RESEARCH ARTICLE

Pseudomonas aeruginosa induces cellular senescence in lung tissue at the early stage of two-hit septic mice

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ABSTRACT

We presume that severe secondary Pseudomonas aeruginosa (PA) infection can lead to cellular senescence in lung tissue and thus contribute to high mortality. We established a two-hit mouse model using cecal ligation and puncture (CLP) followed by sublethal PA lung infection. In lung tissue, increased infiltration of inflammatory cells, elevated lung injury and augmented cellular senescence was shown in mice with CLP followed by sublethal PA infection, and these observations reached a higher rank when higher (H) loads PA (PAO1) were administered to CLP mice (CLP + PAO1-H). Accordingly, oxidative stress-related element gp91phox and inflammation regulator NF-κB were greatly activated in CLP + PAO1-H mice compared to others. There was no obvious inflammation or cellular senescence in sham control, PAO1-infected mice. Consequently, CLP + PAO1-H mice had the highest expression levels of inflammatory cytokines IL-6, TNFα and iNOS among those groups. There was lower bacterial clearance ability in CLP + PAO1-H mice than in other mice. CLP + PAO1-H only had approximately 10% survival after 7 days of investigation and was much lower than others. In conclusion, higher mortality due to increased lung inflammation and cellular senescence are observed in mice with increased loads of PA infection secondary to CLP.

Keywords: Pseudomonas aeruginosa; cecal ligation and puncture; cellular senescence; NADPH oxidase; NF-κB

INTRODUCTION

Pseudomonas aeruginosa (PA) is an opportunistic pathogen and accounts for high incidences of acute and chronic pulmonary infections, including nosocomial infection, long-term colonization with chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF) and non-CF bronchiectasis (Lovewell, Patankar and Berwin 2014; Kho et al. 2016). According to the National Healthcare Safety Network (NHSN), PA is the second most common pathogen causing approximately 16.6% ventilator-associated pneumonia (VAP) in patients in the USA (Sievert et al. 2013). Furthermore, 3% to 7% of all bloodstream infections (BSIs) and 23% to 26% of Gram-negative bacteremias are associated with PA. The mortality rates due to PA BSIs remain high, ranging from 27% to 48% (Hattemer et al. 2013). PA infection prefers patients with underlying immune deficiencies, including elderly populations, neutropenia due to cancer chemotherapy, or immunosuppression due to organ transplant. Additionally, mortality in those patients is relatively higher than that in immune competent patients (Gellatly and Hancock 2013). Some researchers have studied why there are differences in mortality among these individuals. In aged mice, the hyper-reactive systemic inflammatory response observed after lipopolysaccharide administration...
may contribute to the higher mortality because of the exacerbated pulmonary inflammatory response (Gomez et al. 2007). This may partly explain why the elderly population has higher mortality than the younger population. It seems that aging individuals are highly prone to hyperinflammation in response to alien insults, and the weakened immune system due to aging exhibits immunosenescence, which presents as host defense attrition (Brandenberger and Mühlfeld 2017). Cellular senescence is a state of irreversible growth cycle arrest accompanied by impaired cellular function, increased production of reactive oxygen species (ROS) and activated pro-inflammatory signaling and elevated expression of senescence-associated molecules such as β-galactosidase, p16, p53 or p21 (Campisi 2013). Except for telomere-dependent growth arrest, inflammation and oxidative stress also incur senescence.

