Innate immune effectors modulate antibiotic efficacy against *Pseudomonas aeruginosa*

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Muco-obstructive airway diseases (MADs) are a collection of diseases characterized by dehydration and accumulation of airway mucus. The accumulation of mucus leads to airway obstruction, inflammation, and provides a niche for many pathogens. *Pseudomonas aeruginosa* is the dominant pathogen in MADs. Inflammation in the MADs airway is a result of heavy neutrophil infiltration and the release of antimicrobial effectors. The most abundant effectors in the MADs airway are neutrophil elastase (NE), a serine protease, and calprotectin (CP), and zinc and manganese (Zn/Mn) chelator. However, despite such high concentrations of NE and CP, *Pa* remains resilient.

A common feature of chronic *Pa* infection in MADs is antibiotic treatment failure. Antibiotic tolerance is thought to be a major driver of treatment failure in the MADs lung environment. In this study, we set out to identify the impact of NE and CP towards antibiotic tolerance. To study *Pa* antibiotic tolerance in the context of MADs, we use a defined synthetic mucus medium (SMM) that recapitulates the MADs airway environment. We grew *Pa* in our SMM containing 2% mucin, supplemented with NE or CP, then treated with tobramycin and assessed bacterial survival. We found that the addition of NE and CP gave differential responses as NE increased survival and CP decreased survival to tobramycin.

It was previously reported that NE degrades *Pa* flagella. Thus, we investigated how loss of flagella impacts antibiotic tolerance. We found that a flagellar mutant exhibits increased tolerance to tobramycin. Further, we found that co-culture of a flagellar mutant with neutrophils increases neutrophil extracellular trap formation and a flagellar mutant better resists neutrophil killing compared to wild-type. We also screened clonally related clinical isolate pairs for differences in tolerance. We found that the isolate with the higher tolerance had lower swimming motility. These data suggest that loss of flagella provides a fitness advantage both in resisting neutrophil killing and tolerating antibiotics and provides a rationale for the strong selection of flagellar mutants seen in the clinical space.

To investigate how CP impacts antibiotic tolerance, we added excess Zn/Mn to our SMM and found that repletion of these metals restored the antibiotic tolerance phenotype in the presence of CP back to normal SMM levels. Measurements of Zn/Mn in our SMM revealed that our base media, without mucus, contains fleeting amounts of Zn/Mn, whereas the addition of mucus increases Zn/Mn levels comparable to that found in diseased mucus. Addition of Zn/Mn to our base media significantly increased antibiotic tolerance. Taken together, this suggests Zn/Mn play a significant role in antibiotic tolerance. Overall, this study highlights the complexity of the MADs lung environment and how the innate immune system contributes to *Pa* antibiotic tolerance.