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Original Article

Promoted neutrophil respiratory burst makes elimination of mucoid *Pseudomonas aeruginosa* biofilms more difficult by enhancing alginate production

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Abstract: Background: *Pseudomonas aeruginosa* (*P. aeruginosa*) is one of the most common causes of nosocomial infections. This pathogen usually forms biofilms (BFs) and undergoes mucoid conversion during chronic lung infections in patients with ventilator-associated pneumonia (VAP) or cystic fibrosis (CF). These infections can result in excessive inflammation, leading to immune-mediated tissue damage, particularly through the action of recruited white blood cells, known as neutrophils. Most studies have focused on multiple strategies of *P. aeruginosa* BFs in overcoming these deleterious conditions in the host. However, the effects of immune response on *P. aeruginosa* BFs have received little attention. Objective: The current study sought to determine whether the neutrophil respiratory burst, a key part of neutrophil antibacterial activities, affects mucoid *P. aeruginosa* BFs. Methods: Using *in vivo* models of BF infections, this study evaluated the impact of an enhanced neutrophil respiratory burst on mucoid *P. aeruginosa* BFs, investigating the structure and bacterial counts of *P. aeruginosa* BFs, pathological changes, and inflammatory response in the lungs and alginate synthesis. Conclusion: Results showed that infections were more severe with more robust BFs. There was more severe tissue damage and immune response, along with greater alginate production, when the neutrophil respiratory burst was enhanced. This suggests that a promoted neutrophil respiratory burst may make the elimination of mucoid *P. aeruginosa* BFs more difficult by enhancing alginate production.

Keywords: Neutrophil respiratory burst, mucoid *P. aeruginosa* BFs, alginate

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a major pathogen responsible for nosocomial pneumonia, associated with high morbidity and mortality rates in immunocompromised patients [1]. Infections with *P. aeruginosa* are difficult to eradicate after biofilms (BFs) form. They enable bacterial survival despite aggressive antibiotic therapy and an exuberant immune response [2]. Following persistent infections, *P. aeruginosa* undergoes significant phenotypic and genetic changes to adapt to harsh environmental conditions, such as mucoid conversion, slow growth, antibiotic resistance development, motility minimization, loss of quorum sensing (QS), and cell envelope alteration [3]. The typical mucoid phenotype is characterized by an overproduction of alginate. This phenotype is usually found in chronic BF-infected patients and is more difficult to eliminate than the wild strain [3, 4]. Neutrophils are the most abundant innate immune cells, playing an indispensable role in surveying the body for signs of infection and inflammation [5]. Activation of neutrophil NADPH oxidase (NOX_2), as well as the subsequent generation of superoxide, hydrogen peroxide, and other reactive oxygen species (ROS), plays a central role in the antibacterial activities of neutrophils [6]. However, ROS fail to clear bacterial aggregates in BFs. They have been, instead, considered to play a major role in tissue damage, worsening patient conditions [7]. In contrast, Mathee reported

that ROS released from activated neutrophils can facilitate the generation of mucoid variants during planktonic *P. aeruginosa* infections [8]. Mucoid conversion and BF formation render *P. aeruginosa* resistant to most antimicrobial effector mechanisms [9], facilitating persistent infections. Most studies have focused on multiple strategies against *P. aeruginosa* BFs in overcoming these deleterious conditions in the host. However, effects of the immune response on *P. aeruginosa* BFs have received little attention [10]. Considering the influence of ROS on hosts and *P. aeruginosa* cells, the current study examined the roles of ROS in mucoid *P. aeruginosa* BFs in BF infection-related diseases. Accordingly, this study further explored the effects of the respiratory burst of neutrophils on BFs of mucoid *P. aeruginosa* strain FRD1 and alginate production *in vivo*. This work may provide a new insight into the mechanisms of persistent infections caused by mucoid *P. aeruginosa*.

Materials and methods

Bacterial strains

All experiments were performed with a mucoid *P. aeruginosa* FRD1 strain (ΔmucA mutant), kindly provided by Dr. Luyan Z. M. (State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China).