The mortality rate after PA infection has also been shown to be related to bacterial loads in the lungs. This suggests that a higher bacterial load leads to higher mortality (Restagno et al. 2016). As previous reports indicated, inflammation triggered by bacterial infection is highly related to bacterial loads (Mesquita et al. 2016; Wohlwend et al. 2016), and the activated pro-inflammatory milieu initiates host disorders, including senescence, which is called ‘inflammaging’ (Lloyd and Marsland 2017). Hence, it is reasonable to presume that high loads of bacterial colony counts can motivate the inflammatory pathway and thus lead to cellular senescence of lung tissue, finally contributing to higher mortality. Recently, PA infection after cecal ligation and puncture (CLP) was manipulated in different ways to produce a two-hit model that mimics PA secondary infection in the clinic (Hoetzenecker et al. 2011; Restagno et al. 2016; Deng et al. 2017). We wondered whether there were differences in the levels of cellular senescence due to increased lung inflammation caused by elevated bacterial loads. These changes may contribute to deficient host defense and higher mortality. To test our hypothesis, a two-hit mouse model using increased loads of sublethal PA infection secondary to peritonitis sepsis was performed. Because nuclear factor κ B (NF-κ B) controls inflammation and ROS production is maintained by NADPH oxidase activity during infection, we measured NF-κ B and NADPH oxidase element gp91phox in the lung from different mouse models. In this report, mouse lung tissue exhibited elevated inflammation and cellular senescence depending on PA bacterial loads administered after CLP surgery. Consequently, the NF-κ B and NADPH oxidase pathways were also involved in the increased inflammation procedure. Deficient host defense against bacteria and higher mortality were observed in the CLP mice with higher bacterial load infection of PA.

**MATERIAL AND METHODS**

**Preparation of *P. aeruginosa* strain PAO1**

PAO1 (a kindly gift from Dr. Zizhong Xiong) colonies were grown on a Luria-Bertani (LB) agar plate at 37°C overnight. The colonies were inoculated into 10 ml fresh LB medium and grown overnight at 37°C with low-speed shaking. After another inoculation of PAO1 to fresh LB broth, this medium was cultured to reach an optimal density and adjust to a final concentration of 5×10^6 CFU/ml. These cultures were used for all experiments at different CFUs.

**Animals**

Male C57BL/6 mice between 8 and 12 weeks of age were used in all experiments. All mice were housed under specific pathogen-free conditions. They were allowed to accustom to the lab environment for at least 1 week prior to any intervention in a temperature and humidity-controlled housing with a 12-hr light: dark diurnal cycle. Food and water was provided *ad libitum* for the entire duration of the experiment. The animal ethics committee of the Affiliated Drum Tower Hospital of Nanjing University Medical School approved all experimental protocols (No. 20 150 602).

**Cecal ligation and puncture (CLP)**

CLP operation was reproducible and stable animal model for sepsis investigation, since Chaudry and Wichterman et al. described this procedure in detail (Chaudry, Wichterman and Baue 1979). And the operation was conducted as a previous report with minor revisions. After mice were anesthetized with isoflurane (2%–3%), the surgical area was disinfected with betadine and 70% alcohol, and a midline laparotomy was performed. The cecum was then identified, and the distal 50% of the cecum was ligated with 4–0 silk suture followed by puncture with a pass of a 21-gauge. Then, a small amount of stool was squeezed out through both holes. The peritoneal cavity was closed using 5–0 nylon sutures. After surgery, mice were taken out of the anesthesia device and kept warm, and 0.5 ml of warm sterile normal saline was administered by intraperitoneal injection for resuscitation. Sham laparotomy controls underwent the same surgical procedure without CLP. Four hours after CLP, PAO1 (approximately 5×10^3 and 5×10^5 bacterial cells in early logarithmic phase) suspended in sterile PBS or an equivalent volume of vehicle (without PAO1) was administered through tracheal instillation to produce different severities of lung infection for the re-anesthetized CLP mice. Meanwhile, mice with CLP alone and PAO1 lung infection at different bacterial loads of 5×10^3 and 5×10^5 CFU were set up, simultaneously. And postoperative pain control was achieved with once 0.5 mg/kg buprenorphine injection after operations. Before sacrificing 24 hr after infection, mice were kept on a thermostatic environment of 25°C. There were 5 to 10 mice for BAL study and molecular experiment, and three mice for histological study in each group.

**Histological experiments**

CLP mice were sacrificed 24 hr after PAO1 infection. The procedure was performed as described previously (Li et al. 2013). After euthanasia, lungs were perfused with normal saline via vena cava and inflated with 1 ml of 10% neutral buffered formalin. The left lung was then removed en bloc after tracheal ligation, preserved in 10% neutral buffered formalin for 24 hr at room temperature, and subsequently embedded in paraffin. Hematoxylin and eosin (H&E) stains were performed using a standard protocol. And results were evaluated by an investigator who was blinded to the group assignment. The degree of parenchymal distortion in the alveolar tissue was assessed on 20 sequential high-power fields per lung section and graded as follows: 0: normal; 1: < 50% of interalveolar septa (IAS) have increased thickness due to edema and/or inflammatory cell infiltration; 2: > 50% of IAS have increased thickness; 3: > 50% of IAS have increased...
thickness and inflammatory cells are present within alveolar space; 4: consolidated infiltrate with distortion of normal alveolar architecture. The mean score was reported per section.

