Fusobacterium Nucleatum Promotes Pseudomonas Aeruginosa Invasion to Modulate the Inflammatory Cytotoxicity of Pulmonary Epithelial Cells

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Research

Keywords: Pseudomonas aeruginosa, Fusobacterium nucleatum, chronic obstructive pulmonary disease, pulmonary epithelial cells, inflammation, cytotoxicity

Posted Date: November 11th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-102369/v1>

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Abstract

Background

Chronic obstructive pulmonary disease (COPD) is one of the most common and costly respiratory disease and is a leading cause of mortality globally. Inflammatory damage induced by bacterial infections is an important contributor to the etiology of COPD. *Fusobacterium nucleatum*, a recognized periodontal pathogen, frequently coexists with *Pseudomonas aeruginosa* in the airway of COPD patients, its amount is negatively correlated with lung function of COPD patients coinfecting with *P. aeruginosa*, but the mechanism of this is still unclear. This study intends to reveal the effect of *F. nucleatum* on *P. aeruginosa*-induced inflammatory cytotoxicity of pulmonary epithelial cells to illustrate the underlying mechanism of the above clinical phenomenon.

Methods

A549 cells were infected with *F. nucleatum* and *P. aeruginosa* simultaneously or sequentially. Bacterial aggregation assay and scanning electron microscopy (SEM) were used to evaluate the interaction between *F. nucleatum* and *P. aeruginosa*. Antibiotic protection assay was used to detect bacterial adhesion and invasion. The cellular proliferation and cytotoxic damage were examined through CCK-8 assay, lactate dehydrogenase (LDH) leakage assay and calcein acetoxymethyl ester/propidium iodide (Calcein-AM/PI) staining. Enzyme-linked immunosorbent assay (ELISA) was performed to observe IL-1 β , IL-6 and TNF- α expressions.

Results

P. aeruginosa mainly induces IL-1 β and IL-6 associated inflammatory cytotoxicity of pulmonary epithelial cells, *F. nucleatum* mainly initiates high inflammatory proliferation of pulmonary epithelial cells. When pulmonary epithelial cells are simultaneously infected with *P. aeruginosa* and *F. nucleatum*, *F. nucleatum* coaggregates with *P. aeruginosa* to synergistically invade into pulmonary epithelial cells and transiently resists *P. aeruginosa*-induced cytotoxic damage to amplify IL-6 and TNF- α associated inflammation in pulmonary epithelial cells. Furthermore, *F. nucleatum* maintains or even aggravates *P. aeruginosa*-induced inflammatory cytotoxicity of pulmonary epithelial cells, no matter which were pretreated or subsequently infected with *F. nucleatum*.

Conclusions

F. nucleatum modulates *P. aeruginosa*-induced inflammatory cytotoxicity of pulmonary epithelial cells, which may contribute to persistent exacerbation of COPD patients accompanied with *P. aeruginosa* and *F. nucleatum* coinfection.

Background

Chronic obstructive pulmonary disease (COPD) is the third leading cause of mortality worldwide, characterized by persistent airflow limitation and respiratory symptoms including dyspnea, cough and/or sputum production[1]. 54.7% of patients with COPD exacerbations admitted to the hospital are associated with respiratory bacterial infection, [2]. *Pseudomonas aeruginosa*, a ubiquitous opportunistic pathogen, is one of the most prevalent bacteria responsible for respiratory infection in COPD patients[3]. *P. aeruginosa* is associated with prolonged hospitalization, increased exacerbation rate and poor long-term prognosis in COPD patients[4-6].

In 1999, Dr. Scannapieco first proposed that oral bacteria are closely related to respiratory infections[7]. *Fusobacterium nucleatum*, an oral commensal and periodontal pathogen, is ubiquitous in the oral cavity of healthy and diseased individuals. Recent studies demonstrate that *F. nucleatum* colonizes in the respiratory tract, not only leads to respiratory infection in patients with chronic diseases such as tumors and diabetes[8-10], but also causes endobronchial lesion in healthy children[11]. Meanwhile, our previous study finds that *F. nucleatum* and *P. aeruginosa* frequently coexisted in the respiratory tract of patients with COPD exacerbation, and as the number of *F. nucleatum* in the respiratory tract microbiota increases, their lung function declines[12]. These results suggest that *F. nucleatum* and *P. aeruginosa* may play a synergistic role in respiratory infection of COPD patients, but the mechanism underlying this is still unclear.