Bacteria from frozen stocks were plated on Luria-Bertani (LB) agar (Sigma, St. Louis, MO), then inoculated into an LB liquid medium. It was incubated at 37°C with agitation (200 rpm). FRD1 BFs were formed in Jensen's Medium at 37°C [11].

Preparation of endotracheal tubes precoated with bacteria

Tubes for endotracheal intubation, sterile plastic scalp acupuncture tubes with a 3.0-mm diameter, were cut to 1 cm in length. They were immersed in the bacterial suspensions (OD600=0.1) for 3 days at 37°C. BFs formed on both the inner and outer surfaces of these inoculation tubes. BFs on the outer surface of the tubes were carefully cleared with sterile gauze, as described in a previous study [12]. To estimate the bacterial counts in these BFs, the bacteria were detached from the tubes by placing the tubes in an ultrasound bath (Tomy UD-201, Tokyo, Japan) for 30 minutes and a concussion machine (Shanghai Facility Factory; UR 513) for 5 minutes. Afterward, the bacteria were serially diluted and plated on LB plates. The plates were incubated at 37°C overnight and inspected for colonies of *P. aeruginosa*. The number of bacteria at 3 days after incubation and before endotracheal intubation was 6.58±0.57 log10 CFU/tube (means ± SD; n=8).

Animals

Specific pathogen-free male Sprague-Dawley (SD) rats (6-8 weeks of age, 180-220 g in weight) were obtained from the Experimental Animal Center of Chongqing Medical University. The rats were housed in a pathogen-free environment and received sterile food and water in the Laboratory Animal Center of the Children's Hospital of Chongqing Medical University. The experimental protocol was approved by the Animal Care and Use Committee of Chongqing Medical University.

Rat BF infection model and drug administration

The BF infection model was established through a tracheostomy, as previously described [12]. The rats were randomly divided into two groups, with 8 rats given phorbol myristate acetate (PMA) and 8 rats given saline (controls). PMA, a positive modulator of the neutrophil oxidative burst [13], was obtained from Sigma. It was dissolved in dimethyl sulfoxide at a concentration of 1 mg/100 μl, then diluted in saline at the time of treatment. On the second day of intervention, PMA (100 μg per rat) or saline (100 μl per rat) was instilled intratracheally [14]. All rats were sacrificed on the third day after intubation.

Histopathology analysis

Lung tissues were removed from the rats at the time of necropsy. Lung samples were fixed in 10% buffered formalin for 24 hours, embedded in paraffin, cut into 5-μm-thick slices, and stained with hematoxylin-eosin (H&E) [15]. At least ten images of each group were obtained using light microscopy (Nikon Eclipse 55i, Tokyo, Japan).

Table 1. Primers for genes amplified by RT-PCR

Gene	Primer direction	Sequence (5'-3')
algD	Forward	GAGTCCAGCACAGCCTTCTT
	Reverse	ATCAACCAGGGCAAGTCG
algB	Forward	ACGCCAACATCCTCATCCT
	Reverse	TTCGCTTTCCATCAGTTCG
algR	Forward	CGAGGCGTTGAAGAAAGC
	Reverse	TACTTGTGGTCGGCAATGAA
algU	Forward	GATTGATCGTGCGGTTCGTG
	Reverse	AAGATCCGCGACCGTACCGT
ropD	Forward	GGTGTGGTCGGTGTTCATGT
	Reverse	CCGCAAGGTACTGAAGATCG

Electron microscopic studies of endotracheal tubes

The intubation tubes were removed aseptically and washed with sterile phosphate-buffered saline (PBS), removing mucus and planktonic bacteria.

Next, the samples were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer for 2 hours at 4°C. This was followed by refixation in 1% osmium acid in the same buffer for 2 hours at 4°C, dehydration in a series of aqueous ethanol solutions (50-100%), and freeze-drying. The specimens were coated with platinum-palladium using an ion sputtering technique and observed using a JSM-35C scanning electron microscope (SEM) (JEOL, Tokyo, Japan).

Bacterial counts of the tubes

Bacteria, detached from the intubation tubes by ultrasound and vortex treatments, were serially diluted and plated on LB plates. The plates were then incubated overnight at 37°C and inspected for colonies of *P. aeruginosa*.

