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Lactobacillus priming of the respiratory tract: Heterologous immunity and protection against lethal pneumovirus infection

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ABSTRACT

We showed previously that wild-type mice primed via intranasal inoculation with live or heat-inactivated *Lactobacillus* species were fully (100%) protected against the lethal sequelae of infection with the virulent pathogen, pneumonia virus of mice (PVM), a response that is associated with diminished expression of proinflammatory cytokines and diminished virus recovery. We show here that 40% of the mice primed with live *Lactobacillus* survived when PVM challenge was delayed for 5 months. This robust and sustained resistance to PVM infection resulting from prior interaction with an otherwise unrelated microbe is a profound example of heterologous immunity. We undertook the present study in order to understand the nature and unique features of this response. We found that intranasal inoculation with *L. reuteri* elicited rapid, transient neutrophil recruitment in association with proinflammatory mediators (CXCL1, CCL3, CCL2, CXCL10, TNF-alpha and IL-17A) but not Th1 cytokines. IFNγ does not contribute to survival promoted by *Lactobacillus*-priming. Live *L. reuteri* detected in lung tissue underwent rapid clearance, and was undetectable at 24 h after inoculation. In contrast, *L. reuteri* peptidoglycan (PGN) and *L. reuteri* genomic DNA (gDNA) were detected at 24 and 48 h after inoculation, respectively. In contrast to live bacteria, intranasal inoculation with isolated *L. reuteri* gDNA elicited no neutrophil recruitment, had minimal impact on virus recovery and virus-associated production of CCL3, and provided no protection against the negative sequelae of virus infection. Isolated PGN elicited neutrophil recruitment and proinflammatory cytokines but did not promote sustained survival in response to subsequent PVM infection. Overall, further evaluation of the responses leading to *Lactobacillus*-mediated heterologous immunity may provide insight into novel antiviral preventive modalities.

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1. Introduction

Respiratory syncytial virus (RSV; family Paramyxoviridae; genus Pneumovirus) is the most common cause of severe lower respiratory tract disease among infants and young children and is an emerging pathogen of the elderly (Tregoning and Schwarze, 2010; Falsey and Walsh, 2005). In most cases the disease is self - limiting but in some infants it progresses to severe bronchiolitis and respiratory compromise, resulting in more than 100,000 hospitalizations each year in the US alone. Current management for otherwise healthy infants and children hospitalized with severe RSV is supportive care only; there is no available anti-RSV vaccine, although several are under study (Wright and Piedimonte, 2011). While monoclonal anti-RSV prophylaxis is available for identified high-risk infants (Shadman and Wald, 2011), a recent study by Hall and colleagues (Hall et al., 2009) documented that most children hospitalized with severe RSV infection had no identified predisposing risk factors, a finding that highlights the need for more effective management strategies for this disease.

The most severe forms of RSV infection are associated with significant inflammatory pathology: this is clear from human post mortem studies, analyses of samples from the airways of mechanically ventilated infants, and from various animal infection models (Rosenberg and Domachowske, 2012; Dyer et al., 2012). In order to explore the pathogenesis of RSV disease in vivo, we have developed a mouse model using the related pathogen, pneumonia virus of mice (PVM, family Paramyxoviridae, genus Pneumovirus). PVM is a natural rodent pneumovirus that replicates in bronchial epithelial cells and elicits severe inflammatory pathology in most inbred

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strains of mice (Bem et al., 2011; Rosenberg and Domachowske, 2008). Findings at peak morbidity include high virus titer, prominent neutrophil influx, and edema, similar to that described by Welliver and colleagues for fatal RSV infection (Welliver et al., 2008). We have used this model to explore various immunomodulatory therapies for pneumovirus infection, including those targeting the cytokine CCL3 and its receptor, CCR1, strategies which limit neutrophil influx and result in diminished mortality (Bonville et al., 2003; Bonville et al., 2004).

In our previous work, we documented the immunomodulatory properties of *Lactobacillus* strains, specifically, their ability to modulate the antiviral inflammatory response to acute PVM infection (Gabryszewski et al., 2011). Specifically, we found that wild-type mice primed via intranasal inoculation with live or heat-inactivated *Lactobacillus plantarum* or *Lactobacillus reuteri* were completely protected against lethal sequelae of this infection, with significant protection (60% survival) persisting even when virus infection was initiated three months after initial priming with live *L. plantarum*. Priming with live lactobacilli resulted in diminished granulocyte recruitment and diminished expression of multiple proinflammatory cytokines characteristic of acute PVM infection. These findings represent an original and robust example of heterologous immunity, or "innate imprinting," a concept introduced by Hussell and colleagues (Goulding et al., 2007; Didierlaurent et al., 2007; Hussell and Cavanagh, 2009) to explain the increased resistance or susceptibility to an unrelated pathogen generated upon recovery from a primary innate or inflammatory response. As some examples of this concept, Nguyen et al. (2008) found that primary infection with murine gammaherpesvirus infection resulted in diminished recovery of mouse adenovirus-1 upon challenge with this unrelated pathogen up to three weeks later. Similarly, Williams et al. (2004) elicited protection against virus and fungal pathogens by intranasal administration of an *E. coli*-derived heat-labile antigen. Likewise, Li et al. (2010) documented the protective effects of intranasal administration of live *Bordetella pertussis* (BPEZI, pertussis-toxin inactivated) against subsequent infection with influenza, although curiously, no protection was observed in response to inoculation with similar, toxin-gene-deleted (ΔPT *B. pertussis*) strain (Ayala et al., 2011). Of note, not all of these heterologous responses result in positive outcomes or are directed toward promoting homeostasis; several molecular mechanisms have been proposed to explain the well-known increased susceptibility to bacterial pneumonia observed clinically in individuals recovering from acute severe influenza infection (reviewed in Ballinger et al., 2010; Steinberg et al., 2012).