The ability to adhere and invade host cells is a virulence property of certain bacteria. Although *P. aeruginosa* is usually considered an extracellular pathogen, some studies have demonstrated that *P. aeruginosa* can invade a variety of epithelial cells via an endocytic process dependent on actin microfilaments[13, 14]. The invasive *P. aeruginosa* induces membrane blebs in epithelial cells, which are utilized as a niche for bacterial intracellular replication and motility[15]. In contrast, *F. nucleatum* adheres to and invades epithelial cells via a zipping mechanism relying on its outer membrane proteins to bind to the cell-surface receptors[16]. At the same time, *F. nucleatum* can coaggregate with almost all of the oral bacteria dependent on its outer membrane proteins[17-19]. *F. nucleatum* not only enhances the adhesion and invasion of invasive bacteria such as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* to tissue cells[20], but also transports noninvasive bacteria such as *Streptococcus cristatus* and *Streptococcus sanguis* into epithelial cells[21]. These data suggest that *F. nucleatum* may enhance the adhesion and invasion of *P. aeruginosa* to pulmonary epithelial cells due to interspecies coaggregation, thereby aggravate pulmonary tissue damage.

Mucosal epithelium is not only the first physical barrier between host and bacteria, but also an active participant in natural immunity and inflammation[22]. Vos et al. found that *P. aeruginosa* infection of human bronchial epithelial cells can significantly induce the expression of IL-1 family members IL-1 β and IL-1F9[23]. *P. aeruginosa*-derived flagellin induces IL-6 and IL-8 production in bronchial epithelial cells through the phosphorylation of p38, ERK and JNK, which partially explains the underlying mechanism of *P. aeruginosa* causing acute exacerbation of COPD[24]. Hayata et al. report that *F. nucleatum* induces the productions of proinflammatory cytokines IL-6 and IL-8 by the bronchial and pharyngeal epithelial cells, which may trigger exacerbation of COPD[25]. IL-1 β is considered a biomarker of bacteria-associated

exacerbation of COPD, and TNF- α and IL-6 are the potential markers of IL-1 β -associated exacerbation of COPD[26, 27]. These data indicate that coinfection of *P. aeruginosa* and *F. nucleatum* may modulate proinflammatory cytokines IL-1 β , IL-6 and TNF- α production in pulmonary epithelial cells, and then lead to acute exacerbation of COPD.

On the basis of the above thinking and hypothesis, we first investigated the effect of bacterial coaggregation on bacterial adhesion and invasion ability, and then explore the effect of the combined infection with *P. aeruginosa* and *F. nucleatum* on cellular viability and inflammatory response of pulmonary epithelial cells through establishing bacterial simultaneous and sequential infection models, in order to elaborate the mechanism by which *F. nucleatum* cooperates with *P. aeruginosa* to exacerbate COPD.

Methods

Bacteria culture.

F. nucleatum (ATCC 25586) and *P. aeruginosa* PAO1 strain (ATCC BAA-47) were obtained from American Type Culture Collection (ATCC). *F. nucleatum* was grown in tryptic soy broth (TSB, Becton, Dickinson and Company, Sparks, MD, USA) supplemented with 5% defibrinated sheep blood, 10mg/ml hemin and 5mg/ml menadione in anaerobic atmosphere (10% H₂, 10% CO₂, 80% N₂) at 37 °C. *P. aeruginosa* was cultured in TSB under aerobic conditions at 37 °C.

Bacterial aggregation assay.

P. aeruginosa and *F. nucleatum* were standardized in sterile coaggregation buffer (150 mM NaCl, 1 mM Tris, 0.1 mM CaCl₂ and 0.1 mM MgCl₂) to give a final cell density of 1×10⁹ colony-forming units per mL (CFUs/mL). Equal numbers of single- or dual-species bacterial cells were suspended and vortexed for 30 s in a reaction tube (T=0 h). The tubes were incubated at room temperature for 1 h to allow aggregation (T=1 h). A visual rating scale of 0-4 was used to grade the reaction. "0" indicates an evenly turbid suspension with no visible aggregates, meaning no coaggregation; "1+" indicates turbid supernatant with finely dispersed coaggregates; "2+" indicates definite coaggregates that do not precipitate immediately; "3+" indicates slightly turbid supernatant with formation of large precipitating coaggregates; and "4+" indicates complete sedimentation with a clear supernatant[28, 29]. In addition, a spectrophotometric assay was employed to determine the percentage of bacterial auto- and coaggregation[19, 28]. The optical density of bacterial suspensions at 600 nm wavelength (OD₆₀₀) were measured at the time points of 0 and 1 h. Percentage aggregation was calculated using the following equation: % autoaggregation or coaggregation = (OD₆₀₀(T₀)-OD₆₀₀(T₁))/ OD₆₀₀(T₀) ×100. Percentage aggregation were classified as high (more than 40%), intermediate (30-40%) and low aggregation (less than 30%).

Cell culture.