ELISA for IL-10 and IFN-γ

Two milliliters of fresh blood were obtained. Serum was extracted by centrifugation (13,200 g for 10 minutes). Interferon-γ (IFN-γ) and interleukin-10 (IL-10) levels in the serum were determined using enzyme-linked immunosorbent assay kits (ELISA) (Beijing 4A Biotech Corporation, Beijing, China), according to manufacturer instructions.

Alginate assay

Bacteria detached from the intubation tubes were collected by centrifugation and clustered in 10 mL of LB, with rapid aeration at 37°C for 20-22 hours. Alginates were collected from the cultures and levels were determined, as previously outlined [16, 17]. Concentrations of alginate were determined using sodium alginate (Sigma, St. Louis, MO) as a standard. They were normalized to the total cell weight.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Expression of genes involved in alginate biosynthesis was investigated with reverse transcriptase-polymerase chain reaction (RT-PCR). The tubes were treated by the same methods described earlier. After vortexing, the bacteria were collected by centrifugation and clustered in 2.5 mL of LB, with rapid aeration at 37°C for 14 hours. Total RNA was extracted and purified using TRIzol, according to manufacturer protocol (Takara). Reverse transcription was performed with a ReverTra Ace RT-qPCR kit (Takara). Resultant cDNAs were used as templates for RT-PCR with primers designed to detect *algD, algB, algR,* and *algU* genes, with parallel amplification of the *rpo*D gene as an internal control, as previously described [9, 18] (**Table 1**). Real-time PCR was carried out using the SsoFast Evagreen Supermix Kit (Bio-Rad, CA, USA) with a Bio-Rad real-time PCR instrument. After RT-PCR amplification was performed, the comparative threshold method (ΔΔCt analysis) was applied to evaluate relative changes in gene expression. GenEx (Bio-Rad) and Excel (Microsoft) were used to solve the following equation: ΔΔCt = ΔCt sample - ΔCt reference (Bio-Rad).

Results

Structure and bacterial counts of P. aeruginosa BFs

The current study examined the microstructure of BFs on the inner wall of the tubes *in situ* with an SEM. BFs with some mucosal mass and mixed inflammatory cells on their surfaces were observed in both groups (**Figure 1**). However, BFs in the PMA-treated group were thicker (**Figure 1A**) than those in the control group (**Figure 1B**). Additionally, after 3 days of cultur-

