further explore the potential molecular mechanism of how *P. gingivalis* aggravates COPD progression, serum proteomic analysis of rats was performed and the results suggested that Hsp90α/MLKL-involved necroptosis pathway played an important role in. As the one of isoforms of Hsp90 cytosolic molecular chaperone, Hsp90α is associated with COPD progression [30,48]. It has been found that Hsp90α is involved in necroptosis by regulating the phosphorylation, oligomerization and membrane transport of MLKL, the inhibition of Hsp90 decreases the expression of MLKL [24,25]. In our study, Hsp90α and MLKL were up-regulated in COPD+P.g group compared to COPD and Control group, which was positive correlated with MLI. At the same time, we also noticed that the changes of Hsp90α were not significant in COPD group versus Control group. Study has found that the persistent airway goblet cell metaplasia (a common pathological manifestation of respiratory diseases) is related to Hsp90 activity, the inhibition of Hsp90 can reverse goblet cell metaplasia in vivo and in vitro [49]. Therefore, Hsp90 may be a target for COPD therapy. An in vitro study has reported that *P. gingivalis*-LPS can promote the expression of Hsp90α in human gingival fibroblasts to cause inflammation [50]. Our previous research has revealed that *P. gingivalis*-LPS can induce necroptosis of oral epithelial cells and release a large number of DAMPs to further activate macrophages [51]. So, we next conduct that *P. gingivalis*-induced COPD aggravation is related to the up-regulated Hsp90α and MLKL. On the other hand, our in-vitro experiments in supplementary materials suggested that *P. gingivalis* had a direct and complex pathogenic mechanism for COPD, which was similar in peridontits [52]. However, whether it is the indirect effect of *P. gingivalis* on COPD progression needs further verification.

In conclusion, *P. gingivalis* could colonize and be enriched in the lungs of COPD rats, alter lung microbiota and lead to aggravation of COPD. Proteomic analysis of serum suggested that Hsp90α/MLKL-meditated necroptosis was up-regulated in *P. gingivalis*-induced COPD aggravation. The detections of Hsp90α and MLKL in serum and lung tissue verified that Hsp90α/MLKL was up-regulated in COPD+P.g group, which might provide a new idea for the molecular mechanism of the link between *P. gingivalis* and COPD progression.

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## Disclosure statement

No potential conflict of interest was reported by the author(s).

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## References


[1] Gomes-Filho IS, Cruz SSD, Trindade SC, et al. Periodontitis and respiratory diseases: a systematic review with meta-analysis. Oral Dis. 2020;26 (2):439–446. doi: 10.1111/odi.13228

[2] Zeng X-T, Tu M-L, Liu D-Y, et al. Periodontal disease and risk of chronic obstructive pulmonary disease: a meta-analysis of observational studies. PLoS One. 2012;7(10):e46508. doi: 10.1371/journal.pone.0046508

[3] 2020 global strategy for prevention, diagnosis and management of COPD. Available from: https://goldcopd.org/gold-reports/

[4] Viniol C, Vogelmeier CF. Exacerbations of COPD. Eur Respir Rev. 2018;27(147):27. doi: 10.1183/16000617.0103-2017

[5] Ritchie AI, Wedzicha JA. Definition, causes, pathogenesis, and consequences of chronic obstructive pulmonary disease exacerbations. Clin Chest Med. 2020;41(3):421–438. doi: 10.1016/j.ccm.2020.06.007

[6] Kelly N, Winning L, Irwin C, et al. Periodontal status and chronic obstructive pulmonary disease (COPD) exacerbations: a systematic review. BMC Oral Health. 2021;21(1):21. doi: 10.1186/s12903-021-01757-z

[7] Pragman AA, Kim HB, Reilly CS, et al. The lung microbiome in moderate and severe chronic obstructive pulmonary disease. PLoS One. 2012;7(10):e47305. doi: 10.1371/journal.pone.0047305

[8] Erb-Downward JR, Thompson DL, Han MK, et al. Analysis of the lung microbiome in the "healthy" Smoker and in COPD. PLoS One. 2011;6(2):6. doi: 10.1371/journal.pone.0016384

[9] Melo-Dias S, Valente C, Andrade L, et al. Saliva as a non-invasive specimen for COPD assessment. Respir Res. 2022;23(1):23. doi: 10.1186/s12931-022-01935-9

[10] Bui FQ, Almeida-da-Silva CLC, Huynh B, et al. Association between periodontal pathogens and systemic disease. Biomed J. 2019;42(1):27–35. doi: 10.1016/j.bj.2018.12.001

[11] Takahashi T, Muro S, Tanabe N, et al. Relationship between periodontitis-related antibody and frequent exacerbations in chronic obstructive pulmonary disease. PLoS One. 2012;7(7):e40570. doi: 10.1371/journal.pone.0040570

[12] Tan L, Wang H, Li C, et al. 16S rDNA-based metagenomic analysis of dental plaque and lung bacteria in patients with severe acute exacerbations of chronic obstructive pulmonary disease. J Periodontal Res. 2014;49(6):760–769. doi: 10.1111/jre.12159