**BAL fluid cell counts and lung tissue harvest**

The other cohort of animal was used for bronchoalveolar lavage (BAL) fluid and lung tissue harvest. Specifically, the right bronchus was bundled after opening the mice chest, and then lung lavage was performed from the left lung. Right lung was resected, the upper right lung was used for bacterial counts, and the remaining right lung was stored at -70°C immediately for further molecular study. BAL fluid was centrifuged at 400 g for 10 min to separate cells from supernatant. Cells were suspended in cold PBS and total cell counts were measured on a grid hemocytometer.

**Lung bacterial counts study**

The right upper lobe was removed and homogenized in 5 ml of cold PBS. The homogenate was diluted appropriately and plated for enumeration on a blood agar plate by using double dilution methods. Following incubation for 48 hr, the colonies were counted.

**Survival studies**

Mice were subjected to laparotomy without CLP (Sham), with CLP, laparotomy with CLP followed by different colony counts of PAO1 lung infection (5 × 10³ and 5 × 10⁵ bacterial cells), and PAO1 infection alone (5 × 10³ and 5 × 10⁵ bacterial cells, respectively). Mice were evaluated and were euthanized at 7 days after CLP or earlier if they appeared moribund or in distress. Specifically, according to Herndon et al.’s methods (Herndon et al. 2016), after the CLP, all mice were observed every 12 hr for general health. Mice were observed for movement, interaction with conspecifics, posture and amount of time spent focused at the surgical site. Using a clinical condition scale with a complete description of all clinical signs, the score of mice health status was recorded, and the maximal total condition score was 30. Mice that scored greater than 12 or that were unable to right themselves were euthanized by CO₂ asphyxiation or in distress. Specifically, before staining, paraffin sections were dewaxed and hydrated. Then, the section was stained step-by-step using the manufacturer's instructions. According to the manufacturer's instructions, lung tissue was set up using a classic peritonitis model, and a nosocomial lung infection secondary to sepsis was subsequently performed using intratracheal administration with sublethal colony counts of PAO1 after CLP operation. After 24 hr, lung injury, including increased infiltration of inflammatory cells and increased alveolar wall thickness, was greatly elevated in CLP mice with a higher bacterial load of PAO1 infection (5 × 10⁵ CFU; CLP + PAO1-H) compared to other groups, including CLP mice with a lower (L) load of PAO1 infection (5 × 10³ CFU; CLP + PAO1-L) (P < 0.0001, ANOVA test). Mice with CLP only exhibited mild inflammation in the lung tissue. There was no obvious inflammation in the lung tissue from mice with sham operation, and PAO1 infection (Fig. 1A). For BAL fluid cell counts, total inflammatory cells were counted. Compared to the sham control, CLP + PAO1-H mice had significantly increased inflammatory cells. BAL cell counts from CLP mice that received a lower load of PAO1 infection were not significantly different from CLP alone. Additionally, CLP mice with or without a lower load of PAO1 infection had higher BAL cell counts than sham control mice and mice with PAO1 infection alone. There was no significant difference in BAL cell counts among sham-operated mice and PAO1-infected mice (Fig. 1B). Accordingly, analysis of lung injury score (LIS) calculated by degree of parenchymal distortion in the alveolar tissue showed a significant increase in LIS in CLP + PAO1-H mice and a comparable LIS between CLP mice and CLP + PAO1-L mice. There was no obvious LIS for mice infected with sublethal PAO1 (Fig. 1C).

**Western blot**

Primary anti-NF-κB p65, gp91phox, p16ink4a, Na-K-ATPase, β-actin and histone H3 antibodies were purchased from Santa Cruz Biotech Company. Western blotting was performed on cell extracts, membrane proteins and nuclear proteins. β-actin was used as a control for whole cell extract protein, histone H3 was used as a control for nuclear protein, and Na-K-ATPase was used as a control for membrane protein.