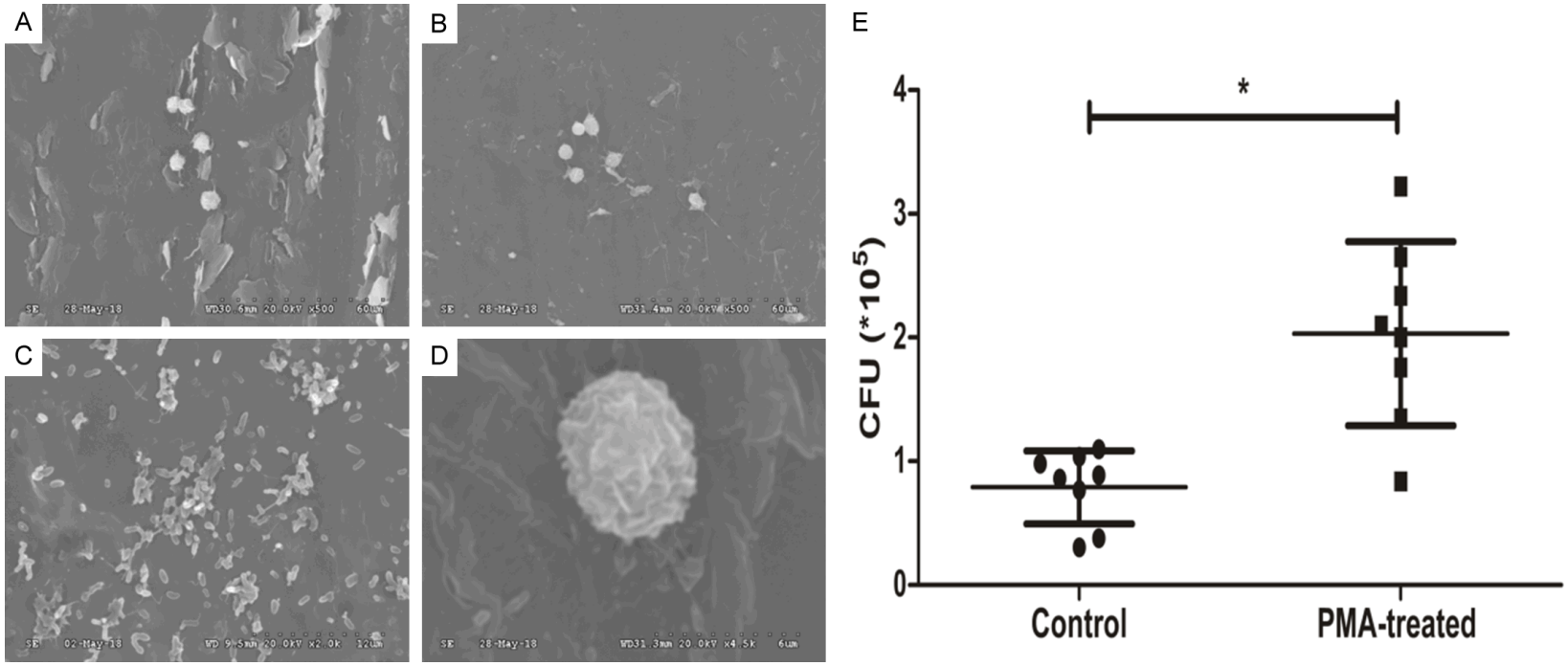

Figure 1. Scanning electron microscopy of the inner surface of the tubes and quantitative bacteriology. (A) PMA-treated group, (B) Control group, (C) BF formation on the third day, and (D) Neutrophil interaction with BFs. *P. aeruginosa* BFs on the inner wall of the tube in the PMA-treated group tube were significantly thicker than those in the control group. (E) Logarithm of colony-forming units of the two experimental groups. Differences were statistically significant between control and PMA-treated groups (*P<0.05). CFU data are representative of three independent experiments, with means ± SD from all experiments in each tube.

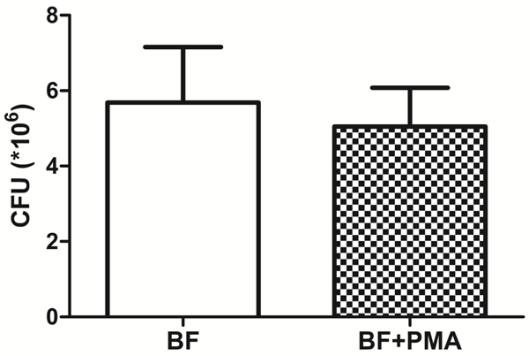

Figure 2. Effects of PMA on viable cells of biofilms of mucoid *P. aeruginosa* FRD1. The numbers of viable cells in biofilms are expressed in colony-forming units (CFUs). Error bars indicate standard deviations. BF, biofilm without PMA; BF+PMA, biofilm treated with PMA (100 μg). Differences were not statistically significant between the two groups (P>0.05). Data are presented as means ± SD (n=3 in each treatment).

ing, mature BFs formed, as shown in **Figure 1C**. It was observed that neutrophils were the major host cells on the surfaces of BFs and most of the cells were round (**Figure 1D**). The number of live *P. aeruginosa* cells in BFs was examined after treatment with PMA or saline. Bacterial counts of the PMA-treated group were significantly higher than those of the control group (P=0.001, Student's t-test, **Figure 1E**). *In vitro*, this study also examined viable cells in BFs treated with same amount of PMA used in the *in vivo* experiment. Viable cells in BFs did not differ significantly between the two groups (P=0.481, Student's t-test, **Figure 2**).