Human pulmonary epithelial cell line A549 was obtained from ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM, HyClone Laboratories, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, HyClone Laboratories, Logan, UT, USA) in a humidified 37°C incubator with 5% CO₂.

Bacterial infection of pulmonary epithelial cells.

A549 cells were incubated with *F. nucleatum* and *P. aeruginosa* alone or together at multiplicity of infection (MOI) of 10, 50 or 100. For bacterial simultaneous infection model, A549 cells were incubated with both *F. nucleatum* and *P. aeruginosa* at the same times. For bacterial sequential infection model, A549 cells were treated with *F. nucleatum* or *P. aeruginosa* for 12 h, and then infected with the other bacteria for 12 h.

Antibiotic protection adhesion and invasion assay

Assays of bacterial adherence and invasion were performed using methods previously described[30]. Briefly, bacteria were harvested, washed, and resuspended in DMEM without antibiotics. A549 cells were infected with bacteria for 3 h, and washed with phosphate-buffered saline (PBS). For determining total adhesion and invasion levels (attachment levels), cells were lysed with sterile distilled water for 30 min, then dilutions of the lysate were plated and cultured anaerobically on TSB agar supplemented with 5% defibrinated sheep blood, 10mg/ml hemin and 5mg/ml menadione to determine CFUs for *F. nucleatum*, or cultured aerobically on TSB agar to determine CFUs for *P. aeruginosa*. For invasion assay, extracellular bacteria were killed with 200 µg/mL gentamicin (Sigma, St. Louis, MO, USA) and 300 µg/mL metronidazole (Sigma, St. Louis, MO, USA) for 1 h. Cellular lysate were diluted and cultured to determine CFUs. The efficiency of bacterial attachment or invasion is equal to CFUs divided by the number of cells.

Scanning electron microscopy (SEM)

A549 cells were infected with *P. aeruginosa* and *F. nucleatum* alone or together for 3 h, washed with PBS, and fixed with 2.5% glutaraldehyde (BioChemika, Fluka, Switzerland). A549 cells were gradually dehydrated with ethanol and smeared onto copper plates followed by gold sputtering, and images were acquired using SEM (Inspect F50, FEI Company, USA) to observe the bacterial adhesion and cell morphology.

CCK-8 cell viability assay

Cell counting kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to assess cell proliferation according to the manufacturer's manual. Briefly, A549 cells were infected with *P. aeruginosa* and *F. nucleatum* alone or together for 4, 8, 16 and 24 h. 10 µL of CCK-8 solution was added to 100 µL of medium solution and incubated for 1 h at 37°C. OD450 was measured with a microplate reader (Infinite M200, Tecan, Austria) to reflect the cell proliferation.

Cytotoxicity assessment by Lactate dehydrogenase (LDH) leakage assay

LDH activity was monitored by the Pierce™ LDH cytotoxicity assay kit (Thermo Scientific, USA) in accordance with the manufacturer's recommendations. A549 cells were exposed to *P. aeruginosa* and *F. nucleatum* alone or together for the desired time, the culture supernatant was harvested and centrifuged at 12000 g for 5 min, and 50 µL supernatant was mixed with 50 µL Reaction mixture and incubated at room temperature in the dark for 30 min. 50 µL stop solution was added to stop reaction. OD490 and OD680 were measured with a microplate reader (Infinite M200, Tecan, Austria). LDH activity = OD490-OD680.

Live/dead assay by calcein acetoxymethyl ester/propidium iodide (Calcein-AM/PI) staining

The effects of *P. aeruginosa* and *F. nucleatum* on the viability or cytotoxicity of pulmonary epithelial cells were evaluated using the Calcein-AM/PI Double Stain Kit (Molecular Probes, Eugene, OR, USA). In brief, A549 cells were exposed to *P. aeruginosa* and *F. nucleatum* alone or together for the desired time, washed with PBS. Cells were stained with 200 µL Calcein-AM/PI stain working solution at 37 °C for 15 min. Living cells (green cytoplasmic fluorescence) and dead cells (red nucleus fluorescence) were immediately observed by fluorescence microscope.

Enzyme-linked immunosorbent assay (ELISA)

A549 cells were exposed to *P. aeruginosa* and *F. nucleatum* alone or together for the desired time, the culture supernatant was harvested and centrifuged at 12000 g for 5 min. IL-1β, IL-6 and TNF-α levels in the culture supernatant were determined via ELISA kits (Invitrogen, Cambrillo, USA) according to the manufacturer's protocol. After the procedure, plates were read on the spectrometer at 450 nm wavelength. The results were converted to numeric values using standard curves.

Statistical analysis

All experiments were performed in triplicate and repeated three times. All values were presented as mean ± standard deviation (SD) and analyzed by analysis of variance followed by Dunnett's multiple comparisons test using GraphPad Prism version 7.00 (GraphPad Software, La Jolla, CA, USA). Differences were considered statistically significant at $P < 0.05$.