**Inflammatory cytokine production**

Total RNA was extracted from homogenized lung tissue using the TRIzol method (Invitrogen, Karlsruhe, Germany). Real-time PCR was used to detect the gene expression levels of TNFα, IL-10 and IL-6. The primers were used as follows: tfβa (forward: 5’-AGG ATA ACT GGA ACA CAG ACA-A3’; reverse: 5’-TGG GAG ACA TAC AAG CA-3’), iNOS (forward: 5’-GAA CTG TAG CAC AGC ACA GGA AAT-3’; reverse: 5’-CGT ACC GGA TGA GCT GTG AAT-3’), il-6 (forward: 5’-GCC TTC CCT ACT TCA CAA-3’; reverse: 5’-ACA ACT CTT TTC TCA TTT CCA C-3’), gapdh (forward: 5’-GCA GTA AAC AGT CCA TCT ACA A A A-3’; reverse: 5’-GTC TCC TTC ATC AAC CCT-3’). The results were calculated as 2^-ΔΔCT.

**Statistical analysis**

Analyses were performed with GraphPad Prism 5.0 software using an unpaired t-test (two groups) or one-way ANOVA (multiple groups) followed by the scheffe post-test. The results are represented as the mean ± SEM. Two-tailed P-values < 0.05 were considered significant. Log-rank test was used for the survival study.

**RESULTS**

**Pseudomonas aeruginosa PAO1 infection leads to CLP mice with elevated inflammation and tissue senescence in the lungs**

We set up septic animals using a classic peritonitis model, and a nosocomial lung infection secondary to sepsis was subsequently performed using intratracheal administration with sublethal colony counts of PAO1 after CLP operation. After 24 hr, lung injury, including increased infiltration of inflammatory cells and increased alveolar wall thickness, was greatly elevated in CLP mice with a higher bacterial load of PAO1 infection (5 × 10⁵ CFU; CLP + PAO1-H) compared to other groups, including CLP mice with a lower (L) load of PAO1 infection (5 × 10³ CFU; CLP + PAO1-L) (P < 0.0001, ANOVA test). Mice with CLP only exhibited mild inflammation in the lung tissue. There was no obvious inflammation in the lung tissue from mice with sham operation, and PAO1 infection (Fig. 1A). For BAL fluid cell counts, total inflammatory cells were counted. Compared to the sham control, CLP + PAO1-H mice had significantly increased inflammatory cells. BAL cell counts from CLP mice that received a lower load of PAO1 infection were not significantly different from CLP alone. Additionally, CLP mice with or without a lower load of PAO1 infection had higher BAL cell counts than sham control mice and mice with PAO1 infection alone. There was no significant difference in BAL cell counts among sham-operated mice and PAO1-infected mice (Fig. 1B). Accordingly, analysis of lung injury score (LIS) calculated by degree of parenchymal distortion in the alveolar tissue showed a significant increase in LIS in CLP + PAO1-H mice and a comparable LIS between CLP mice and CLP + PAO1-L mice. There was no obvious LIS for mice infected with sublethal PAO1 (Fig. 1C).

**PA infection secondary to CLP in septic mice induces lung tissue due to aging**

Because lung injury and inflammation were greatly activated in CLP mice with PAO1 infection, we investigated the aging exhibited by lung tissue using SA-β-gal staining and the senescent biomarker p16ink4a. As shown in Fig. 2A and B, CLP +
Figure 1. Secondary PA infection caused lung inflammation after CLP in a colony-dependent manner, as indicated as follows: (A), histological study of lung tissue, (B), cell counts in BAL, (C), lung injury scores 24 hr after surgery in control mice treated with sham laparotomy (Ctrl mice that underwent CLP followed by high colony counts of PAO1 infection (CLP + PAO1-H), mice with CLP followed by low colony counts of PAO1 infection (CLP + PAO1-L), mice underwent CLP alone (CLP), with high colony counts of PAO1 infection (PAO1-H), and low colony counts of PAO1 infection (PAO1-L). *** = P < 0.0001 for comparison among groups using ANOVA test. For histological study, three mice used in each group. For BAL and molecular study, five samples were obtained from sham control, PAO1-infected mice and CLP + PAO1-H mice; seven samples were obtained from CLP + PAO1-L mice and 10 samples withdrawn from CLP mice.