Pathological morphology of rat lungs infected with P. aeruginosa BFs

Lung tissues in both groups with BF-covered tubes showed signs of necrosis, edema, abscesses, and a large area of lung consolidation (**Figure 3**). In the PMA-treated group, more inflammatory cells infiltrated around small- and medium-sized tracheas and blood vessels (**Figure 3A-C**). More severe histological injuries were observed in lung tissues, with more signs of necrosis, edema, abscesses, and a larger area of lung consolidation (**Figure 3A-C**). However, less necrosis and smaller areas of lung consolidation were observed in the control group (**Figure 3E-G**). Moreover, the control group showed less inflammatory infiltration than the PMA-treated group (**Figure 3E-G**). Additionally, few changes were noted in the lungs of rats intubated with a sterile tube (**Figure 3D**, **3H**).

Cytokine assay

Responses of IL-10 and IFN-γ after infection were determined by ELISA. No significant differences in IL-10 levels were found between the PMA-treated group and control group (P=0.959, Mann-Whitney U-test, **Figure 4A**). However, levels of IFN-γ in the PMA-treated group were significantly higher than those in the control group (P<0.001, Student's t-test, **Figure 4B**). Additionally, the ratios of IFN-γ to IL-10 were significantly higher in the PMA-treated group than those in the control group (P=0.004, Student's t-test, **Figure 4C**).

Alginate synthesis of P. aeruginosa BFs

Alginate is the main extracellular matrix component of mucoid *P. aeruginosa* BFs. Thus, the current study investigated the effects of the neutrophil respiratory burst on alginate production by the mucoid *P. aeruginosa* strain FRD1. Alginate was produced in both groups. Alginate production was significantly higher in the PMA-treated group than that in the control group (P=0.031, Student's t-test, **Figure 5A**). RT-PCR was used to evaluate the effects of the neutrophil respiratory burst on expression levels of genes (*algD, algB, algR* and *algU*) involved in alginate biosynthesis in mucoid *P. aeruginosa* FRD1 BFs. Expression levels of *algD, algB, algR* and *algU* were significantly upregulated in the PMA-treated group, compared to those in the control group (P=0.001, P=0.001, P=0.007, P=0.002, Mann-Whitney U-test, Mann-Whitney U-test, Mann-Whitney U-test, Student's t-test, respectively, **Figure 5B-E**).

Discussion

P. aeruginosa is an opportunistic gram-negative bacterium associated with various types of human infections, including ventilator-associated pneumonia (VAP), cystic fibrosis (CF), burn wounds, and urinary tract infections [19]. Once established, *P. aeruginosa* infections become incredibly difficult to treat. This is due to the development of antibiotic-tolerant aggregated communities known as BFs [20]. BFs are organized by microorganisms, containing a matrix of extracellular DNA, proteins, and polysaccharides. *P. aeruginosa* is particularly adept at creating biofilms [38]. *P. aeruginosa* mucoid conversion, defined by an overproduction of the exopolysaccharide alginate, is correlated with accelerated disease progression in patients