[13] Whiteside SA, McGinniss JE, Collman RG. The lung microbiome: progress and promise. J Clin Invest. 2021;131(15):131. doi: 10.1172/JCI150473

[14] Budden KF, Shukla SD, Rehman SF, et al. Functional effects of the microbiota in chronic respiratory disease. Lancet Respir Med. 2019;7(10):907–920. doi: 10.1016/S2213-2600(18)30510-1

[15] Wang L, Hao K, Yang T, et al. Role of the lung microbiome in the pathogenesis of chronic obstructive pulmonary disease. Chinese Med J. 2017;130 (17):2107–2111. doi: 10.4103/0366-6999.211452

[16] Yan Z, Chen B, Yang Y, et al. Multi-omics analyses of airway host–microbe interactions in chronic obstructive pulmonary disease identify potential therapeutic interventions. Nat Microbiol. 2022;7(9):1361–1375. doi: 10.1038/s41564-022-01196-8

[17] Wang Z, Locantore N, Haldar K, et al. Inflammatory endotype–associated airway microbiome in chronic obstructive pulmonary disease clinical stability and exacerbations: a multicohort longitudinal analysis. Am J Respir Crit Care Med. 2021;203(12):1488–1502. doi: 10.1164/rccm.202009-3448OC

[18] Leung JM, Tiew PY, Mac Aogain M, et al. The role of acute and chronic respiratory colonization and infections in the pathogenesis of COPD. Respirology. 2017;22(4):634–650. doi: 10.1111/resp.13032

[19] Armitage MN, Spittle DA, Turner AM. A systematic review and meta-analysis of the prevalence and impact of pulmonary bacterial colonisation in stable state chronic obstructive pulmonary disease (COPD). Biomedicines. 2022;10(1):10. doi: 10.3390/biomedicines10010081

[20] Kamio N, Hayata M, Tamura M, et al. Porphyromonas gingivalis enhances pneumococcal adhesion to human alveolar epithelial cells by increasing expression of host platelet-activating factor receptor. FEBS Lett. 2021;595(11):1604–1612. doi: 10.1002/1873-3468.14084

[21] Okabe T, Kamiya Y, Kikuchi T, et al. Porphyromonas gingivalis Components/Secretions synergistically enhance pneumonia caused by streptococcus pneumoniae in mice. Int J Mol Sci. 2021;22(23):22. doi: 10.3390/ijms222312704

[22] Li Q, Pan C, Teng D, et al. Porphyromonas gingivalis modulates Pseudomonas aeruginosa-induced apoptosis of respiratory epithelial cells through the STAT3 signaling pathway. Microbes Infect. 2014;16(1):17–27. doi: 10.1016/j.micinf.2013.10.006

[23] Barnes PJ. Inflammatory mechanisms in patients with chronic obstructive pulmonary disease. J Allergy Clin Immunol. 2016;138(1):16–27. doi: 10.1016/j.jaci.2016.05.011

[24] Jacobsen AV, Lowes KN, Tanzer MC, et al. HSP90 activity is required for MLKL oligomerisation and membrane translocation and the induction of necroptotic cell death. Cell Death Dis. 2016;7(1):e2051–e2051. doi: 10.1038/cddis.2015.386

[25] Zhao XM, Chen Z, Zhao JB, et al. Hsp90 modulates the stability of MLKL and is required for TNF-induced necroptosis. Cell Death Dis. 2016;7(2):e2089–e2089. doi: 10.1038/cddis.2015.390

[26] Samson AL, Zhang Y, Geoghegan ND, et al. MLKL trafficking and accumulation at the plasma membrane control the kinetics and threshold for necroptosis. Nat Commun. 2020;11(1):3151. doi: 10.1038/s41467-020-16887-1

[27] Tang D, Kang R, Vanden Berghe T, et al. The molecular machinery of regulated cell death. Cell Res. 2019;29(5):347–364. doi: 10.1038/s41422-019-0164-5

[28] Dondelinger Y, Declercq W, Montessuit S, et al. MLKL compromises plasma membrane integrity by binding to phosphatidylinositol phosphates. Cell Rep. 2014;7(4):971–981. doi: 10.1016/j.celrep.2014.04.026

[29] Pasparakis M, Vandenabeele P. Necroptosis and its role in inflammation. Nature. 2015;517 (7534):311–320. doi: 10.1038/nature14191

[30] Hacker S, Lambers C, Hoetzenecker K, et al. Elevated HSP27, HSP70 and HSP90 alpha in chronic obstructive pulmonary disease: markers for immune activation and tissue destruction. Clin Lab. 2009;55(1–2):31–40.