Figure 2. Cellular senescence in lung tissue. (A), SA-β-gal staining positive cells in the lung tissue after 24 hr of secondary PA infection for CLP mice and (B), OD value of positive staining was calculated by ImageJ Software (National Institutes of Health, USA); (C), the expression of senescence marker p16ink4a (upper line, β-actin was used as control); (D), the densitometry analysis of p16ink4a.

PAO1-H mice had more prominent cellular senescence (blue staining) than CLP + PAO1-L mice and CLP mice, and the latter two groups were comparable for SA-β-gal staining. Barely, no positive staining in the lungs from sham mice and sublethal PAO1-infected mice. Meanwhile, as shown in Fig. 2C, the senescence-associated biomarker p16ink4a was significantly increased in the lung tissue from CLP + PAO1-H mice, CLP + PAO1-L mice and CLP mice; of those, CLP + PAO1-H mice had the highest level of p16ink4a expression. PAO1 infection alone in mice caused a mild increase in p16ink4a expression, and there was no significant difference between the two groups (see Fig. 2D).

Activation of oxidative stress and inflammatory pathways in the lungs of two-hit septic mice

Because cellular senescence was shown in this animal model, we then examined oxidative stress and the inflammatory pathway in the lung tissue by investigating the expression of NADPH oxidase elements gp91phox and nuclear NF-κB. As indicated in Fig. 3A and C, gp91phox protein extracted from the lung tissues exhibited higher levels of expression in CLP + PAO1-H mice than in CLP + PAO1-L mice or CLP mice. In sham mice and
PAO1-infected mice, gp91phox expression was not obviously elevated after 24 hr of treatment. Regarding the inflammatory pathway, NF-κB subunit p65 increased in the nucleus in the lungs of CLP mice with different colony counts of PAO1 infection, and in accordance with the trend of gp91phox, CLP mice with severe PAO1 infection had the highest expression level of p65 in the nucleus compared to others (Fig. 3B and D).

Severe secretory phenotypes shown in CLP mice with higher secondary PA infection

Because there was an increase in gp91phox and NF-κB p65 activation in CLP mice with higher PA bacterial load infection, we then examined inflammatory cytokine gene expression, including IL-6, TNFα and iNOS, to study the milieu of the lungs of those animals. As shown in Fig. 4A, B and C, there was a significant difference among these groups using one-way ANOVA (P < 0.0001). Of those, CLP + PAO1-H mice had the highest secretory phenotypes on IL-6, TNFα and iNOS and exhibited 13–28 times of elevation on their expression level above baseline. CLP + PAO1-L mice also had elevated inflammatory cytokine production compared to CLP mice, PA infected alone or baseline mice. This suggests that secondary PA infection after CLP triggers more severe inflammation in the lungs and that the severity of inflammation is related to the bacterial load of secondary infection.

Higher bacterial loads of PA infection secondary to CLP cause deteriorated host clearance of bacteria in the early stage

We then wanted to determine the outcomes of these elevated inflammation and senescence cells in the lung from different groups of mice. As shown in Fig. 5A, 24 hr after PAO1 inoculation, mice were sacrificed, and bacterial load in the lung was determined in those groups of animals. Despite initial infection with different colony counts of PA, after 24 hr, PAs were nearly cleared in the lung from mice that received sublethal PAO1 infection only. However, mice inoculated with PA after CLP had worse clearance of bacteria in the lungs, especially for CLP mice with higher bacterial loads of PA infection. After 24 hr of PA infection, the bacterial count in the lung reached 8 log CFU in CLP + PAO1-H mice, which was approximately 2–3 log higher than that in CLP + PAO1-H and CLP mice, respectively. This suggests that CLP mice with a secondary infection of PA display a decreased ability for bacterial clearance in the lungs.

PAO1 infection secondary to CLP affect survival rate based on the severity of PAO1 colony counts

For the survival study, 20 mice per group were subjected to CLP + PAO1-H, CLP + PAO1-L, CLP alone, PAO1-H alone, PAO1-L alone or sham laparotomy and investigated for one week. At 7 days after CLP, all mice in the sham control, PAO1-H and PAO1-L groups survived, compared to 18/20 deaths in the CLP + PAO1-H group, 14/20 deaths in the CLP + PAO1-L group, and 8/20 deaths in the CLP alone group (Fig. 5B). There was a significant difference among those groups for survival (Log-rank test P < 0.0001). This study shows that high colony counts of PAO1 infection secondary to CLP result in a trend towards worse survival in mice, suggesting that severity of PAO1 infection in this two-hit model is correlated with worse prognosis.