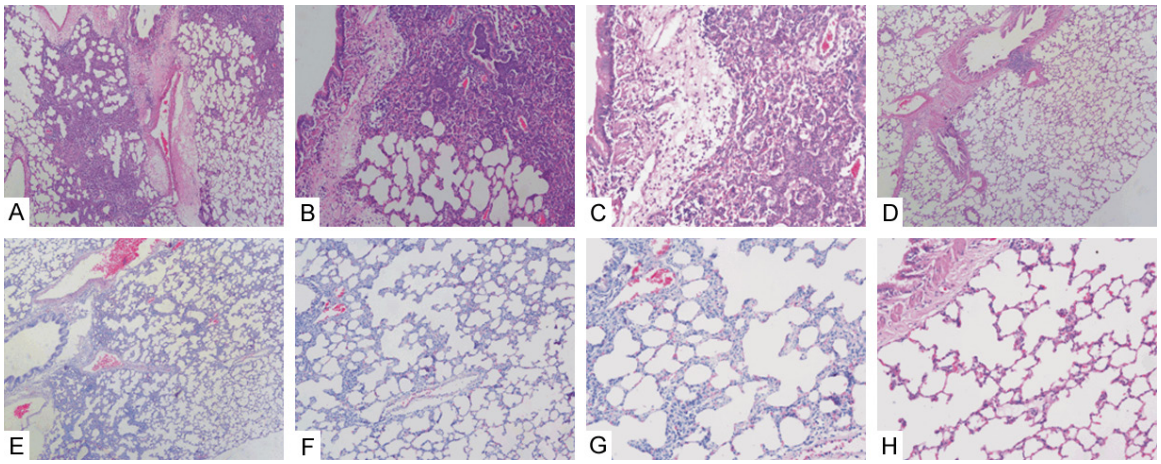

Figure 3. Histopathological changes in the lungs of rats, as indicated by hematoxylin-eosin (H&E) staining. (A-C) PMA-treated group, (E-G) Control group, (D and H) Sterile tube group. (A, D and E) Original magnification 40×; (B and F) Original magnification 100×; and (C, G and H) Original magnification 200×.

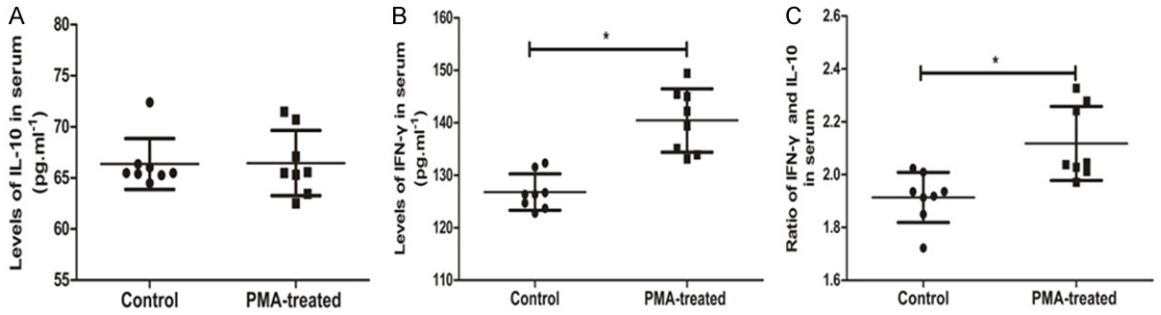

Figure 4. Quantitative assays for protein levels of IL-10 and IFN-γ in serum. A. IL-10 levels in the two experimental groups. B. IFN-γ levels in the two experimental groups. C. Ratios of IFN-γ to IL-10 in the two experimental groups. Differences in IL-10 levels were not statistically significant between the PMA-treated group and control group ($P>0.05$). Differences in IFN-γ levels and ratios of IFN-γ to IL-10 were statistically significant between the two groups ($*P<0.05$). Results are representative of three independent experiments.

[21]. Mucoidy contributes to the formation of BFs by *P. aeruginosa*, prevents antibiotic penetration, and inhibits phagocytosis and activation of the complement system [22]. This phenotype is usually found in patients with persistent BF infections. It is more difficult to eliminate than the wild strain [3, 4].

Neutrophils are the first line of defense against bacterial infections. The generation of ROS is a key part of the neutrophil arsenal [23]. However, a key characteristic of BF-based infections is the inability of the immune system, particularly neutrophils, to eliminate infections caused by BFs [24]. Furthermore, an aggressive and prolonged inflammatory immune response dominated by neutrophils causes progressive damage to the airway and lungs [24, 25]. Most studies have focused on multiple strategies against *P. aeruginosa* BFs in overcoming these deleterious conditions in the host. However, effects of the immune response on *P. aeruginosa* BFs have received little attention [10]. QS has been implicated in the differentiation, architecture, and virulence factors of *P. aeruginosa* BFs [7]. Pacheco and his colleagues demonstrated that the presence of neutrophils can upregulate the synthesis of some QS-controlled virulence factors, including rhamnolipids, in wild-type *P. aeruginosa* [26]. Another study indicated that exposure of *P. aeruginosa* PAO1 to activated neutrophils characterized by a respiratory burst substantially affected metabolism and bacterial virulence pathways [27]. Therefore, the neutrophil respiratory burst may have complicated effects on

