

Comparative Transcriptional Profiles of the Pulmonary Innate Immune Response to Isogenic Antibiotic-Susceptible and Multidrug-Resistant *P. aeruginosa*

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ABSTRACT

BACKGROUND: *Pseudomonas aeruginosa* is a common cause of pneumonia in critically-ill and immunocompromised patients who can rapidly progress to sepsis, multiorgan failure and death if appropriate antibiotic therapy is not administered early in the course of infection. However, clinicians have long recognized that multidrug-resistant (MDR) strains of *P. aeruginosa* rarely progress to fulminate sepsis in the first 72 hours, possibly due to reduced fitness of these strains *in vivo* relative to the fully-antibiotic susceptible strains. We hypothesized that differences in the clinical presentation of MDR *P. aeruginosa* may result from altered patterns of activation of the host innate immune responses compared to antibiotic-susceptible strains.

METHODS: We compared the transcriptional response of 84 genes involved in the innate immune response in the lungs of mice infected with matched strains of antibiotic-susceptible and multidrug resistant *P. aeruginosa*. Wild type *P. aeruginosa* (PAO1) and an isogenic matched strain that over-expresses a multidrug efflux pump and loss of outer membrane porin (Δ MexR/ Δ OprD PAO1). Antibiotic susceptibility and pathogen growth rates were confirmed prior to infection. Isolates were instilled in the trachea (1×10^8 CFU) of anesthetized intubated Balb/c mice. At serial timepoints (1, 24 and 72 hours after infection), groups of 5 mice were euthanized and lungs were excised for isolation of total lung RNA (n=3) or histological examination (n=2). Total lung RNA quality was verified using Agilent RNA 6000 Bioanalyzer kits and reverse transcribed (1 μ g) into cDNA. Expression of 84 genes involved in the inflammatory response, pathogen recognition and defense, cell signaling and sepsis were then analyzed using real-time-PCR arrays (SA Biosciences, Fredrick, MD). Gene expression was calculated in relation to uninfected control mice and 5 housekeeping genes using software provided by the manufacturer ($\Delta\Delta$ CT method).

RESULTS: Antibiotic susceptibility to 3 antibiotic classes was confirmed in the Δ MexR/ Δ OprD PAO1 strain versus wildtype PAO1, although the growth rates of both strains *in vitro* were equivalent. Infection with the WT-PAO1 was associated with increased expression of genes involved in the recognition of the Gram negative bacterial cell wall (TLR2, Pglyrp3, Pglyrp3), inflammation (TNF α , IL-1, IL-6), chemokines (CCL1, CCL6), complement and collectin production (C3a, C1p, Dmbt1), and sepsis (Nos2, Protein C). Although pathogen recognition and proinflammatory cytokine signatures were similar in the Δ MexR/ Δ OprD PAO1 infected animals, significantly lower expression levels of complement, collectin (C3a, C1p, Dmbt1) and sepsis markers (Nos2, Protein C) were found in animals infected with the MDR strain. Histologically, lungs from both animal groups exhibited evidence of pneumonia with acute inflammatory necrosis and consolidation. However, inflammation and consolidation was more widespread in animals infected with the antibiotic-susceptible versus the MDR strain.

CONCLUSIONS: Expression of common antibiotic resistance mechanisms (efflux, porin loss) in *P. aeruginosa* was associated with altered patterns of innate immune system activation in experimental pneumonia. Attenuated responses in complement activation may explain why patients with MDR *P. aeruginosa* are less likely to present with fulminate pneumonia and sepsis in the first 72 hours of infection. Further studies are underway to explore differences in complement binding between antibiotic susceptible and MDR *P. aeruginosa*.

