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Age-Related Differences in the Neutrophil Response to Pulmonary *Pseudomonas* Infection

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Abstract

Background—*Pseudomonas aeruginosa* pneumonia is more common and more lethal in the elderly. The immunologic underpinnings of this increased incidence and mortality has not been evaluated, however is assumed to be a complication of age-associated immune dysfunction.

Methods—Young (10–12 week old) and aged (18–20 month old) BALB/c mice were subjected to intratracheal infection of *Pseudomonas aeruginosa*. Animals were sacrificed 24 hours after inoculation. Lungs were collected for analysis of lung pathology, chemokine levels, neutrophil counts, and myeloperoxidase activity.

Results—Pulmonary levels of the neutrophil chemokine KC are significantly higher in aged mice relative to young following *P. aeruginosa* infection. Despite this, neutrophil counts are higher in young mice compared to aged mice after infection. Furthermore, the neutrophils are predominantly found in the air space of young infected mice. This correlated with increased myeloperoxidase activity from bronchoalveolar lavage specimens of young mice relative to aged mice after infection.

Conclusions—Neutrophil migration into the lungs is impaired in aged mice 24 hours after intratracheal infection despite elevated chemokine levels, suggesting that immunosenescence is impairing neutrophil migration.

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Keywords

Aging; *Pseudomonas aeruginosa*; infection; pneumonia; inflammation; neutrophil; inflammaging

1. Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is the second most common bacterial organism to cause hospital-acquired and ventilator-associated pneumonia in the U.S. and worldwide, accounting for nearly one in five bacterial pneumonias (Jones 2010). The incidence of *P. aeruginosa* pneumonia increases with advancing age (Venier and others 2011), as does its associated mortality (Tumbarello and others 2013). This combination makes it a particularly problematic disease in the elderly. *Pseudomonas* pneumonias induce an intense pro-inflammatory response in the first 24 hours after infection, largely characterized by neutrophil infiltration and activation (McConnell and others 2010). This crucial early innate immune response depends not only on neutrophil activation and phagocytic ability, but also on mobilization and directed migration to the site of infection. Given the changes that occur to the immune system with advancing age (Plackett and others 2004), it is likely that this immune response may be significantly altered in the elderly, accounting for their increased susceptibility to *P. aeruginosa* infections. A greater understanding of the mechanisms of age-related innate immune dysfunction in the setting of pneumonia is required to address this growing at-risk patient population. Clinical evidence and animal studies have repeatedly demonstrated diminished neutrophil function with advanced age though the precise mechanisms of neutrophil impairment may depend on the context and nature of the insult. The studies herein investigate the neutrophilic response in aged and young mice to the clinically relevant setting of a pulmonary infection with *P. aeruginosa*.

2. Methods

2.1. Animals

Young (10–12 week) and aged (18–20 month) female BALB/c mice were obtained from the National Institute of Aging colony at Charles River Laboratories (Wilmington, MA) and allowed to acclimate at the Loyola University Chicago Animal Care Facility. Mice were housed under specific pathogen-free conditions on a 12-hour light and dark cycle with free access to food and water. All animal studies were performed with strict accordance to and approval of Loyola's Animal Care and Use Committee.

2.2. Intratracheal infection

Mice were anesthetized with an intraperitoneal injection of a combination of ketamine (100 mg/kg) and xylazine (10 mg/kg). Animals then received an intratracheal inoculation with *Pseudomonas aeruginosa* (ATCC #19660) as previously described (Davis and others 2004; Murdoch and others 2008) with minor modifications. Briefly, mice were placed in a supine position and a small incision was made to expose the trachea. The inoculum was given by instilling 100 μ L of a predetermined concentration of *P. aeruginosa* followed by 100 μ L of air directly into the trachea. The survival analysis was performed using *P. aeruginosa* inoculums of 10,000, 40,000, and 100,000 colony forming units (CFU). All other analyses

were performed using a concentration of 40,000 CFU of *P. aeruginosa*. The incision was closed with wound clips and the animals were placed on their abdomen on a 30° incline while recovering from anesthesia. Body temperature was maintained at physiologic levels by placing the cages on heating pads until the animals were fully awake and ambulating. Sham animals were treated in an identical manner except they were injected with 100 µL of sterile saline followed by 100 µL of air.