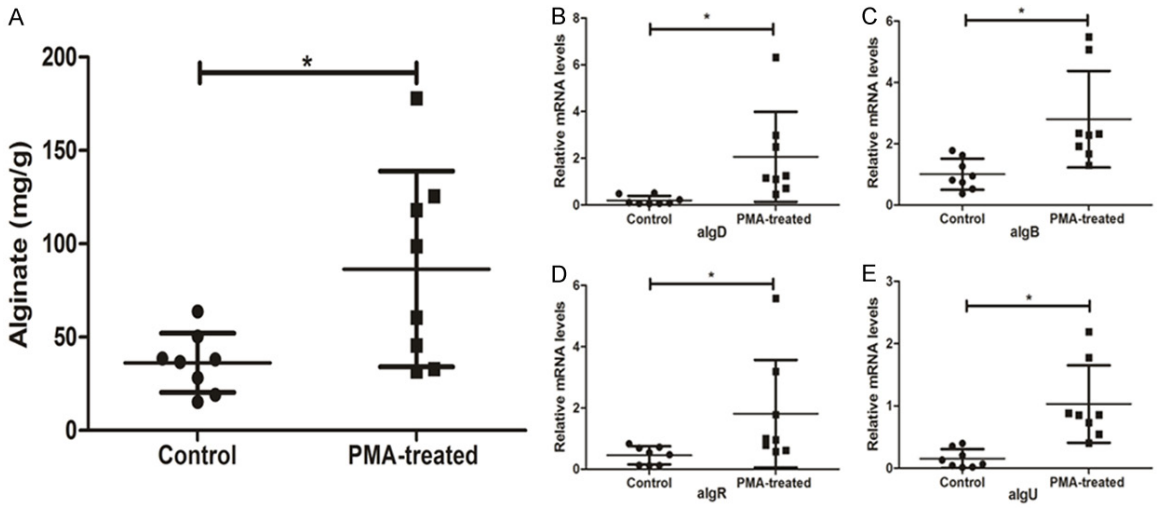

Figure 5. Alginate synthesis in the two experimental groups. A. Alginate production in the two groups. B-E. Comparison of the mRNA levels of *algD, algB, algR* and *algU* between the PMA-treated group and control group. Gene expression levels in the PMA-treated group were calculated after normalizing the signals to *ropD* and are presented as the fold change relative to the control. Alginate production was higher in the PMA-treated group than the control group (*P<0.05). *Alg* gene expression levels were significantly higher in the PMA-treated group than those in the control group (*P<0.05). The experiment was repeated three times.

P. aeruginosa. Unfortunately, previous studies have focused on the effects of activated neutrophils or ROS on wild-type *P. aeruginosa*. Few studies have investigated the effects of neutrophils or their active products on mucoid *P. aeruginosa* BFs, even though most strains isolated from chronic BF-infected patients have a mucoid colony morphology and are more difficult to eliminate [28]. Therefore, the current study investigated the influence of the neutrophil respiratory burst on mucoid *P. aeruginosa* BFs. Recently, in an *in vitro* study, the present research group found that with persistent recruitment of neutrophils and H_2O_2, the main ROS formed during the oxidative burst, promoting the formation and development of mucoid *P. aeruginosa* BFs. However, conditions *in vivo* are more complex than those *in vitro*. Therefore, the present study carried out an *in vivo* investigation of the effects of the neutrophil respiratory burst on mucoid *P. aeruginosa* BFs.

Rat models of BF infections were developed using hollow tubes, enabling visualization of BF development, neutrophil infiltration, and matrix material *in vivo* [29, 30]. Several types of positive modulators of the neutrophil oxidative burst existed, including N-formylmethionyl-leucyl-phenylalanine (fMLP) [31] and PMA [32]. Moreover, fMLP produced by bacteria, such as *Escherichia coli* and *Staphylococcus aureus*, has often been used as a chemotactic agent or a priming agent for neutrophil stimulation. It may even reduce the respiratory burst of neutrophils [33, 34]. PMA is often used as a soluble stimulus for ROS production [33]. Therefore, PMA was selected to stimulate neutrophils. This was coincided with the *in vitro* study. To ensure an enhanced neutrophil respiratory burst, concentrations of PMA, previously described in other studies, were used [14]. These are much greater than those used *in vitro*. With SEM images and quantitative bacteriology, the thickness of the BFs and the number of bacteria were increased in mature FRD1 BFs in vivo when the neutrophil respiratory burst was enhanced. A previous vitro study showed that hydrogen peroxide and activated neutrophils promoted mucoid *P. aeruginosa* BF formation and development *in vitro* [35]. However, *in vivo* conditions are considerably more complex than *in vitro* conditions. The phenomenon observed *in vivo* indicated that the neutrophil respiratory burst may play a negative role in BF clearance but a positive role in BF maintenance or development.