Results

1. The interaction between *F. nucleatum* and *P. aeruginosa*: *F. nucleatum* coaggregates with *P. aeruginosa* and adheres to pulmonary epithelial cells.

To detect the relationship between *P. aeruginosa* and *F. nucleatum*, bacterial aggregation assay was performed. The profiles of microbial aggregation of *P. aeruginosa* and *F. nucleatum* were showed in Figure 1A-B. *P. aeruginosa* displayed a poor autoaggregation with an autoaggregation score of "0" and a low percentage autoaggregation ($8.1 \pm 3.66\%$). *F. nucleatum* showed a strong autoaggregation with an autoaggregation score of "4" and a high percentage autoaggregation ($72.99 \pm 9.43\%$). Moreover, *F.*

nucleatum and *P. aeruginosa* were intermediate coaggregators with an autoaggregation score of “2” and a percentage autoaggregation of $30.52 \pm 7.28\%$.

To explore whether bacterial coaggregation affect the form of bacterial adherence to host cells, bacterial adhesion assay and SEM were performed. Figure 1C demonstrated that *P. aeruginosa* mainly attached to the cellular junction of pulmonary epithelial cells, which shrunk and became round (Figure 1C). *F. nucleatum* auto-aggregated to form a bacterial network and attached to the cellular surface of pulmonary epithelial cells, which remained stretched (Figure 1C). When *P. aeruginosa* and *F. nucleatum* coinfecting pulmonary epithelial cells, *F. nucleatum* coaggregated with *P. aeruginosa* and adhered to the cellular junction and surface of pulmonary epithelial cells, which shrunk and became round (Figure 1C).

2. The model of pulmonary epithelial cells simultaneously infected with *P. aeruginosa* and *F. nucleatum*.

2.1 Coinfection with *P. aeruginosa* and *F. nucleatum* promotes each bacterial invasion of pulmonary epithelial cells.

To explore the effect of *P. aeruginosa* and *F. nucleatum* coinfection on bacterial attachment and invasion efficiencies, pulmonary epithelial cells were infected with *P. aeruginosa* and/or *F. nucleatum* at different MOI (10, 50, 100). Figure 2A-B demonstrated that both *P. aeruginosa* and *F. nucleatum* could adhere to and invade the pulmonary epithelial cells, and as the bacterial MOI value increased, the attachment and invasion efficiencies of *P. aeruginosa* and *F. nucleatum* increased. When the pulmonary epithelial cells were simultaneously infected with *P. aeruginosa* and *F. nucleatum* at different MOI (10, 50, 100), *F. nucleatum* did not affect the attachment efficiency of *P. aeruginosa* to pulmonary epithelial cells, but *F. nucleatum* (MOI=100) significantly enhanced the invasion efficiency of *P. aeruginosa* into pulmonary epithelial cells (Figure 2C). Meanwhile, *P. aeruginosa* did not affect the attachment efficiency of *F. nucleatum* to pulmonary epithelial cells, but *P. aeruginosa* (MOI=100) significantly enhanced the invasion efficiency of *F. nucleatum* into pulmonary epithelial cells (Figure 2D).

2.2 *F. nucleatum* coinfection transiently resists *P. aeruginosa*-induced cytotoxicity of pulmonary epithelial cells.

To exploit the effect of *P. aeruginosa* and *F. nucleatum* coinfection on the biological activity of pulmonary epithelial cells, cell proliferation and cytotoxicity were examined by CCK8 assay and LDH activity assay, respectively. Compared with the control (A549), *P. aeruginosa* (MOI 10, 50, 100) significantly inhibited the proliferation of pulmonary epithelial cells (Figure 3A). When the MOI of *P. aeruginosa* were 50 and 100, *P. aeruginosa* significantly induced cytotoxicity of pulmonary epithelial cells, and the cytotoxicity increased with the extension of infection time (Figure 3A). In contrast, *F. nucleatum* had no effect on the cytotoxicity of pulmonary epithelial cells, but enhanced the proliferation of pulmonary epithelial cells, which were infected by *F. nucleatum* at MOI of 100 at 8 h (Figure 3B). However, when pulmonary epithelial cells were simultaneously infected with *P. aeruginosa* and *F. nucleatum*, cell proliferation activity was slightly lower than the control but higher than the *P. aeruginosa*-infected group after 12 h (Figure 3C). Meanwhile, the

combined infection of *P. aeruginosa* and *F. nucleatum* significantly enhanced the cytotoxic response compared to the control group, but the cytotoxicity level of the combined infection group was lower than that of the single *P. aeruginosa* group after 12 h (Figure 3C).