2.3. Circulating granulocyte counts

Blood was collected via cardiac puncture immediately after sacrifice and put into capillary tubes containing EDTA to prevent coagulation. Peripheral blood samples were then analyzed for differential blood counts using a Heska Multivet veterinary blood analyzer (Heska, Loveland, CO).

2.4. Pulmonary Chemokine Levels

Animals were sacrificed 24 hours after infection. Necropsy was performed and animals with visible tumors were excluded from further analysis. The right lung lobes were removed and snap frozen in liquid nitrogen. The lung lobes were stored at -80°C until ready for further analysis. Frozen tissue was homogenized in BioPlex cell lysis buffer (BioRad, Hercules, CA) according to manufacturer's instructions and analyzed for chemokine (KC) with a BioPlex multiplex bead array according to the manufacturer's specifications. Results were normalized to total protein measured by the BioRad Protein Assay.

2.5. Histopathology

After sacrificing the animal, the upper left lung lobe was inflated with 10% formalin, fixed overnight and embedded in paraffin before being sectioned (5 µm) and stained with hematoxylin and eosin (H&E) as previously described (Patel and others 1999). In a blinded fashion, 10 high power fields (400x) per animal were assessed for neutrophil infiltration into the pulmonary interstitium and air space.

2.6. Myeloperoxidase Activity

To confirm neutrophil counts from the pulmonary intersitium and air space, myeloperoxidase (MPO) activity was determined in bronchoalveolar lavage (BAL) fluid and the subsequent bronchoalveolar lavaged (BAL) lung tissue. BAL fluid was collected by lavaging the lungs with five aliquots of 0.2 mL of RPMI-1640 media as described elsewhere (Davis and others 2004). Lavaged lung lobes were homogenized in phosphate buffer containing 0.5% hexadecyl-trimethylammonium. The samples were then sonicated and the supernatants cleared by centrifugation before incubation with *o*-dianisidine hydrochloride and hydrogen peroxide. MPO content in the samples was determined based on optical density readings from the MPO standard (Sigma Aldrich, St Louis, MO), which was run in parallel. Results were normalized to the total protein content of each sample. Data are listed as MPO activity per mg of protein.

2.7. Statistical Analysis

One-way analysis of variance (ANOVA) was used to determine differences between young and aged mice with and without infection, and Tukey's post-hoc test once significance was achieved. A student's t-test was used to compare MPO levels between young and aged infected mice. A p-value of <0.05 was considered significant. Data are reported as mean values $\pm$ standard error of the mean.

3. Results

3.1. Survival

Optimization of the infection model was balanced to maximize the size of inoculum and maintain 100% survival of young and aged mice. Intratracheal administration of 10,000 CFU is associated with 100% survival of young and aged mice for the first three days after infection (Figure 1). Likewise, 40,000 CFU was associated with 100% survival between both groups at 3 days after infection. Increasing the inoculum to 100,000 CFU is associated with 50% survival in young mice at 3 days after infection and yielded 100% mortality within 24 hours after inoculation in aged mice.

3.2. Circulating Granulocytes

A significant increase in circulating granulocytes was observed in aged mice given infection compared to saline-treated controls (Figure 2). In comparison, no significant change was observed between young mice with and without infection after 24 hours. The continued leukocytosis observed in the aged animals but absent in the young animals 24 hours after bacterial inoculation may indicate the response to a continuing active infection versus a resolving infection, respectively.

3.3. KC Levels

Pulmonary levels of the neutrophil chemokine KC, also known as CXCL1, were measured in lung homogenates and normalized for total protein content of the homogenate. As seen in Figure 3, the concentration of lung KC was not significantly different between young versus aged saline-treated mice. At 24 hours after inoculation with *P. aeruginosa*, pulmonary levels of KC were significantly greater than that of saline-treated mice regardless of age ($p<0.05$). Additionally, levels of KC were significantly higher in aged mice infected with *P. aeruginosa* relative to young mice infected with the bacteria ($p<0.001$).

