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***Pseudomonas aeruginosa*: breaking down barriers**

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Abstract

Many bacterial pathogens have evolved ingenious ways to escape from the lung during pneumonia to cause bacteremia. Unfortunately, the clinical consequences of this spread to the bloodstream are frequently dire. It is therefore important to understand the molecular mechanisms used by pathogens to breach the lung barrier. We have recently shown that *Pseudomonas aeruginosa*, one of the leading causes of hospital-acquired pneumonia, utilizes the type III secretion system effector ExoS to intoxicate pulmonary epithelial cells. Injection of these cells leads to localized disruption of the pulmonary-vascular barrier and dissemination of *P. aeruginosa* to the bloodstream. We put these data in the context of previous studies to provide a holistic model of *P. aeruginosa* dissemination from the lung. Finally, we compare *P. aeruginosa* dissemination to that of other bacteria to highlight the complexity of bacterial pneumonia. Although respiratory pathogens use distinct and intricate strategies to escape from the lungs, a thorough understanding of these processes can lay the foundation for new therapeutic approaches for bacterial pneumonia.

Keywords

Pseudomonas aeruginosa; pneumonia; bacterial dissemination; type III secretion; ExoS

Introduction

Pseudomonas aeruginosa is one of the leading causes of nosocomial infections, including hospital-acquired pneumonia (HAP), bloodstream, urinary tract, wound, and burn infections (Stryjewski 2003). In the context of HAP, *P. aeruginosa* accounts for up to 20% of all cases and has an attributable mortality rate of ~30% (Fagon et al. 1989; George et al. 1998; Heyland et al. 1999; Rello et al. 1997). During HAP *P. aeruginosa* has the ability to disrupt the lung barrier leading to *P. aeruginosa* bacteremia, which is a particularly poor prognostic sign for clinical outcomes (Magret et al. 2011). However, the mechanism by which *P. aeruginosa* breaches the epithelial and endothelial barriers has remained a mystery until recently. This review highlights recent advances in our understanding of *P. aeruginosa* pneumonia and compares the mechanism of lung barrier disruption to those of other bacterial pathogens. Knowledge of the molecular mechanisms of disease for multiple pathogens could provide valuable insights for clinical treatment of bacterial pneumonia.

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Of the many virulence factors employed by *P. aeruginosa* to cause disease, the type III secretion system (T3SS) has garnered much attention due to its near ubiquity among *P. aeruginosa* clinical isolates and the epidemiological link between T3SS attributes and worse clinical outcomes and higher mortality rates in humans (El-Solh et al. 2012; Pena et al. 2013). Recent studies have also highlighted the T3SS contribution to pathogenesis in multiple animal models of disease, including acute pneumonia, bacteremia, burn infections, keratitis, and peritonitis (Hauser 2009). Understanding the molecular mechanisms of type III-mediated disease pathogenesis could allow for the design of novel preventatives and therapeutics to combat *P. aeruginosa* infections.

The T3SS in *P. aeruginosa* consists of 36 genes, which together provide for the elaboration and regulation of a supramolecular “needle” structure that extends away from the bacterium and can penetrate the host cell membrane (Hauser 2009). The needle structure spans the inner and outer membranes and peptidoglycan layer of the bacterium and provides a conduit through which effector proteins can be secreted directly from the cytosol of the bacterium into the host cell. To date four effector proteins have been identified: ExoS, ExoT, ExoU, and ExoY. While nearly all strains harbor the genes encoding the T3SS needle apparatus, clinical isolates vary in their carriage of the different effector genes (Feltman et al. 2001).

ExoS is a bi-functional toxin encoded by the *exoS* gene, which is found in approximately 70–80% of *P. aeruginosa* clinical isolates. Containing both GTPase activating protein (GAP) and ADP-ribosyltransferase (ADPRT) domains, ExoS has a multi-pronged approach to disrupt the host. The GAP domain targets the small GTPases Rho, Rac, and Cdc42, keeping them in the inactive GDP-bound form, which serves to disrupt the actin cytoskeleton of the host cell and inhibit phagocytosis of the bacteria in cell culture experiments (Goehring et al. 1999; Pederson et al. 1999). The ADPRT domain becomes activated after binding to a eukaryotic cofactor and causes host cell death, disruption of the actin cytoskeleton, inhibition of endocytosis and disruption of vesicular trafficking (Barbieri et al. 2001; Fraylick et al. 2001; Pederson and Barbieri 1998; Rocha et al. 2003). Interestingly, studies examining epithelial monolayers under cell culture conditions showed that type III secretion in general and ExoS in particular facilitated movement of *P. aeruginosa* across the monolayer (Heiniger et al. 2010; Soong et al. 2008). In addition to its pathogenic effects on cells grown in culture, ExoS causes decreased survival and greater bacterial dissemination from the lung in a mouse model of acute pneumonia (Shaver and Hauser 2004). Likewise, a functional type III secretion system was necessary for transversal of *P. aeruginosa* across a corneal epithelium (Sullivan et al. 2015). However, until recently the mechanism behind enhanced bacterial dissemination and the cell types targeted by ExoS during pneumonia still remained unclear.

