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# Immunobiotic *Lactobacillus* administered post-exposure averts the lethal sequelae of respiratory virus infection

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ABSTRACT

We reported previously that priming of the respiratory tract with immunobiotic *Lactobacillus* prior to virus challenge protects mice against subsequent lethal infection with pneumonia virus of mice (PVM). We present here the results of gene microarray which document differential expression of proinflammatory mediators in response to PVM infection alone and those suppressed in response to *Lactobacillus plantarum*. We also demonstrate for the first time that intranasal inoculation with live or heat-inactivated *L. plantarum* or *Lactobacillus reuteri* promotes full survival from PVM infection when administered within 24 h *after* virus challenge. Survival in response to *L. plantarum* administered after virus challenge is associated with suppression of proinflammatory cytokines, limited virus recovery, and diminished neutrophil recruitment to lung tissue and airways. Utilizing this post-virus challenge protocol, we found that protective responses elicited by *L. plantarum* at the respiratory tract were distinct from those at the gastrointestinal mucosa, as mice devoid of the anti-inflammatory cytokine, interleukin (IL)-10, exhibit survival and inflammatory responses that are indistinguishable from those of their wild-type counterparts. Finally, although *L. plantarum* interacts specifically with pattern recognition receptors TLR2 and NOD2, the respective gene-deleted mice were fully protected against lethal PVM infection by *L. plantarum*, as are mice devoid of type I interferon receptors. Taken together, *L. plantarum* is a versatile and flexible agent that is capable of averting the lethal sequelae of severe respiratory infection both prior to and post-virus challenge via complex and potentially redundant mechanisms.

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## 1. Introduction

Inflammation in response to severe respiratory virus infection can be complex and difficult to manage (Rosenberg and Domachowske, 2012). While antivirals are available for the treatment of specific respiratory viruses, they offer little benefit once symptoms become severe (Chan-Tack et al., 2015) suggesting that virus-induced inflammation contributes broadly to disease pathogenesis. Previous studies of the innate host response to viral lung infections have identified biomarkers of severe disease, highlighting key pathways for the study of anti-inflammatory and/or immunomodulatory intervention (Schwarze and Mackenzie, 2013; Tabarani et al., 2013; Welliver, 2008).

Toward this end, there is currently significant interest in the immunomodulatory properties of probiotic bacteria (reviewed in Bron et al., 2011; Lebeer et al., 2010). While most of the literature on this subject features bacteria of the genera *Lactobacillus* and *Bifidobacteria* and their beneficial actions at the gastrointestinal mucosa, our group has focused on the impact of both live and heat-inactivated preparations of *Lactobacillus* species administered directly to the respiratory tract. Specifically, we have reported that priming of the respiratory tract of inbred strains of mice with live or heat-inactivated *Lactobacillus plantarum* results in robust and

*Abbreviations:* PVM, pneumonia virus of mice; *Lp*, *Lactobacillus plantarum*; *Lr*, *Lactobacillus reuteri*.

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sustained protection against a subsequent lethal respiratory virus infection in association with profound inflammatory suppression (Gabryszewski et al., 2011; Garcia-Crespo et al., 2013; Percopo et al., 2014a). This set of observations is a unique example of heterologous immunity (also known in other contexts as trained immunity, innate imprinting, and innate memory), which are concepts that explain how the innate immune system alters its responsiveness and offers cross-protection against unrelated pathogens after a primary inflammatory or infectious event (reviewed in Levy and Netea, 2014; Netea et al., 2011; Wissinger et al., 2009; Locati et al., 2013; Ifrim et al., 2014). Using the defined pneumonia virus of mice model of severe pneumovirus infection (PVM; Family *Paramyxoviridae*, genus Pneumovirus; reviewed in Dyer et al., 2012; Bem et al., 2011), we have reported that *Lactobacillus* species introduced to the lung are cleared rapidly, and do not colonize the respiratory tract (Garcia-Crespo et al., 2013). In contrast to live or inactivated bacteria, *Lactobacillus* genomic DNA introduced alone cannot elicit protection against lethal virus infection (Garcia-Crespo et al., 2013). Furthermore, neither B cells nor antibodies are crucial elements of *Lactobacillus*-mediated protection against the lethal sequelae of this infection (Percopo et al., 2014a). Orally-delivered *L. plantarum* also has no impact in this setting (Percopo et al., 2014a), a finding that is consistent with the current clinical literature on the role of oral supplementation and its impact on respiratory virus infection (Hao et al., 2011; Esposito et al., 2014; Ozen et al., 2015).

In this manuscript, we demonstrate for the first time that intranasal inoculation with live or inactivated *L. plantarum* also promotes full survival from acute PVM infection when administered within 24 h *after* virus challenge, a finding that is also associated with suppression of virus replication and diminished expression of virus-induced proinflammatory cytokines. We have utilized this extended and clinically important means of eliciting protection to explore features of *Lactobacillus*-mediated protection at the respiratory tract that are unique and distinct from those taking place at gastrointestinal mucosa.