3.4. Neutrophil Migration and Compartmental Localization

Representative histologic images of young and aged mice treated with either saline or *P. aeruginosa* are shown in Figure 4. The overall neutrophil counts per 10 high power fields were comparable between young and aged saline-treated mice (Figure 5). The number of neutrophils in the lungs of both groups of infected mice were significantly above those of saline-treated mice regardless of age ($p<0.01$). Additionally, young infected mice had significantly more neutrophils in their lungs than aged infected mice ($p<0.001$).

Differences in the location of neutrophils after infection is visually apparent in Figure 4. This was quantified by counting the number of neutrophils per 10 high power fields in the

air space versus the lung parenchyma (Figure 6). Neutrophil counts are nearly negligible in young and aged saline-treated animals. The number of neutrophils in the airspace of young infected mice was significantly greater than all other groups ($p < 0.001$) (Figure 6A). In contrast, the neutrophil accumulation in the airspace of aged infected mice was increased, however this difference was not statistically significant from the saline-treated groups.

A different pattern is seen with parenchymal neutrophils (Figure 6B). While no difference was noted in parenchymal neutrophils between young saline-treated and aged saline-treated mice, there was a trend towards increased parenchymal neutrophils in young infected mice that did not reach statistical significance. The number of neutrophils in the parenchyma of aged infected animals was significantly above that of all other groups ($p < 0.05$).

3.5. Myeloperoxidase Activity

Functional neutrophil activity in infected animals was quantified in terms of respiratory burst and oxidation capacity utilizing MPO levels as a surrogate (Table 1). MPO levels in lung homogenates were similar between young and aged infected mice. In contrast, MPO levels of BAL samples were significantly higher ($p < 0.0001$) in young than in aged infected mice.

4. Discussion

The neutrophil response to intratracheal inoculation of *P. aeruginosa* 24 hours after infection was dependent on the age of the animal. Significant neutrophil accumulation into the air space of younger mice and a resultant higher level of respiratory burst capacity compared to aged mice occurred despite higher pulmonary levels of chemokine KC in aged mice. This apparent inability of aged neutrophils to respond appropriately to chemotactic stimuli is a serious immunologic deficit as neutrophils are a key component in the initial immune response to pulmonary infection. This was demonstrated by Tsai et al where drug-induced neutropenia or CXCR2 blockade resulted in increased susceptibility to intratracheal *P. aeruginosa* infection in young mice. Interestingly, neutrophil depletion or CXCR2 blockade in young mice demonstrated the phenotype of our aged mice after an infectious pulmonary challenge, with a decrease in pulmonary neutrophil accumulation and increased mortality 24 hours after infection compared to controls. While this highlights the important role of early neutrophil chemotaxis in bacterial pneumonia it also suggests that neutrophil dysfunction plays a predominant role in the increased susceptibility of aged mice to pulmonary infections.

In agreement with these findings, *ex vivo* neutrophils from aged mice have been shown to lack directional migration and are less responsive to increasing levels of KC than neutrophils from young mice (Nomellini and others 2012; Nomellini and others 2008). Similarly, neutrophils from aged humans exhibit an impairment in directed migration as well (Sapey and others 2013). This correlates with our findings that after an infection, despite a peripheral blood leukocytosis and increased pulmonary chemotactic stimuli, there is a paucity of neutrophils in the lung, suggesting an appropriate mobilization of neutrophils but an inability in performing directed chemotaxis. One possible explanation could be the age-dependent dysfunction of triggering receptor expressed on myeloid cell-1 (TREM-1)

described by Fortin et al, which is essential for transepithelial migration of neutrophils into the lungs (Klesney-Tait and others 2013). Previous studies using a model of intraperitoneal injection of lipopolysaccharide (LPS) also found heightened levels of KC in the lungs of aged animals relative to sham (Gomez and other 2007; Gomez and others 2009). In contrast to our current work, however, this increase in KC was accompanied by an increase in pulmonary neutrophils. Differences between these studies could be explained by the systemic nature of an LPS injection versus the localized insult of bacteria into the lungs, requiring more directed chemotaxis. This is supported by a skin wound infection model utilizing *Staphylococcus aureus*, where lower numbers of neutrophils were found within the wound of aged mice despite an elevation of KC levels over younger counterparts (Brubaker and others 2013).