In order to observe the cell viability or cytotoxicity intuitively, pulmonary epithelial cells were labeled with Calcein-AM and PI dyes. Figure 2D demonstrated that pulmonary epithelial cells infected with *F. nucleatum* were mainly live, but the *P. aeruginosa* alone or combination with *F. nucleatum* groups were mainly dead cells at 24 h.

2.3 *P. aeruginosa* and *F. nucleatum* coinfection enhances IL-6 and TNF- α expression in pulmonary epithelial cells compared with single *P. aeruginosa* infection.

To detect the effect of *P. aeruginosa* and *F. nucleatum* coinfection on inflammatory response of pulmonary epithelial cells, the expressions of IL-1 β , IL-6 and TNF- α were examined by ELISA. Compared with the control group (A549), *P. aeruginosa* significantly increased the secretion of IL-1 β and IL-6 in pulmonary epithelial cells, but there was not significant difference in IL-1 β and IL-6 secretion levels between different MOI groups (Figure 4A). *P. aeruginosa* had no significant effect on TNF- α secretion of pulmonary epithelial cells (Figure 4A). Figure 4B showed that compared with the control group (A549), *F. nucleatum* (MOI 100) significantly enhanced the secretion of IL-1 β , IL-6 and TNF- α , and the secretion levels of IL-1 β , IL-6 and TNF- α in *F. nucleatum* MOI 100 group were higher than those of *F. nucleatum* MOI 10 and/or MOI 50 groups. Moreover, *P. aeruginosa* and *F. nucleatum* coinfection significantly enhanced the secretion of IL-1 β , IL-6 and TNF- α in pulmonary epithelial cells, and the secretion levels of IL-6 and TNF- α in the combined infection group were significantly higher than those of *P. aeruginosa* alone group (Figure 4C).

3. The model of pulmonary epithelial cells pretreated with *P. aeruginosa*: *F. nucleatum* subsequential infection maintains *P. aeruginosa*-induced inflammatory cytotoxicity of pulmonary epithelial cells.

To determine the role of *F. nucleatum* in the case where respiratory *P. aeruginosa* infection has already occurred, pulmonary epithelial cells were pretreated with *P. aeruginosa* followed with *F. nucleatum* infection (Figure 5A). Figure 5B-C demonstrated that no matter with *F. nucleatum* subsequential infection or not, *P. aeruginosa* significantly inhibited the proliferation and induced cytotoxicity of pulmonary epithelial cells. Although single *F. nucleatum* infection significantly induced the secretions of IL-1 β , IL-6 and TNF- α in pulmonary epithelial cells, the expression levels of IL-1 β , IL-6 and TNF- α in *P. aeruginosa* and *F. nucleatum* sequential infection group were consistent with those of the single *P. aeruginosa* infection group (Figure 5D).

4. The model of pulmonary epithelial cells pretreated with *F. nucleatum*: *F. nucleatum* pretreatment enlarged TNF- α associated cytotoxicity of pulmonary epithelial cells subsequentially infected with *P. aeruginosa*.

In order to explore whether the presence of *F. nucleatum* would affect the progress of respiratory *P. aeruginosa* infection, pulmonary epithelial cells were pretreated with *F. nucleatum* followed with *P. aeruginosa* infection (Figure 6A). Figure 6B-C demonstrated that *P. aeruginosa* subsequent infection significantly inhibited the proliferation and induced cytotoxicity of pulmonary epithelial cells, no matter which were pretreated with *F. nucleatum* or not. *P. aeruginosa* alone significantly upregulated the secretions of IL-1 β and IL-6, but it did not affect TNF- α expression (Figure 6D). However, *P. aeruginosa* subsequent infection significantly enhanced TNF- α secretion in pulmonary epithelial cells pretreated with *F. nucleatum*, compared with the single *F. nucleatum* infection group (Figure 6D).

Discussion

Bacterial autoaggregation and coaggregation were defined as the adherence of bacteria belonging to the same strain and different bacterial strains, respectively, which are essential for the development of multispecies biofilm communities. This study demonstrated that *F. nucleatum*, a Gram-negative obligate anaerobe in the oral cavity, was a strong autoaggregator, which is consistent with Karched's study [31]. *F. nucleatum* plays a crucial role in the development and maturation of dental plaque biofilm due to its strong ability to coaggregate with all of the other oral bacteria, such as *P. gingivalis* and streptococci. That may be the reason that *P. aeruginosa* coaggregates with *F. nucleatum* moderately, although *P. aeruginosa* is a poor autoaggregator.