DISCUSSION

In the current study, we showed that at the early stage of the two-hit mouse model, accelerated inflammation and cellular senescence in the lung tissue is due to higher bacterial loads of sublethal PA infection secondary to CLP surgery. Specifically, secondary PA infection after CLP causes elevated lung inflammation and cellular senescence in a bacteria load-dependent
Figure 4. (A) TNF-α, (B) IL-6, (C) iNOS expression in the lung tissue. The results are summarized from 5 ~ 10 animals for each group. ***P < 0.0001 using ANOVA test for those groups. Above the sham control, relative TNF-α production in PAO1-H, PAO1-L, CLP + PAO1-H, CLP + PAO1-L and CLP was 1.83 ± 0.37 (mean ± SD), 1.38 ± 0.35, 28.42 ± 4.93, 14.35 ± 2.95, 6.08 ± 2.08, respectively; relative IL-6 in every group was 1.84 ± 0.48, 1.45 ± 0.47, 23.04 ± 6.286, 11.50 ± 1.55 and 5.57 ± 0.92, respectively; and relative iNOS production in the above groups was 2.83 ± 0.40, 1.67 ± 0.40, 13.86 ± 2.15, 8.63 ± 1.06 and 5.96 ± 0.64, respectively.

Figure 5. (A) Bacteria clearance 24 hr after secondary PAO1 infection in CLP mice, sham control, PA infection alone or CLP alone mice; (B) mortality analysis. Kaplan-Meier curves were plotted for control (Ctrl) mice subjected to sham laparotomy, CLP + PAO1-H mice, CLP + PAO1-L mice, CLP mice alone, PAO1-H and PAO1-L mice. Each group had 20 mice. There was a significant difference among those groups for survival rated (Log-rank (Mantel-Cox) Test, P < 0.0001).

Oxidative stress-related elements NADPH oxidase gp91phox and nuclear NF-κB and its dependent cytokines are activated in this two-hit animal model, and the degree of activation seems in relation to the severity of PA infection. CLP mice with higher bacteria loads of PA infection exhibit higher colony counts after bacteria isolation and higher mortality.

According to Migiyama’s report, mortality in immunocompromised patients tended to be higher than in immunocompetent patients and showed a ratio of 7-day mortality of 30% vs. 8% (Migiyama et al. 2016). Except for immune incompetent patients, PA pneumonia is more common and lethal in the elderly. In aged mice, neutrophil migration into the lungs was impaired with PA infection despite elevated chemokine levels, suggesting that immunosenescence contributes to worse mortality (Chen et al. 2014). In the current study, although it is unknown which type of cell was dominantly exhibited senescence in the lung tissue after PA infection secondary to CLP, we still clearly investigated a phenomenon in which mice with increased cellular senescence in the lungs had impaired bacterial clearance and higher mortality. Further study is needed to clarify which type of senescent cells at this early stage of infection contribute to poor outcome.

In this study, we used a sublethal bacterial load of a PA secondary infection model to determine how PA affects the host inflammatory status under critical conditions. PA infection did not kill non-operated mice either at loads of 5 × 10³ or at 5 × 10⁵ CFU. According to a previous report, 10⁷ CFU of PAO1 showed lethal power for C3Heb/FeJ mice or C3H/HeJ mice (George et al., 1993). From our data, 5 × 10³ and 5 × 10⁵ CFU of PAO1 were sublethal for C57BL/6 mice, and they only incur a slight increase in lung inflammation. In addition, 5 × 10⁵ CFU PA intratracheal infection was cleared by 24 hr after inoculation. In Restagno’s experiment, intratracheal inoculation of the PA strain to C57BL/6J at 5 × 10⁶ CFU caused only one CLP mouse to die, which is obviously different from the current study (Restagno et al. 2016). However, the design of our experiment is different from their experiment, and PA was instilled 4 hr after CLP, which was much earlier than that of Restagno (5 days after CLP). Meanwhile, CLP operation could greatly influence the experimental results. Recently, a two-hit mouse model using sublethal CLP...
plus 24 hr post-infection with $1 \times 10^5$ CFU was shown to cause approximately 60% death, which was closer to our report (Song et al. 2015). Compared to their experiment, the death in our study was elevated, which suggested that survival was closely related to the primed bacterial loads. Also, the time point of secondary infection for the animal model was earlier in our study than in others, this may lead to higher mortality due to overlapped severe injury on the very early stage of sepsis. Decades ago, CLP was set up in mice to study the mechanisms of sepsis and its related complications, including lung injury. The occurrence of mortality for CLP mice is mainly on the first 3 days after surgery (Baker et al. 1983; Muenzer et al. 2006). The majority death of mice in our experiment was also within 3 days after surgery and PA administration.

