Antimicrobial peptides alter early immune response to influenza A virus infection in C57BL/6 mice

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ABSTRACT

Influenza is a disease of the respiratory system caused by single stranded RNA viruses with varying genotypes. Immunopathogenesis to influenza viruses differs based on virus strain, dose, and mouse strain used in laboratory models. Although effective mucosal immune defenses are important in early host defense against influenza, information on the kinetics of these immune defense mechanisms during the course of influenza infection is limited. We investigated changes to antimicrobial peptides and primary innate immune cells at early time points after infection and compared these variables between two prominent H1N1 influenza A virus (IAV) strains, A/CA/04/2009 and A/PR/08/1934 in C57BL/6 mice. Alveolar and parenchymal macrophage ratios were altered after IAV infection and pro-inflammatory cytokine production in macrophages was induced after IAV infection. Genes encoding antimicrobial peptides, β-defensin (*Defb4*), bactericidal-permeability increasing protein (*Bpifa1*), and cathelicidin antimicrobial peptide (*Camp*), were differentially regulated after IAV infection and the kinetics of *Defb4* expression differed in response to each virus strain. Beta-defensin reduced infectivity of A/CA/04/2009 virus but not A/PR/08/1934. Beta defensins also changed the innate immune cell profile wherein mice pre-treated with β-defensin had increased alveolar macrophages and CD103^{+} dendritic cells, and reduced CD11b^{+} dendritic cells and neutrophils. In addition to highlighting that immune responses may vary based on influenza virus strain used, our data suggest an important role for antimicrobial peptides in host defense against influenza virus.

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1. Introduction

Influenza is an infectious disease of the pulmonary system caused by viruses of the *Orthomyxoviridae* family. Influenza A virus (IAV) can undergo changes over a period of time in the form of gradual mutations (antigenic drift) or abrupt changes in surface proteins (antigenic shift) which may render the virus highly infectious and/or transmissible thereby reducing the efficacy of vaccines. IAV infections have resulted in four pandemics since 1900, of which the 1918 pandemic alone claimed over 50 million lives worldwide (Taubenberger and Morens, 2006).

Influenza pathogenesis is a combination of viral virulence and host responses. Early defenses in the lung after virus infection have a strong influence on host protection and antiviral immunity. Primary immune defense strategies in the lungs against environmental pathogens are multilayered, consisting of a physical barrier (epithelial cells), mucosal liquid containing antimicrobial peptides (AMPs), and immune cells (macrophages and neutrophils) (Nicod, 2005). Antimicrobial peptides are an important primary host defense mechanism in the respiratory mucosa and are generally classified by the presence or absence of disulfide bonds. Most AMPs found in the lung mucosa are produced by epithelial and innate immune cells, and their functions are better characterized in defense against bacteria than viruses wherein AMP-induced clumping and trapping of bacteria abrogate their ability to invade host cells (Boyton and Openshaw, 2002). Epithelial AMP expression is regulated by pathogen exposure and innate mediators (Tecle et al., 2010). Defensins, AMPs that contain three disulfide bonds, are produced by epithelial cells and leukocytes in the mucosa (Risso, 2000) and are increased during acute inflammation (Bals et al., 2001). Beta-defensins can activate immature DCs (Biragyn et al.,

2002) and inhibit *Haemophilus influenzae* infections (Moser et al., 2002), and recently shown to inhibit IAV (Zhao et al., 2016). As one of the most abundant proteins in the airways, bactericidal-permeability-increasing (BPI) proteins inhibit bacteria and fungi from colonizing the lungs (Britto and Cohn, 2015; Seshadri et al., 2012). BPI family member SPLUNC can inhibit the growth of bio-film forming bacteria (Gally et al., 2011; Lukinskiene et al., 2011). Cathelicidin antimicrobial peptide (CAMP) deficient mice have increased susceptibility to skin infections (Nizet et al., 2001), and human cathelicidin, LL-37, inhibits IAV infectivity by damaging the viral envelope (Tripathi et al., 2013), and pre-treatment of mice with LL-37 resulted in lower lung viral burden and pro-inflammatory cytokines (Barlow et al., 2011). Since AMPs may have therapeutic potential against viral disease, understanding how they regulate early immune responses is important.

Macrophages play important roles in host defense against inhaled pathogens through phagocytic clearance and production of mediators that enhance immune responses. Numerous studies have established that IAV infects macrophages by binding to lectin-type receptors (Reading et al., 2000; Upham et al., 2010) although effective viral replication does not occur within these cells (Rodgers and Mims, 1981; Tate et al., 2010). Pro-inflammatory cytokines produced by macrophages during IAV infection (Lee et al., 2012) may promote inflammation and help control viral replication and clearance. IAV can directly impact macrophage function by altering the expression of *SOCS-1* and *RIG-I* and these responses differ by IAV strain (Ramirez-Martinez et al., 2013). Alveolar macrophage depletion in swine prior to IAV infection resulted in blunted adaptive immune responses (Kim et al., 2008), and alveolar macrophages are reduced during IAV infection of mice (Ghoneim et al., 2013). The reduction of macrophage populations in the lungs and associated alterations of functional capacity may significantly impair host defenses.

