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Pneumolysin expression by *streptococcus pneumoniae* protects colonized mice from influenza virus-induced disease

Amaya I. Wolf [a], Maura C. Strauman [a], Krystyna Mozdzanowska [a], Katie L. Williams [a], Lisa C. Osborne [b], Hao Shen [b], Qin Liu [a], David Garlick [a], David Artis [b], Scott E. Hensley [a], Andrew J. Caton [a], Jeffrey N. Weiser [b], Jan Erikson [a,*]

[a] *The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104, United States of America*
[b] *University of Pennsylvania, Philadelphia, PA 19104, United States of America*

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ABSTRACT

The response to influenza virus (IAV) infection and severity of disease is highly variable in humans. We hypothesized that one factor contributing to this variability is the presence of specific respiratory tract (RT) microbes. One such microbe is *Streptococcus pneumoniae* (*Sp*) that is carried asymptomatically in the RT of many humans. In a mouse co-infection model we found that in contrast to secondary bacterial infection that exacerbates disease, *Sp* colonization 10 days prior to IAV protects from virus-induced morbidity and lung pathology. Using mutant *Sp* strains, we identified a critical role for the bacterial virulence factor pneumolysin (PLY) in mediating this protection. Colonization with the PLY-sufficient *Sp* strain induces expression of the immune-suppressive enzyme arginase 1 in alveolar macrophages (aMø) and correlates with attenuated recruitment and function of pulmonary inflammatory cells. Our study demonstrates a novel role for PLY in *Sp*-mediated protection by maintaining aMø as "gatekeepers" against virus-induced immunopathology.

Introduction

Respiratory tract (RT) infections remain a major health problem worldwide. Influenza A viruses and *Streptococcus pneumoniae* (*Sp*) are two pathogens most often associated with RT co-infections (Mccullers, 2006), yet the sequence in which humans are infected with influenza virus and *Sp* is not known. McCullers et al. were first to show in a mouse co-infection model that the order of pathogen exposure critically determines beneficial versus detrimental outcomes (McCullers and Rehg, 2002); when *Sp* preceded infection with influenza virus, mortality was reduced, whereas *Sp* infection following a viral infection exacerbated disease. Secondary bacterial infections have been well-studied. Excessive lung tissue pathology has been ascribed to post-influenza suppression of innate immune cell functions and inhibition of bacterial clearance. In turn, bacterial outgrowth is thought to lead to excessive cell infiltrations into the lung and increased inflammatory responses (Mccullers, 2006; Short et al., 2012).

How *Sp* as a RT colonizing microbe impacts immunity to influenza infection has not been investigated. Depending on geographics and socio-economics, *Sp* is carried asymptomatically in the RT of up to 60% of children and 10–40% of adults (Mccullers, 2006). We, and others have shown in mouse studies that elimination of bacteria colonizing mucosal surfaces of the respiratory and intestinal tract with antibiotic treatment can have detrimental effects on anti-influenza immune responses (Abt et al., 2012; Ichinohe et al., 2011). Furthermore, bacterial components in the form of pathogen-associated molecular patterns (PAMPs) recognized by toll-like receptors (TLRs) as well as synthetic TLR ligands can elicit anti-viral resistance and attenuate disease (Evans et al., 2010).

Alveolar macrophages (aMø) play a critical role in lung homeostasis and in protective immunity to viral and bacterial infections (Hussell and Bell, 2014). Depletion of aMø with clodronate-liposomes in both influenza virus and *Sp* infection models results in increased morbidity and exaggerated inflammatory responses (Knapp et al., 2004; Murphy et al., 2011; Schneider et al., 2014; Tate et al., 2010; Tumpey et al., 2005). AMø have potent phagocytic properties facilitating pathogen clearance, and they orchestrate host defenses through the production of inflammatory cytokines and chemokines that activate and attract inflammatory monocytes, neutrophils, and effector T cells to the lungs. These cell populations are necessary for local defense and pathogen clearance, but they can also contribute to tissue injury (La Gruta et al., 2007). Studies have demonstrated that influenza induces TLR desensitization (Didierlaurent et al., 2008) and depletion of aMø

* Corresponding author.
E-mail address: jan@wistar.org (J. Erikson).

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(Ghoneim et al., 2013), which then contributes to increased susceptibility to secondary bacterial infection. The effect of bacterial exposure on Mø function and how this could impact a subsequent influenza infection is less well studied. Recent evidence from a mouse model with *Staphylococcus aureus (S. aureus)* suggest that bacterial exposure induces alternatively activated Mø that mediate immune protection against influenza-induced disease (Wang et al., 2013).

The role of bacterial virulence factors in modulating the host response to influenza virus co-infections has not been studied. The streptococcal virulence factor pneumolysin (PLY) is an important determinant of the inflammatory immune response to *Sp* (Kadioglu et al., 2008; Marriott et al., 2008). PLY is a member of the cholesterol-dependent toxins with pore-forming, lytic and complement activating properties and can act as a ligand for TLR2, TLR4 and NLRP3 (Dessing et al., 2009; Malley et al., 2003; McNeela et al., 2010; van Rossum et al., 2005; Witzenrath et al., 2011). Importantly, PLY has been shown to induce type I IFNs and inflammatory cytokines that in context of viral co-infection could exert anti-viral functions or exacerbate immune responses.

Here, we investigated the *in vivo* host response to influenza virus in the context of *Sp* co-infection. We identified a critical role for PLY-expressing *Sp* in modulating virus-induced disease. Our findings provide a molecular basis for the protective signals from a common RT microbe regulating the sequelae of influenza virus infection.