Respiratory mucosal epithelium mediates the crosstalk between respiratory system and external environment. The adhesion and invasion of bacteria to host cells is a prerequisite for their virulence and infection. Studies have reported that both *P. aeruginosa* and *F. nucleatum* have the ability to adhere and invade into host cells [13, 14, 16]. *F. nucleatum* not only enhances the adhesion and invasion of *P. gingivalis* and *A. actinomycetemcomitans* [20], but also carries *S. cristatus* and *S. sanguis* into the oral epithelium with its coaggregation function [21]. Consistent with these results, this study found that both *P. aeruginosa* and *F. nucleatum* were able to adhere to and invade into pulmonary epithelial cells, as the MOI of bacteria increased, the number of bacteria that adhered to and invaded into pulmonary epithelial cells increased. When pulmonary epithelial cells were coinfecting with *P. aeruginosa* and *F. nucleatum*, *P. aeruginosa* and *F. nucleatum* coaggregated with each other to attach to the cell surface and increase each bacterial invasion into pulmonary epithelial cells.

Some bacteria activate anti-apoptotic pathway to replicate and survive within the cells in order to avoid host immune defense, certain bacteria directly induce cell death and aggravate tissue damage. Previous studies show that *P. aeruginosa* directly acts on tissue cells through its type III secretion system or toxic factors such as pyocyanin to induce apoptosis [32, 33]. This study demonstrated that *P. aeruginosa* attached to and destroyed the cell junction of pulmonary epithelial cells, significantly inhibited cell proliferation, and induced cytotoxic death of pulmonary epithelial cells. As distinct from *P. aeruginosa*, *F. nucleatum* promoted the proliferation of pulmonary epithelial cells without inducing cytotoxicity. However, the presence of *F. nucleatum* transiently alleviated the inhibitory effect of *P. aeruginosa* on cell proliferation and weakened *P. aeruginosa*-induced cytotoxicity of pulmonary epithelial cells, which were

simultaneously infected by *P. aeruginosa* and *F. nucleatum*. Our previous study has pointed out that periodontal pathogen *P. gingivalis* could transiently inhibit *P. aeruginosa*-induced apoptosis of pulmonary epithelial cells through the STAT3 signaling pathway [34]. These results indicate that *F. nucleatum* promotes *P. aeruginosa* invasion and transiently resists *P. aeruginosa*-induced damage of pulmonary epithelial cells, which may be a common characteristic of periodontal anaerobic pathogen to cause persistent pulmonary infection of *P. aeruginosa*.

It is well-known that the acute exacerbation of COPD is closely related to the persistent airway inflammation induced by bacterial infection. The expression of IL-1 β in patients with COPD is upregulated, and IL-1 β level is correlated with the increase of airway neutrophils and the decline of lung function[35]. Airway (sputum) IL-1 β is considered to be a biomarker for acute exacerbation of COPD associated with bacteria[26]. Demera et al. analyzed the sputum proteomic characteristics that associated with increased IL-1 β levels and bacterial exacerbations of COPD, found that the content of *P. aeruginosa* in the sputum was positively correlated with IL-1 β level, and TNF- α and IL-6 are IL-1 β signature and associated with IL-1 β -mediated COPD exacerbation[27]. This study showed that both *P. aeruginosa* and *F. nucleatum* could significantly induce IL-1 β and IL-6 secretion, *F. nucleatum* also induce TNF- α secretion in pulmonary epithelial cells.

In addition, the simultaneous infection with *P. aeruginosa* and *F. nucleatum* significantly enhance IL-6 and TNF- α expressions in pulmonary epithelial cells compared with single *P. aeruginosa* infection group, which were similar to respiratory syncytial virus that enhanced the release of inflammatory factors IL-6 and IL-8 in bronchial epithelial cells coinfecting with *P. aeruginosa*[36]. Fu et al. pointed out that sputum IL-1 β and serum IL-6 were higher in the frequent exacerbators with COPD, which activated the IL-1 β -system inflammation axis to increase the risk of frequent exacerbations of COPD patients[37]. Therefore, these results suggest that the simultaneous infection with *F. nucleatum* and *P. aeruginosa* enhances pro-inflammatory cytokines IL-6 and TNF- α expressions in pulmonary epithelial cells, which may contribute to increase the susceptibility of COPD exacerbation.