Mechanism of *P. aeruginosa* dissemination from the lung

In a recent study by our group, we adapted the CCF2-AM/ β -lactamase reporter assay to develop a novel imaging approach for monitoring the contribution of type III injection of ExoS to disease pathogenesis during acute murine pneumonia (Rangel et al. 2015). In this study, intact lungs from mice infected with a strain of *P. aeruginosa* secreting ExoS fused to β -lactamase were incubated with the β -lactam substrate CCF2-AM before being fixed,

frozen, and sectioned for viewing by fluorescence microscopy. Injected cells were identified and quantified by TissueQuest software, which detected the blue fluorescence emitted by the cleaved CCF2. Unlike previous studies, this adaptation allowed for viewing and quantification of injected cells in the context of the normal tissue architecture.

Using this technique, we discovered that ExoS is preferentially injected into phagocytes early during infection (first 12 hours), with almost no injection of type I or type II pneumocytes. However, by 18–24 hours post-infection, type I pneumocytes accounted for nearly 40% of all cells injected with ExoS. Interestingly, injection of ExoS occurred in discrete areas of the lung termed “fields of cell injection” (FOCI) that became larger as the infection progressed. Expansion of the FOCI was accompanied by cell death and disruption of the epithelial barrier of the lung, all of which were dependent on the ADPRT activity of ExoS but not the GAP activity. Loss of integrity of the epithelial barrier allowed bacteria to cross into the bloodstream and disseminate throughout the body (Rangel et al. 2015).

These findings provide a missing piece that combined with previous data in the field allows for a holistic model of how *P. aeruginosa* disseminates from the lung into the bloodstream (see figure). First, *P. aeruginosa* uses the type III secretion machinery to inject invading leukocytes, with the ADPRT activity of ExoS preventing phagocytosis of the bacteria (Rangel et al. 2014). Second, ExoS is injected into type I pneumocytes, creating FOCI composed of dead and compromised cells, resulting in disruption of the epithelial barrier (Rangel et al. 2015). Third, *P. aeruginosa* elaborates a type II secretion system to export LasB, an extracellular protease that cleaves extracellular matrix components, as well as vascular endothelium cadherin (VE-cadherin). LasB cleavage of VE-cadherin disrupts the homotypic interactions between VE-cadherin subunits and disrupts adherens junctions, which provide anchors between neighboring endothelial cells (Golovkine et al. 2014). Injection of type III effectors leads to the rapid retraction of endothelial cells and further disruption of the endothelial barrier (Golovkine et al. 2014; Huber et al. 2014). Thus, LasB and type III effectors work synergistically to bypass the final barrier preventing *P. aeruginosa* from entering the bloodstream and disseminating throughout the body. (Golovkine et al. 2014).

Mechanistic comparison to other bacterial pathogens

Several other bacterial pathogens are known to breach the epithelial and endothelial barriers to disseminate throughout the body and cause disease. *Staphylococcus aureus* is one of the leading causes of nosocomial pneumonia and has increasingly become associated with community-acquired infections (Klebens et al. 2007; Lowy 1998). Work over the last five years has highlighted the ability of *S. aureus* to breach the epithelial and endothelial barriers, allowing for bacterial entry into the bloodstream. Mechanistically, *S. aureus* accomplishes this via its secreted virulence factor α -toxin (α -hemolysin, Hla) in a mechanism with similarities to that employed by *P. aeruginosa*. Hla is a pore-forming toxin that has been shown to be required for disease pathogenesis in *S. aureus* pneumonia (Bubeck Wardenburg et al. 2007; Bubeck Wardenburg and Schneewind 2008). Secreted as a water-soluble monomer, Hla binds to host cells via its cellular receptor A Disintegrin And Metalloprotease 10 (ADAM10), which is a zinc- dependent metalloprotease that serves as a cellular sheddase

to regulate cell-surface levels of host proteins (Berube and Bubeck Wardenburg 2013; Saftig and Hartmann 2005; Wilke and Bubeck Wardenburg 2010). Upon binding to ADAM10, Hla oligomerizes into a heptameric structure and forms a 2–3 nm β -barrel pore that leads to the upregulation of ADAM10 metalloprotease activity and untimely cleavage of ADAM10 substrates including E- and VE-cadherin leading to the disruption of both the epithelial and endothelial barriers, which provides direct access to the bloodstream (Gouaux 1998; Inoshima et al. 2011; Inoshima et al. 2012; Song et al. 1996). Thus, *S. aureus* makes use of a single virulence factor to accomplish what *P. aeruginosa* does with both ExoS and LasB. In the context of *P. aeruginosa*, it is interesting that LasB is capable of cleaving VE-cadherin, but is unable to cleave the closely related E-cadherin (Golovkine et al. 2014), necessitating the use of type III effectors to disrupt the epithelial barrier.