We hypothesized that mouse-adapted (A/PR/08/1934) and non-adapted (A/CA/04/2009) H1N1 strains of IAV differentially induce primary mucosal defense mechanisms in the lungs. Early markers of inflammation and AMP expression were regulated during IAV infection, and mouse β-defensin 4 (MBD4) had an impact on viral replication and early immune cell regulation. Taken together, our data show that early immune defenses differ by IAV strain and AMPs alter IAV pathogenesis. This has important ramifications both for our understanding of host immunity and for IAV strain selection in immunological studies.

2. Materials and methods

2.1. Ethics statement

All experiments were approved by the Institutional Animal Use and Care Committees at St. Jude Children's Research Hospital and the University of Tennessee Health Science Center.

2.2. Viruses

A comparison of published protein sequences between the laboratory strain A/PR/08/1934 (PR8) and the 2009 pandemic influenza strain A/CA/04/2009 (CA4) suggested that virus virulence and replication within hosts, and immune responses to these IAV strains may differ (Supplementary Fig. 1). The CA4 strain obtained from Dr. Richard Webby at SJCRH was propagated in Madin-Darby canine kidney (MDCK.2, ATCC, Manassas, VA) cells while our PR8 strain was cultured in embryonated chicken eggs. Both strains were sequence verified to be void of any mutations in hemagglutinin and neuraminidase genes. Effects of virus propagation methods have been extensively investigated and are not the focus of this work.

2.3. Animals

Seventeen-week-old C57BL/6 female mice from Jackson Laboratories (Bar Harbor, ME) were acclimatized for one week in specific pathogen-free ABSL2 facilities with *ad libitum* access to food and water in a 12-h light:dark cycle.

2.4. Influenza model

Both these virus strains can induce severe morbidity and mortality in mice. Since the focus of this study was to investigate innate immune responses to disease, we selected a non-lethal dose of virus to perform the model. Mice were infected intranasally with 1000 TCID$_{50}$ of either CA4 or PR8 virus in 50 μL. In our hands, C57BL/6 mice infected with CA4 at this dose have a weight loss nadir at ~12–15% while PR8 induces a nadir at ~25–27% and no death (cut off set to 30% weight loss or other signs of severe morbidity). Mice in the mock-treated group were administered PBS in place of virus. All animals were weighed prior to infection and every day thereafter until sacrifice. Animals were euthanized by CO_2 asphyxiation followed by cervical dislocation at predetermined time points after infection.

2.5. Sample harvest

Euthanized mice were tracheostomized and bronchoalveolar lavage (BAL) was performed twice with 1 mL of sterile PBS and stored in ice. The middle and accessory lobes of the right lung were snap frozen in liquid nitrogen for RNA analyses. Left lobes were harvested and fixed *ex vivo* by injecting 1 mL of 10% neutral buffered formalin for histological analysis. Whole lungs were harvested and snap frozen in liquid nitrogen from mice for viral titer determination. All samples were stored at −80 °C until use.

2.6. Virus titration

Lungs collected and stored at −80 °C were placed in ice and homogenized in the presence of 1 mL of sterile PBS. Homogenates were centrifuged at 600×g for 10 min at 4 °C and supernatants were stored in aliquots at −80 °C. Thawed aliquots were serially diluted at 1:10 and used to infect MDCK.2 cells grown to confluence in 96-well plates. Cells were washed one hour later and incubated in media containing 1 μg/mL TPCK-trypsin (Worthington Biochemical, Lakewood, NJ) at 37 °C/5% CO_2. Viral titers were read 72 h later by hemagglutination assay in the presence of 0.5% chicken red blood cells and calculated by the Reed-Muench method.

2.7. BAL cell analysis

BAL contents were centrifuged at 600×g for 10 min at 4 °C and supernatants stored at −80 °C. BAL cells were re-suspended in 0.2 mL of PBS and cytospun onto a glass slide. Slides were differentially stained (Quik-Dip, Mercedes Medical, Sarasota, FL) for morphometric analyses. Slides were observed under high power magnification (1250×) of a light microscope to enumerate ciliated epithelial cells in five randomly selected fields by an investigator blinded to the study groups.

2.8. Histological analysis

Formalin fixed left lungs were paraffin embedded and 4 μm sections were affixed onto glass slides, de-paraffinized and stained with Hematoxylin and Eosin. Slides were observed under low power magnification (200×) in a light microscope and peribronchial