Patients admitted to intensive-care units are at high risk of health-care-associated infections, secondary BSIs and pneumonia, which greatly increase mortality and length of stay in intensive-care units (Lambert et al. 2011). In the current study, sublethal PA infection alone or CLP alone did not motivate massive lung inflammation and cellular senescence, while post-infection with sublethal PA greatly increased lung inflammation and cellular senescence in CLP mice. Furthermore, the increased burden of PA bacterial load after CLP caused more severe lung inflammation and senescence, deteriorated bacterial clearance and higher mortality when compared to the lower burden of PA infection secondary to CLP. This result is identical to the clinical findings of human beings in that the severe outcome of PA infection is related to a higher bacterial burden (Zhuo et al., 2008).

For patients with infectious diseases, immunosenescence is identified to be related to clinical outcome and mainly refers to decreased function of immune cells due to natural aging in elderly patients (Agarwal and Busse 2010; Jose et al. 2017). The basis of aging is cellular senescence dependent on telomere and/or non-telomere mechanisms (Brandenberger and Mühlfeld 2017). The occurrence of immunosenescence in elderly populations leads to dysfunction of host defense. For example, peripheral blood mononuclear cells (PBMCs) isolated from old individuals (≥ 65 years) exhibited a delayed and altered response to stimulus (Metcalfe et al. 2015). In the current study, enhanced cellular senescence was found in lung tissue following infection with increased bacterial loads after CLP, suggesting that severe secondary PA infection caused cellular senescence, impaired bacterial clearance and pronounced higher mortality. These results are identical to the finding that immunosenescence is strongly driven by persistent infections and/or tissue inflammation (Lopez-Otin et al. 2013). Recently, the definition of oxidative-inflamm-aging was noted to be accompanied by chronic oxidative and inflammatory stress (Bauer and Fuente MdeL. 2017). The basis of aging is cellular senescence dependent on telomere and/or non-telomere mechanisms (Brandenberger and Mühlfeld 2017).

Inhibition of NF-κB reduces oxidative DNA damage and stress and delays cellular senescence (Tilstra et al. 2012). Further experiments in our study also confirmed the remarkable activation of gp91phox and NF-κB p65 in these two-hit animal models compared to their comparable animals. As a consequence of gp91phox and NF-κB activation, the expression levels of iNOS, TNFα, and IL-6 were greatly enhanced in mice with higher colony counts of PA01 infection secondary to CLP. The bridge molecule between oxidative stress and inflammation is still unknown. Recently, Chan et al. reported that in islets, redox signaling of NADPH oxidase-mediated ROS production would further trigger iNOS and NF-κB-related proinflammatory cytokine production (Chan et al. 2017), supporting ROS might be working as a second messenger. Additionally, the shortcomings of our study include the following: (i) oxidative and inflammation-related senescence is also an observational phenomenon in our study; further study exploring the specific mechanism for cellular senescence should be performed; and (ii) the specific cell type that fulfilled senescence during these two-hit mouse models should be determined.

In conclusion, PA induces lung tissue to senescence in a two-hit mouse model and exhibits a colony count-dependent manner. Coincidently, elevated lung inflammation and increased expression of the oxidative stress regulator gp91phox and the inflammatory regulator NF-κB are also found. Septic mice with higher PA bacterial load infections have compromised clearance of bacteria and higher mortality.

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**Conflict of interest.** None declared.

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