Numerous studies have demonstrated that the immune response to bacterial infections is modulated by various cytokines [36]. IFN-γ [36] and IL-10 [12, 18] are primary proinflammatory and anti-inflammatory cytokines, respectively,

in lung infections. Singh and his colleagues [37] demonstrated that the severity of lung pathology and the deterioration of lung function associated with *P. aeruginosa* infections may be due to an increase in the proinflammatory cytokine IFN-γ and a decrease in the anti-inflammatory cytokine IL-10. Constitutive production of IL-10 may help prevent local tissue destruction by inhibiting the production of proinflammatory cytokine IFN-γ [38]. The current study found no differences in IL-10 levels between the two groups, but higher levels of IFN-γ and higher ratios of IFN-γ to IL-10 were observed in the BFs of the PMA-treated group, compared to those in BFs of the control group. Moreover, several more severe pathological changes in lung tissues of the PMA-treated groups were observed. These findings suggest that the infection was more serious in the PMA-treated group than the control group. Given the effects of the enhanced neutrophil respiratory burst on both BF clearance and the host and the role of BFs in the immune response, it was speculated that an enhanced neutrophil respiratory burst may worsen lung infections by both exacerbating host conditions and decreasing mucoid *P. aeruginosa* BF elimination.

Alginate, the predominant extracellular polysaccharide in mucoid variants, is a virulence factor that increases the resistance of BFs to antibiotics and the immune system [39]. It plays a critical role in the formation of thick and highly structured BFs, which contribute to clogging the lungs of patients [11, 40]. In addition, alginate overproduction can promote *P. aeruginosa* coinfections with other pathogens, such as *S. aureus* [41]. This can also worsen patient conditions and accelerate disease progression. Therefore, the current study investigated the roles of the neutrophil respiratory burst in alginate production by mucoid *P. aeruginosa* BFs. Present data showed that an enhanced neutrophil respiratory burst promoted the production of alginate in FRD1 BFs, consistent with the results of a previous *in vitro* study [35]. The *alg* genes are main genes that regulate and control alginate synthesis [42]. Thus, this study also investigated the effects of the neutrophil respiratory burst on expression levels of genes (*algD*, *algB, algU* and *algR*) involved in alginate biosynthesis in mucoid *P. aeruginosa* FRD1 BFs [9, 16, 18]. Data suggests that an enhanced neutrophil respiratory burst *in vivo* upregulated expression levels of key genes, promoting alginate production. In addition, several groups have demonstrated that ROS production by stimulated neutrophils plays an important role in the generation of mucoid variants during inflammatory responses to *P. aeruginosa* PAO1 [8, 43]. The current study noted that an enhanced neutrophil respiratory burst could also promote alginate synthesis after mucoid mutation by upregulating *alg* genes.

Overall, the present work provides a deeper understanding of the effects of the neutrophil respiratory burst on mucoid *P. aeruginosa* BFs. Results suggest that an enhanced neutrophil respiratory burst may complicate mucoid *P. aeruginosa* BF elimination by promoting alginate production and may induce more severe pathological changes and inflammatory responses in the lungs. Furthermore, the enhanced neutrophil respiratory burst promoted the production of alginate, the predominant extracellular polysaccharide in mucoid variants, by upregulating *alg* genes to advance mucoid *P. aeruginosa* BFs. However, the current study was unable to identify molecular mechanisms in which the neutrophil respiratory burst regulates mucoid *P. aeruginosa*. This requires further immunological and biochemical studies.

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Disclosure of conflict of interest

None.

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