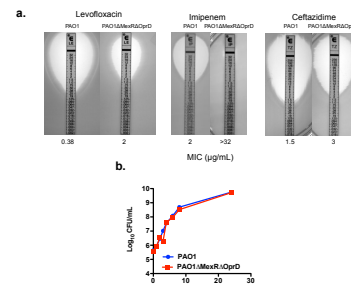
BACKGROUND

- Pseudomonas aeruginosa* is the 5th most commonly isolated bloodstream pathogen in U.S. hospitals and a common cause of pneumonia in mechanically-ventilated patients
- If not detected early and appropriately treated, *P. aeruginosa* pneumonia can rapidly progress to respiratory failure with sepsis that is associated with high rates of morbidity and mortality
- The management of *P. aeruginosa* infection is complicated by the propensity of this bacterial species to develop resistance to multiple classes of antibiotics (e.g., β -lactams, cephalosporins, carbapenems, fluoroquinolones)
- Clinicians have long known that unlike antibiotic-susceptible strains, it is uncommon for patients infected with multi-drug resistant (MDR) strains of *P. aeruginosa* to progress to respiratory failure or sepsis in the first 72 hours of infection. This difference has long been assumed to be a consequence of the impaired "fitness" of the MDR strain
- Because the pathology of acute respiratory failure and sepsis are driven in part by the host immune response, we hypothesized that MDR strains may activate host innate immune responses differently than antibiotic-susceptible strains
- To explore this hypothesis, we compared the transcriptional signatures of the host innate immune response in the lungs of mice infected with isogenic antibiotic-susceptible and MDR *P. aeruginosa*

METHODS

- Isogenetic test isolates**
 - Antibiotic susceptible (PAO1)
 - Multi-drug efflux overexpression, loss of outer membrane porin, OprD (Δ MexR/ Δ OprD PAO1)
 - Subcultured on 5% blood agar before growth in cation-adjusted Mueller-Hinton Broth (10^8 CFU/mL)
 - Cells were centrifuged ($12,000 \times g$) at 4°C before resuspension in sterile 0.85% saline.

Figure 1. Confirmation of isolate susceptibility and *in vitro* fitness



(A) Mean inhibitory concentrations (MICs) of the test isolates were confirmed using Epikermic strips (AB BioRxiv, Solna, Sweden) according to the manufacturer's instructions. Overexpression of the MexA-MexR-OprM multidrug efflux pumps results in elevated MICs for levofloxacin, imipenem, and cefazidime. Loss of outer membrane porin (OprD) impairs imipenem entry into the cell. (B) Comparison of the growth rates of the antibiotic susceptible (PAO1) and the multidrug-resistant strains in cation-adjusted Mueller-Hinton broth. A standardized inoculum (10^8 CFU) of each test isolate was prepared in growth media. At serial timepoints, samples were removed and plated ($50 \mu\text{L}$) on blood agar using a spiral plater for enumeration of viable bacteria. Growth rates were determined to be essentially identical for the antibiotic-susceptible and MDR strains.

Figure 2. Schematic of experimental murine pneumonia model

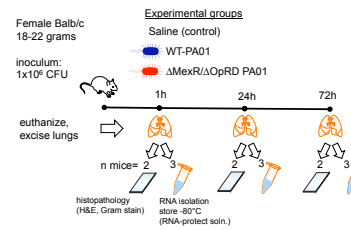
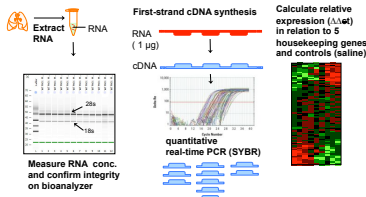
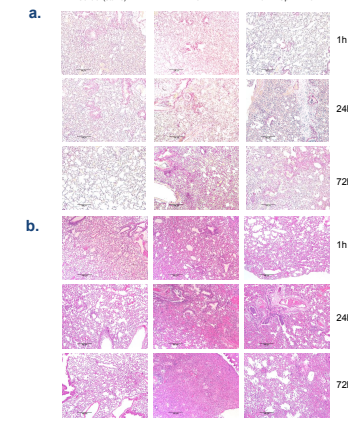


Figure 3. Determination of innate immune response transcriptional profile in mouse lungs



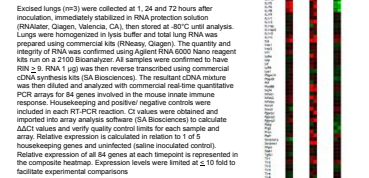
RESULTS

Figure 4. Δ MexR/ Δ OprD PAO1 produces less severe pneumonia in the experimental model relative to the isogenic antibiotic-susceptible PAO1 strain



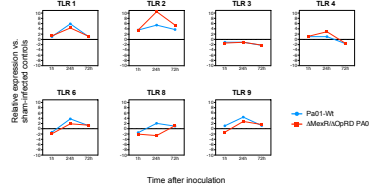
Excised lungs were fixed in 10% formaldehyde, embedded in paraffin and processed for (Fig 4a) Gram staining or (Fig 4b) hematoxylin-eosin staining to assess the severity of pneumonia. Increased retention of the Gram counter stain (nuclei) is evident around bronchioles in the PAO1 vs. Δ MexR/ Δ OprD PAO1 infected animals by 72 hours (1st row, Fig 4a). H&E staining (Figure 4b) demonstrates the presence of pneumonia in both PAO1 and Δ MexR/ Δ OprD PAO1 infected animals. However, inflammation and consolidation of the lung is much more extensive in the PAO1-infected animals.