It is a widely recognized clinical phenomenon that patients with more severe COPD are more likely to have severe periodontal disease[38, 39], and a significantly increased risk of COPD occurs among severe periodontitis subjects[40]. Our previous study finds that 45.3% patients with COPD exacerbation are associated with respiratory infection with *F. nucleatum* and *P. aeruginosa*[12], two types of sequential infection model with *P. aeruginosa* and *F. nucleatum* are established to fully elaborate the role of mixed infection in COPD. The current study demonstrated that no matter with *F. nucleatum* pretreatment or subsequential infection, *P. aeruginosa* significantly induced cytotoxic damage of pulmonary epithelial cells. *F. nucleatum* pretreatment or subsequential infection kept or even amplified *P. aeruginosa*-induced cytotoxicity of pulmonary epithelial cells, which may explain why *F. nucleatum* reduced lung function of COPD patients accompanied with *P. aeruginosa* infection[12]. On the other hand, *F. nucleatum* subsequential infection maintained the secretion levels of IL-1 β , IL-6 and TNF- α in pulmonary epithelial cells compared to the single *P. aeruginosa* group, which may be due to the low cellular viability caused by

P. aeruginosa pretreatment. Nevertheless, *F. nucleatum* pretreatment initiated and enhanced TNF- α associated inflammation of pulmonary epithelial cells, which were subsequently infected by *P. aeruginosa*. These results suggest that *F. nucleatum* can maintain or even aggravate *P. aeruginosa*-induced inflammatory cytotoxicity of pulmonary epithelial cells in the sequential infection models.

Conclusions

Considerable research has focused on the interaction between single bacterium and host cells, while this study highlights the effect of bacterial mixed infection on cellular biological function through establishing bacterial simultaneous and sequential infection models. It demonstrated that *P. aeruginosa* mainly induces IL-1 β and IL-6 associated inflammatory cytotoxicity of pulmonary epithelial cells, *F. nucleatum* mainly initiates high inflammatory proliferation of pulmonary epithelial cells. When pulmonary epithelial cells are simultaneously infected with *P. aeruginosa* and *F. nucleatum*, *F. nucleatum* coaggregates with *P. aeruginosa* to synergistically invade into pulmonary epithelial cells and transiently resists *P. aeruginosa*-induced cytotoxic damage. The simultaneous infection enhances IL-6 and TNF- α associated inflammation in pulmonary epithelial cells in contrast to single *P. aeruginosa* infection and the presence of *F. nucleatum* maintains or even aggravates *P. aeruginosa*-induced inflammatory cytotoxicity of pulmonary epithelial cells in the sequential infection models, which may be the reason that *F. nucleatum* reduced lung function of COPD patients accompanied with *P. aeruginosa* infection and contribute to persistent exacerbation of COPD patients accompanied with *P. aeruginosa* and *F. nucleatum* coinfection. These results suggest that periodontal anaerobic pathogens should never be neglected in the treatment of COPD.

Abbreviations

COPD: Chronic obstructive pulmonary disease; ATCC: American Type Culture Collection; TSB: Tryptic soy broth; CFUs: Colony-forming units; DMEM: Dulbecco's modified Eagle's medium; FBS: Fetal bovine serum; MOI: Multiplicity of infection; PBS: Phosphate-buffered saline; SEM: Scanning electron microscopy; LDH: Lactate dehydrogenase; Calcein-AM: Calcein acetoxymethyl ester; PI: Propidium iodide; ELISA: Enzyme-linked immunosorbent assay; SD: Standard deviation.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

National Natural Science Foundation of China (NO. 81670997), the Plan of the talents for Liaoning Development (NO. XLYC1802129) and the Higher Education Fundamental Research Project of Liaoning Province (NO. LQNK201724).

Author contributions

All authors have made substantial contributions to conception and design of the study. QL designed and performed the experiments, analyzed the data, wrote and revised the draft of the paper. HW, LT, SZ, LL and XT participated the experiments, analyzed the data and revised the article. YP developed the idea for this study and revised the manuscript. All authors read and approved the final manuscript.

Acknowledgments

Not applicable.

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Figures

Figure 1

F. nucleatum coaggregates with *P. aeruginosa* and adheres to pulmonary epithelial cells. (A) Photographs of autoaggregation and coaggregation of *P. aeruginosa* and *F. nucleatum*. The aggregation score of each bacterial group was indicated below the photograph. (B) Percentage aggregation of *P. aeruginosa* and *F. nucleatum*. (C) Representative SEM images of pulmonary epithelial cells infected with *P. aeruginosa* and *F. nucleatum* alone or together. Pa, *P. aeruginosa*; Fn, *F. nucleatum*.

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Figure 2

The adherence and invasion of *P. aeruginosa* and *F. nucleatum* to pulmonary epithelial cells. (A) The attachment and invasion efficiency of *P. aeruginosa* to pulmonary epithelial cells. A549 cells were infected with different MOI (10, 50, 100) of *P. aeruginosa*. (B) The attachment and invasion efficiency of *F. nucleatum* to pulmonary epithelial cells. A549 cells were infected with different MOI (10, 50, 100) of *F. nucleatum*. (C) The effect of *F. nucleatum* on the attachment and invasion of *P. aeruginosa*. A549 cells were simultaneously infected with *P. aeruginosa* (MOI 100) and *F. nucleatum* (MOI 10, 50, 100). (D) The effect of *P. aeruginosa* on the attachment and invasion of *F. nucleatum*. A549 cells were simultaneously infected with *F. nucleatum* (MOI 100) and *P. aeruginosa* (MOI 10, 50, 100). * $P < 0.05$. Pa, *P. aeruginosa*; Fn, *F. nucleatum*.