Streptococcus pneumoniae is another leading cause of pneumonia, but unlike *P. aeruginosa*, it primarily causes community-acquired pneumonia. Bacteremic pneumonia with *S. pneumoniae* occurs in 20–25% of cases with an attributable mortality rate of 10–30% (Kalin et al. 2000; Marfin et al. 1995; Mufson 1981; Torres et al. 1998). Mechanistically, *S. pneumoniae* dissemination into the bloodstream is very different from that caused by *P. aeruginosa* or *S. aureus*. In the case of *S. pneumoniae*, inflammation is the primary driver of lung barrier disruption; immune cell infiltration into the lung is thought to be responsible for dissemination into the bloodstream. Neutrophils, dendritic cells, and mast cells have all been implicated in allowing bacterial translocation across the epithelial and endothelial barriers (Penaloza et al. 2015; Rosendahl et al. 2013; van den Boogaard et al. 2014) with pro-inflammatory factors, such as IL-8, von Willebrand factor, Hepoxilin A₃, and CCAAT/enhancer-binding protein δ (CEBPD)-mediated upregulation of pro-inflammatory cytokines all contributing to bacterial dissemination (Bhowmick et al. 2013; Duitman et al. 2012b; Lutge et al. 2012). Additionally, the anti-inflammatory molecules IL-10 and activated protein C help to prevent bacterial dissemination (de Boer et al. 2014; Penaloza et al. 2015), confirming the primary role of the host inflammatory response in bacterial dissemination.

Finally, *Klebsiella pneumoniae* is another major cause of HAP with mortality rates of up to 50% following bacterial dissemination into the blood (Podschun and Ullmann 1998). To date no virulence factors have been identified that directly contribute to lung barrier disruption, and unlike *S. pneumoniae* inflammation appears to be beneficial to the host in both killing *K. pneumoniae* and blocking bacterial dissemination. Pro-inflammatory mediators such as tumor necrosis factor α (TNF α), gamma interferon, G-CSF, IL-22, myeloid-related protein-14, and CEBPD are all required for host protection against *K. pneumoniae* (Achouiti et al. 2012; Balamayooran et al. 2012; Duitman et al. 2012a; Laichalk et al. 1996; Moore et al. 2002; Xu et al. 2014), while molecules that blunt the inflammatory response, such as interleukin-1 receptor-associated kinase M and thrombospondin-1 enhance bacterial dissemination (Hoogerwerf et al. 2012; Zhao et al. 2015). In the future, it will be important to determine the exact mechanisms by which *K. pneumoniae* causes dissemination and the virulence factors that facilitate this process.

Conclusion

Recent work has contributed substantial knowledge as to how bacterial pathogens can disseminate into the blood during pneumonia. These studies aim to help inform treatment in the clinic by providing basic knowledge of bacterial pathogenesis as well as what constitutes a productive host response. However, the work detailed in this review makes it clear the mechanisms for bacterial virulence and dissemination to the bloodstream can vary substantially among different pathogens. An immune response that protects against one pathogen can be detrimental in the context of another, as is the case with CEBPD-mediated transcriptional regulation, which is host-protective in the case of *K. pneumoniae* infection, but augments pathogenesis of *S. pneumoniae* (Duitman et al. 2012a; Duitman et al. 2012b). Thus, treatment in the clinic will depend on accurate identification of the disease-causing organism, as well as knowledge of the molecular mechanisms of disease. In the future, it will be important to continue to refine our knowledge of disease pathogenesis and to search for novel virulence factors that may play a role in infection. This will allow for targeted approaches to combat bacterial pathogens in an era of continuing evolution of antibiotic resistance.

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Figure 1.

Model of *P. aeruginosa* dissemination from the lung into the bloodstream. 1) Following bacterial entry into the airways, neutrophils are recruited to the alveolar space where they engage *P. aeruginosa* in an attempt to clear the infection. *P. aeruginosa* uses the type III secretion system to inject ExoS into neutrophils, which blocks phagocytosis of the bacteria and allows for bacterial persistence. 2) *P. aeruginosa* attaches to airway epithelial cells. Injection of ExoS into type I pneumocytes leads to the creation of “FOCI” of dead and compromised cells and the disruption of the epithelial barrier. 3) *P. aeruginosa* uses a type II secretion system to secrete LasB, a protease that cleaves VE-cadherin and leads to the disruption of the adherens junction of vascular endothelial cells. 4) Following endothelial cell disruption, *P. aeruginosa* has access to the bloodstream and disseminates throughout the body.