In this manuscript we have characterized the innate inflammatory responses in the airways that are elicited in response to priming with live *Lactobacillus* species as part of an ongoing effort to elucidate the mechanisms that promote this unique and notably robust form of heterologous immunity to respiratory virus infection.

2. Materials and methods

Mice Wild-type BALB/c and C57BL/6 mice were purchased from Division of Cancer Therapeutics, Frederick, MD. IFN$\gamma^{-/-}$ mice (Line 208; Dalton, et al., 1993) are maintained by NIAID-Taconic contract. All mouse studies were carried out in accordance with Animal Study Protocol LAD-8E approved by the National Institutes of Allergy and Infectious Diseases Animal Care Committee.

2.1. Lactobacillus inoculations

Lactobacillus plantarum NCIMB 8826 (ATCC BAA-793) and *L. reuteri* F275 (ATCC 23272) were grown overnight at 37 °C in Difco Lactobacilli MRS Broth (BD Biosciences, Sparks, MD). Correlations between OD_{600} and colony forming units (cfu) were as previously described (Gabryszewski et al., 2011). Bacteria were harvested by centrifugation (5 min, 1500 rpm Sorvall RT6000B centrifuge) washed with sterile tissue-culture grade phosphate-buffered saline (PBS) and re-suspended in PBS supplemented with 1% bovine serum albumin (PBS/BSA) at 2×10^{10} colony forming units (cfu)/mL (*L. plantarum*) or 2×10^9 cfu/mL (*L. reuteri*). Mice were inoculated intranasally with 50 μL PBS/BSA with 10^9 or 10^8 cfu live *L. plantarum* or *L. reuteri* in 50 μL PBS/BSA, respectively, at time points indicated. In some experiments, mice were inoculated intranasally with purified Gram-positive peptidoglycan (100 μg/50 μL PBS/BSA; Invivogen, San Diego, CA).

2.2. Virus preparation and inoculations

PVM strain J3666 is maintained as an in vivo passaged stock; virus copy number per unit volume was determined by a quantitative PCR method (Gabryszewski et al., 2011) that targets the PVM small hydrophobic (SH) gene. All PVM-infected mice were inoculated intranasally with 50 μL of PVM stock (2×10^5 PVM_{SH}/μL) diluted 1:2000 (C57BL/6 mice) or 1:3000 (BALB/c mice) in culture medium while under isoflurane anesthesia.

2.3. Leukocyte recruitment

Lung tissue was harvested and single cell suspensions prepared essentially as described in (Gabryszewski et al., 2011); here, fresh digestion medium was added after first 45 min of incubation, and a 70-micron filter cell strainer was used. Live/dead stain (Invitrogen) was added to the cells and antibody binding to F_c receptors was blocked with anti-mouse CD16/CD32 (BD Biosciences, Durham, NC). Cells were then stained with anti-CD3-PE-Cy5, anti-CD3-Alexa-700, anti-CD19-Alexa-647, anti-CD49b (DX5)-PE, anti-GR1-APC, anti-GR1-V450, anti-CD11b-PE (BD Biosciences); anti-CD11c-Alexa-488, and/or anti-MHC II (I-A/I-E)-APC (eBiosciences, San Diego) in PBS/BSA at 4 °C for 1 h and washed with PBS/BSA. A minimum of 100,000 events was collected on an LSRII flow cytometer (BD Biosciences) and data was analyzed in FlowJo 9.2. Analyses are presented as percentage of live cells.

2.4. Histology

Lung tissue was evaluated as indicated. Lungs were inflated trans-tracheally with 250 μL of 10% phosphate buffered formalin; lungs and heart were removed and stored at 4 °C in 10% phosphate buffered formalin. Samples were paraffin embedded, sectioned and stained with hematoxylin and eosin (Histoserv, Germantown, MD).

2.5. Cytokine and immunoglobulin ELISAs

At selected time points, lungs of mice primed with *L. reuteri* (10^8 cfu), Gram-positive peptidoglycan, *L. reuteri* genomic DNA (see below) followed by PVM infection, or PBS/BSA at time points indicated were collected and blade-homogenized in 1 mL PBS/BSA. Clarified supernatants were aliquoted and stored at −80 °C for later use. ELISAs were performed to evaluate cytokine responses (R&D Systems, Minneapolis, MN) as per the manufacturer's instructions. Cytokine production was normalized to total lung protein determined by a BCA assay (Pierce, Rockford, IL). Cytokines and IgG were measured in sera from mice at selected time points after intranasal priming with *L. reuteri* or PBS/BSA control. Cytokine levels were measured with a mouse cytokine/chemokine Milliplex kit (Millipore, Billerica, MA) following manufacturer's instructions. Serum IgG was measured using a designated kit from Kamiya Biomedical (Seattle, WA). Seroconversion to PVM was

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