Results

The order of bacterial co-infection determines the severity of influenza-induced morbidity and lung pathology

A mouse model of RT co-infection was established using mouse-adapted influenza virus A/PR/8/34 (PR8) and the P1121 strain of *S. pneumoniae* (*Sp*, serotype 23F) originally isolated from the human nasopharynx (van Rossum et al., 2005). Mice were infected with PR8 or with P1121 alone, or co-infected either with PR8 and 5 days later with P1121 (PR8+P1121) or first with P1121 and 10 days later with PR8 (P1121+PR8) as depicted in Fig. 1A.

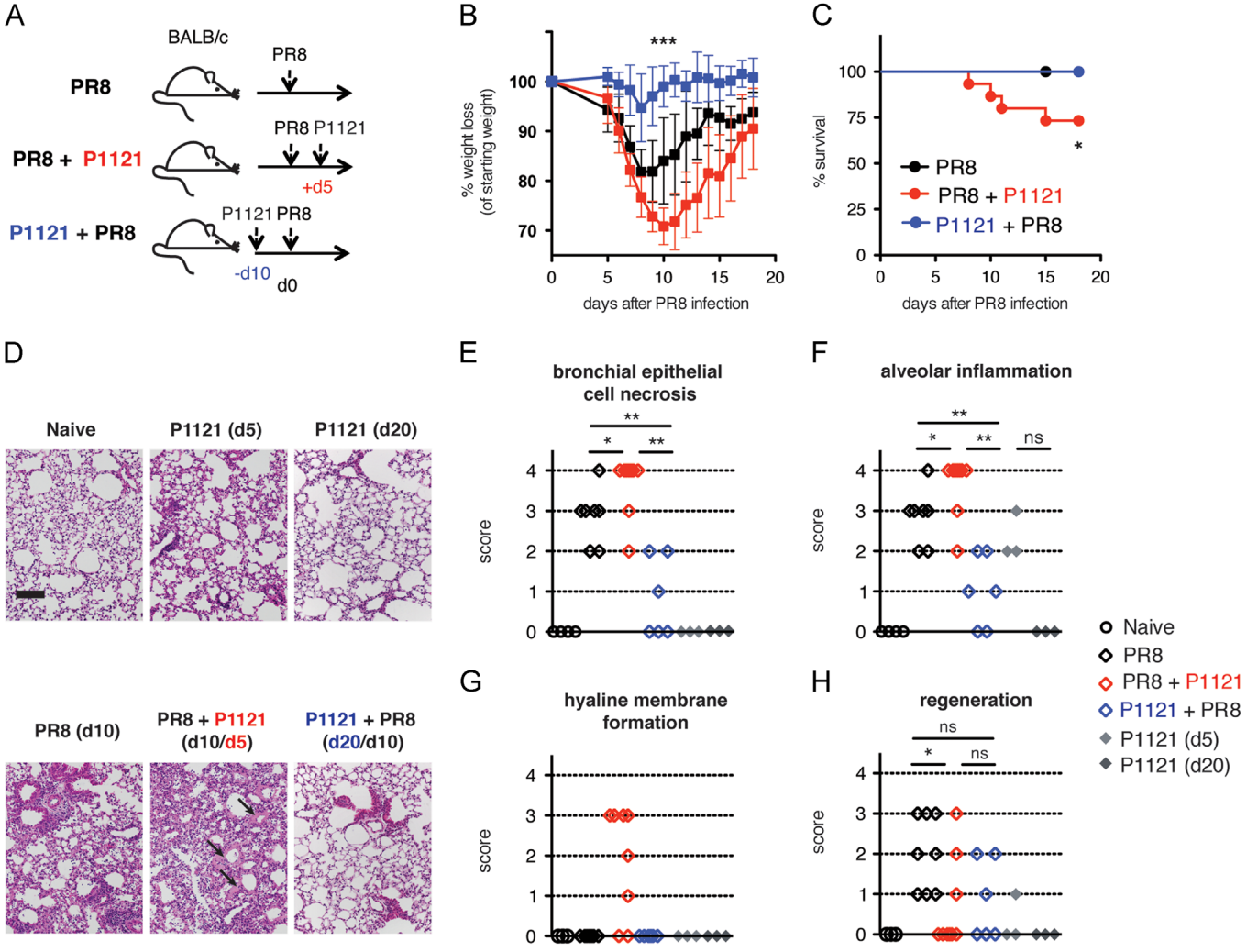

Fig. 1. *Sp* modulates influenza virus-associated morbidity and lung pathology. (A) BALB/c mice were infected with influenza A virus PR8 alone (PR8, black line) or co-infected with *Streptococcus pneumoniae* (*Sp*) P1121 10 days prior to PR8 (P1121+PR8, blue line) and 5 days after PR8 (PR8+P1121, red line). (B) Weight loss from $n=10$–15 mice/group from at least 3 independent experiments combined. Data are expressed as mean ± SD. Co-infected groups were analyzed for statistical significance relative to mice infected with PR8 alone at the peak of weight loss (d10 p.i. PR8). Kruskal–Wallis test. (C) Kaplan–Meier survival ($n=10$–15 mice/group). Log-rank (Mantel–Cox) test. (D) Representative H&E sections from lungs of naive and infected mice at indicated days after infections. Original magnification: 20x. Scale bar: 100 μm. Arrows point to hyaline membrane formation. (E–H) Histopathology scores for bronchial epithelial cell necrosis (E), alveolar inflammation (F), hyaline membrane formation (G) and epithelial cell regeneration (H) corresponding to the time points shown in (D). Results from 3 independent experiments. Mann–Whitney test. NS: not significant $p>0.05$, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.