Figure 5. Δ MexR/ Δ OprD PAO1 elicits a different innate immune response in the lung of mice compared to the isogenic antibiotic-susceptible PAO1 strain



Excised lungs (n=3) were collected at 1, 24 and 72 hours after inoculation, immediately stabilized in RNA protection solution (RNAlater, Qiagen, Valencia, CA), then stored at 80°C until analysis. Lungs were homogenized in lysis buffer and total lung RNA was prepared using commercial kits (RNeasy, Qiagen). The quantity and integrity of RNA was confirmed using Agilent RNA 6000 Nano reagent kits run on a 2100 Bioanalyzer. All samples were confirmed to have RIN ≥ 9 . RNA (1 μ g) was then reverse transcribed using commercial cDNA synthesis kits (SA Biosciences). The resultant cDNA mixture was then diluted and analyzed with commercial real-time quantitative PCR arrays for 84 genes involved in the mouse innate immune response. Housekeeping and positive/negative controls were included in each RT-PCR reaction. $\Delta\Delta$ values were obtained and reported into array analysis software (SA Biosciences) to calculate $\Delta\Delta\Delta$ values and verify quality control limits for each sample and array. Relative expression is calculated in relation to 1 \pm 5 housekeeping genes and uninfected (saline inoculated control). Relative expression of all 84 genes reported is presented in the composite heatmap. Expression levels were limited at ≤ 10 fold to facilitate experimental comparisons.

Figure 6. Δ MexR/ Δ OprD PAO1 elicits similar patterns of PAMP activation as the isogenic antibiotic-susceptible PAO1 strain



CONCLUSIONS

Figure 7. Δ MexR/ Δ OprD PAO1 elicits similar patterns of pro-inflammatory cytokine and chemokine activation as the isogenic antibiotic-susceptible PAO1 strain

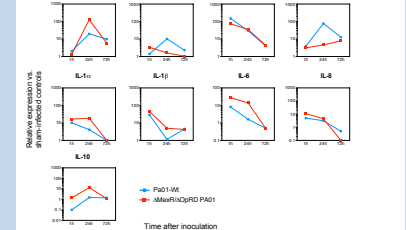
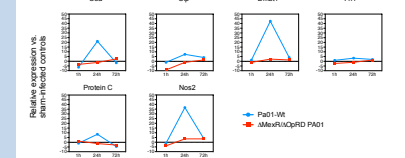
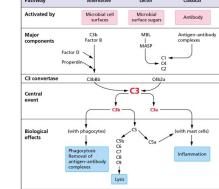


Figure 8. Activation of alternative complement pathway and sepsis markers are attenuated in mice infected with Δ MexR/ Δ OprD PAO1 versus the isogenic antibiotic-susceptible PAO1 strain



CONCLUSIONS

- Similar to previous studies, we found that *P. aeruginosa* elicits a pronounced pro-inflammatory response in the lungs of mice that appears to be elicited through recognition of conserved bacterial PAMP (i.e. LPS, peptidoglycans)
- Despite producing less severe pneumonia in the animals, infection with the Δ MexR/ Δ OprD PAO1 strain was associated with similar patterns and magnitude of pro-inflammatory cytokine, chemokine and PAMP gene activation as the wild-type PAO1 strain
- Expression patterns of several genes involved in complement synthesis and sepsis, however, were markedly different between the isogenic MDR and antibiotic-susceptible strain, suggesting that activation of the alternative complement pathway may be attenuated in the Δ MexR/ Δ OprD PAO1 versus wildtype PAO1. This difference could explain (in part) the difference in clinical syndromes observed between antibiotic-susceptible and MDR strains of *P. aeruginosa*
- We are currently exploring differences in complement activation between the Δ MexR/ Δ OprD PAO1 versus wildtype PAO1 strain as well as clinical isolates



ACKNOWLEDGEMENTS

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All animals used in this study were handled with the highest standards of humane and ethical care in accordance with the University of Houston Institutional Animal Care and Use Committee guidelines.