[31] Lu Z, Van Eeckhoutte HP, Liu G, et al. Necroptosis signaling promotes inflammation, airway remodeling, and emphysema in chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 2021;204 (6):667–681. doi: 10.1164/rccm.202009-3442OC

[32] Zhang D, Cao L, Wang Z, et al. Salidroside mitigates skeletal muscle atrophy in rats with cigarette smoke-induced COPD by up-regulating myogenin and down-regulating myostatin expression. Biosci Rep. 2019;39(11):39. doi: 10.1042/BSR20190440
[33] Qi Y, Shang J-Y, Ma L-J, et al. Inhibition of AMPK expression in skeletal muscle by systemic inflammation in COPD rats. Respir Res. 2014;15(1):15. doi: 10.1186/s12931-014-0156-4
[34] Yang Y, Di T, Zhang Z, et al. Dynamic evolution of emphysema and airway remodeling in two mouse models of COPD. BMC Pulm Med. 2021;21(1):134. doi: 10.1186/s12890-021-01456-z
[35] Tyanova S, Temu T, Cox J. The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. Nat Protoc. 2016;11(12):2301–2319. doi: 10.1038/nprot.2016.136
[36] Chen LJ, Tu ZY, Wang Y, et al. ATP5O Hypo-crotonylation caused by HDAC2 hyper- phosphorylation is a primary detrimental factor for down-regulated phospholipid metabolism under chronic stress. Research. 2022;2022. doi: 10.34133/2022/9834963
[37] Zheng S, Yu S, Fan X, et al. Porphyromonas gingivalis survival skills: Immune evasion. J Periodontal Res. 2021;56(6):1007–1018. doi: 10.1111/jre.12915
[38] Tan L, Tan X, Pan C, et al. Relationship among clinical periodontal, microbiologic parameters and lung function in participants with chronic obstructive pulmonary disease. J Periodontol. 2019;90(2):134–140. doi: 10.1002/JPER.17-0705
[39] Rosa EP, Murakami-Malaquias-da-Silva F, Palma-Cruz M, et al. The impact of periodontitis in the course of chronic obstructive pulmonary disease: pulmonary and systemic effects. Life Sci. 2020;261:261. doi: 10.1016/j.lfs.2020.118257
[40] Suzuki R, Kamio N, Kaneko T, et al. Fusobacterium nucleatum exacerbates chronic obstructive pulmonary disease in elastase-induced emphysematous mice. FEBS Open Bio. 2022;12(3):638–648. doi: 10.1002/2211-5463.13369
[41] Agusti A, Hogg JC, Drazen JM. Update on the pathogenesis of chronic obstructive pulmonary disease. N Engl J Med. 2019;381(13):1248–1256. doi: 10.1056/NEJMra1900475
[42] Mammen MJ, Sethi S. COPD and the microbiome. Respirology. 2016;21(4):590–599. doi: 10.1111/resp.12732
[43] Sethi S, Murphy TF. Current concepts: infection in the Pathogenesis and course of chronic obstructive pulmonary disease. N Engl J Med. 2008;359(22):2355–2365. doi: 10.1056/NEJMra0800353
[44] Mayhew D, Devos N, Lambert C, et al. Longitudinal profiling of the lung microbiome in the AERIS study demonstrates repeatability of bacterial and eosinophilic COPD exacerbations. Thorax. 2018;73(5):422–430. doi: 10.1136/thoraxjnl-2017-210408
[45] Avalos-Fernandez M, Alin T, Metayer C, et al. The respiratory microbiota alpha-diversity in chronic lung diseases: first systematic review and meta-analysis. Respir Res. 2022;23(1):23. doi: 10.1186/s12931-022-02132-4
[46] Wang Z, Singh R, Miller BE, et al. Sputum microbiome temporal variability and dysbiosis in chronic obstructive pulmonary disease exacerbations: an analysis of the COPDMAP study. Thorax. 2018;73(4):331–338. doi: 10.1136/thoraxjnl-2017-210741
[47] Madapoosi SS, Cruickshank-Quinn C, Opron K, et al. Lung microbiota and metabolites collectively associate with clinical outcomes in milder stage chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 2022;206(4):427–439. doi: 10.1164/rccm.202110-2241OC
[48] Zuehlke AD, Beebe K, Neckers L, et al. Regulation and function of the human HSP90AA1 gene. Gene. 2015;570(1):8–16. doi: 10.1016/j.gene.2015.06.018
[49] Pezzulo AA, Tudas RA, Stewart CG, et al. HSP90 inhibitor geldanamycin reverts IL-13– and IL-17–induced airway goblet cell metaplasia. J Clin Investig. 2019;129(2):744–758. doi: 10.1172/JCI123524
[50] Zhang H, Huang J, Fan XS, et al. HSP90AA1 promotes the inflammation in human gingival fibroblasts induced by Porphyromonas gingivalis lipopolysaccharide via regulating of autophagy. BMC Oral Health. 2022;22(1):22. doi: 10.1186/s12903-022-02304-0
[51] Geng F, Liu J, Yin C, et al. Porphyromonas gingivalis lipopolysaccharide induced RIPK3/MLKL-mediated necroptosis of oral epithelial cells and the further regulation in macrophage activation. J Oral Microbiol. 2022;14(1):14. doi: 10.1080/20002297.2022.2041790
[52] Chopra A, Bhat SG, Sivaraman K. Porphyromonas gingivalis adopts intricate and unique molecular mechanisms to survive and persist within the host: a critical update. J Oral Microbiol. 2020;12(1):12. doi: 10.1080/20002297.2020.1801090