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Figure 3

The effect of *P. aeruginosa* and *F. nucleatum* on cellular viability and cytotoxicity. (A) The effect of *P. aeruginosa* on cell viability and cytotoxicity of pulmonary epithelial cells. A549 cells were infected with different MOI (10, 50, 100) of *P. aeruginosa*. * Pa10 compared with A549, † Pa50 compared with A549, ‡ Pa100 compared with A549. (B) The effect of *F. nucleatum* on cell viability and cytotoxicity of pulmonary epithelial cells. A549 cells were infected with different MOI (10, 50, 100) of *F. nucleatum*. * Fn10 compared with A549, † Fn50 compared with A549, ‡ Fn100 compared with A549. (C) The effect of the simultaneous infection of *P. aeruginosa* and *F. nucleatum* on cell viability and cytotoxicity of pulmonary epithelial cells. A549 cells were infected with *P. aeruginosa* and *F. nucleatum* alone or together at a MOI of 100. * Pa100 compared with A549, † Fn100 compared with A549, ‡ PaFn100 compared with A549. */†/‡ $P < 0.05$. (D) Representative fluorescence images of pulmonary epithelial cells infected with *P. aeruginosa* and *F. nucleatum* alone or together. A549 cells were infected with *P. aeruginosa* and *F. nucleatum* alone or together at a MOI of 100 for 24 h. Pa, *P. aeruginosa*; Fn, *F. nucleatum*.

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Figure 4

The effect of *P. aeruginosa* and *F. nucleatum* on IL-1β, IL-6 and TNF-α productions. (A) The effect of *P. aeruginosa* on IL-1β, IL-6 and TNF-α productions of pulmonary epithelial cells. A549 cells were infected with different MOI (10, 50, 100) of *P. aeruginosa* for 24 h. (B) The effect of *F. nucleatum* on IL-1β, IL-6 and TNF-α productions of pulmonary epithelial cells. A549 cells were infected with different MOI (10, 50, 100) of *F. nucleatum* for 24 h. (C) The effect of the simultaneous infection of *P. aeruginosa* and *F. nucleatum* on IL-1β, IL-6 and TNF-α productions of pulmonary epithelial cells. A549 cells were infected with *P. aeruginosa* and *F. nucleatum* alone or together at a MOI of 100 for 24 h. * P<0.05. Pa, *P. aeruginosa*; Fn, *F. nucleatum*.

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Figure 5

The effect of *F. nucleatum* sequential infection on cellular viability, cytotoxicity and inflammation. (A) Schematic representation of cell model. A549 cells were treated with or without *P. aeruginosa* (MOI 100) for 12 h, and then infected with *F. nucleatum* (MOI 100) for other 12 h. (B) The effect of *F. nucleatum* sequential infection on cellular viability of pulmonary epithelial cells. (C) The effect of *F. nucleatum* sequential infection on cytotoxicity of pulmonary epithelial cells. (D) The effect of *F. nucleatum* sequential infection on IL-1 β , IL-6 and TNF- α productions of pulmonary epithelial cells. * $P < 0.05$. Pa, *P. aeruginosa*; Fn, *F. nucleatum*.

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subsequential infection on cellular viability of pulmonary epithelial cells. (C) The effect of *F. nucleatum* subsequential infection on cytotoxicity of pulmonary epithelial cells. (D) The effect of *F. nucleatum* subsequential infection on IL-1 β , IL-6 and TNF- α productions of pulmonary epithelial cells. * $P < 0.05$. Pa, *P. aeruginosa*; Fn, *F. nucleatum*.

Figure 6

The effect of *F. nucleatum* pretreatment on cellular viability, cytotoxicity and inflammation. (A) Schematic representation of cell model. A549 cells were treated with or without *F. nucleatum* (MOI 100) for 12 h, and then infected with *P. aeruginosa* (MOI 100) for other 12 h. (B) The effect of *F. nucleatum* pretreatment on cellular viability of pulmonary epithelial cells. (C) The effect of *F. nucleatum* pretreatment on cytotoxicity of pulmonary epithelial cells. (D) The effect of *F. nucleatum* pretreatment on IL-1 β , IL-6 and TNF- α productions of pulmonary epithelial cells. * $P < 0.05$. Pa, *P. aeruginosa*; Fn, *F. nucleatum*.

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