The increased susceptibility of the elderly to bacterial pneumonia is also confounded by age-associated baseline inflammation and decreased pulmonary function and reserve. Hinjosa et al demonstrated that the chronic inflammation seen in aged mice caused an upregulation of polymeric immunoglobulin receptor (pIgR) and platelet-activating factor receptor (PAFr), two proteins implicit in the attachment and invasion of bacteria in the lung. They also showed that baseline “inflamm-aging” created Toll-like receptor (TLR) dysfunction, further exacerbating the ability of aged mice to clear pulmonary infections. These results were expanded upon in a study by Shivhankar et al where increased expression of bacterial ligands including PAFr in the lungs was correlated with mortality after a pulmonary infectious challenge. Furthermore, they were able to find similar markers of cellular senescence in lung tissue from both elderly humans and mice, demonstrating the clinical relevance of the aged mouse model.

In addition to the age-induced changes at a cellular level that predispose the elderly to pneumonias, patients of advanced age have a myriad of functional deficits that increase their morbidity and mortality in this setting. As reviewed by Lowery et al, an aged phenotype demonstrates decreased pulmonary reserve, cough strength, and mucociliary clearance, all of which contribute to worsened outcomes after pneumonia.

5. Conclusion

These studies demonstrate the effects of age-induced immunosenescence on a *Pseudomonas* tracheopulmonary infection. The results demonstrate that in aged mice, neutrophils fail to accumulate within the lung despite the infectious insult and elevated levels of KC. Further studies will need to be conducted to determine the mechanisms behind this aberrant response.

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Highlights

- Aged mice exhibit greater mortality than young mice after pulmonary bacterial infection
- Despite an increased leukocytosis and pulmonary KC levels, aged mice accumulate less pulmonary neutrophils after infection
- The abated neutrophil response was accompanied by an apparent inability of neutrophils to extravasate into the air space.
- Aging impairs directed neutrophil migration which may contribute to the susceptibility of the elderly to bacterial pneumonia

Figure 1.

Survival after intratracheal infection with *Pseudomonas aeruginosa* at low dose (10,000 CFU) and high dose (100,000 CFU) inoculums. Saline control and mid dose (40,000 CFU) demonstrated 100% survival and is not shown.

Figure 2. Circulating granulocytes. Blood was collected after sacrifice and analyzed for number of granulocytes. N = 4 animals per groups. *p<0.05 compared to young infected and aged saline treated mice.

Figure 3.

KC levels in lung homogenates. Lung lobes were homogenized and analyzed for KC content using multiplex bead array. Results were normalized to the total protein present in each sample. Data are presented as mean $\pm$ standard error of the mean. N = 4 animals per group. *p< 0.01 compared to young saline or aged saline. #p<0.001 compared to young infected.

Figure 4.

Pulmonary histology. H&E stained lung tissue sections from (A) young saline, (B) aged saline, (C) young infected, and (D) aged infected mice. Representative sections of 3 experiments are shown at 200x magnification. Arrows indicate neutrophils in the airspace (C) and pulmonary parenchyma (D).

Figure 5.

Total pulmonary neutrophil count per 10 high power fields (includes air space and parenchyma). Data are presented as mean $\pm$ standard error of the mean, $n = 4$ animals per group. * $p < 0.01$ compared to young saline or aged saline. # $p < 0.001$ compared to young infected.

Figure 6.

Compartmental localization of neutrophils in the lung. (A) Neutrophil count in the air space per 10 high power fields. (B) Neutrophil count in the pulmonary parenchyma per 10 high power fields. Data are presented as mean $\pm$ standard error of the mean. N = 4 animals per group. *p<0.001 compared to all other groups. #p<0.05 compared to all other groups.

Table 1

Pulmonary myeloperoxidase activity.

MPO activity was measured in bronchoalveolar lavage fluid (BALF) from young and aged infected mice, as well as the lavaged lung homogenates obtained from the same animals. Data are presented as mean $\pm$ standard error of the mean. N = 4 animals per group.

Young PA Homogenate	2.6 $\pm$ 1.4
Aged PA Homogenate	4.8 $\pm$ 1.2
Young PA BALF	24.2 $\pm$ 1.6 *
Aged PA BALF	2.8 $\pm$ 1.8

*
p < 0.0001 compared to all other groups.