## 2. Materials and methods

### 2.1. Mice

Wild-type BALB/c and C57BL/6 mice were obtained from Division of Cancer Therapeutics and Charles River Laboratories, Frederick, MD. Interleukin-10 gene-deleted (IL-$10^{-/-}$) and Type I interferon receptor gene-deleted (IFN$\alpha\beta R^{-/-}$) mice are maintained by the NIAID/Taconic consortium. Toll-like receptor 2 gene-deleted mice (#004650) and nucleotide-binding oligomerization domain-containing protein 2 gene-deleted mice (#005763) were obtained from The Jackson Laboratory. All mouse studies were approved by NIAID and carried out in accordance with NIAID ACUC Guidelines.

### 2.2. Virus

Mouse passaged stocks of PVM strain J3666 were prepared and stored in liquid nitrogen as previously described (Domachowske et al., 2000). Mice were inoculated intranasally under isoflurane anaesthesia with 50 μL of a 1:10,000 dilution (0.2 $TCID_{50}$ units for mice on a BALB/c background) or a 1:1000 dilution (2.0 $TCID_{50}$ units for mice on a C57BL/6 background) in Iscove's Modified Dulbecco's medium (IMDM) at time points indicated. Virus titer was determined from cDNA generated from total RNA from mouse lung tissue via a dual standard curve qRT-PCR method targeting the PVM small hydrophobic (SH) gene and mouse GAPDH; this assay generates absolute copy numbers per copy GAPDH ($PVM_{SH}$/GAPDH) as previously described (Percopo et al., 2014b). Influenza A/HK/68 (H3N2) was passaged in BALB/c mice and stored at $10^4$-fold concentrated stocks at −80 °C.

### 2.3. Lactobacillus

Cultures of *L. plantarum* NCIMB 8826 (ATCC BAA-793) or *Lactobacillus reuteri* F275 (ATCC 23272) were grown overnight in MRS broth at 37 °C. For experiments with live bacteria, cells were washed in sterile phosphate buffered saline (pbs) and re-suspended at $2 \times 10^9$ or $2 \times 10^{10}$ colony forming units (cfu)/mL in sterile pbs with 0.1% bovine serum albumin (bsa) for intranasal inoculation under isoflurane anesthesia using the $OD_{600}$ vs. cfu/mL determinations reported previously (Gabryszewski et al., 2011). Each mouse received 50 μL of this dilution (=$10^8$ or $10^9$ cells or cfu) or 50 μL diluent control (pbs/bsa) per inoculation. In other experiments, *L. plantarum* or *L. reuteri* overnight cultures were washed with pbs and heat-inactivated by multiple cycles of freeze/thaw (Gabryszewski et al., 2011) or by heating to 70 °C for 30 min prior to re-suspension at $10^{11}$ cells/mL. Concentrated stocks of inactivated *L. plantarum* and *L. reuteri* were stored at −80 °C or −20 °C prior to dilution (in pbs/bsa) for inoculation as indicated.

### 2.4. Cytokine analysis

Cytokine ELISAs (R&D Systems) were performed on clarified homogenates of lung tissue and corrected for total protein by BCA assay (Pierce) as previously described (Gabryszewski et al., 2011).

### 2.5. Bronchoalveolar lavage (BAL) and cell counts

Cytospin slides (one slide per mouse) were prepared from BAL fluid (1.5 mL in pbs/bsa), fixed and stained with Diff-Quik. Two-hundred (200) to 300 cells were counted per slide.

### 2.6. Histology

Tissue sections prepared from 10% phosphate-buffered formalin-fixed lung tissue were stained with hematoxylin and eosin (H&E; Histoserv, Germantown, MD).

### 2.7. Flow cytometry

Lung tissue was harvested and single-cell suspensions were prepared as previously described (Garcia-Crespo et al., 2013; Percopo et al., 2014a). Live/Dead stain (Invitrogen) was added to the cells and Ab binding to Fc receptors was blocked with anti-mouse CD16/CD32. For analysis of T and B cells, lung suspensions were stained with anti-CD45-eF450 (eBioscience), anti-CD3-AF700 (eBioscience), anti-CD4-FITC (eBioscience), anti-CD8a-PE (BD), and anti-CD19-AF647 (BD) in PBS with 0.1% BSA at 4 °C for 30 min and washed with this buffer prior to analysis. For myeloid cell analysis, cells were stained with anti-CD45-AF700 (BD), anti-CD11c-AF488 (BD), anti-SiglecF-PE (BD), anti-GR1-V450 (BD) and anti-MHCII-APC (eBioscience). Natural killer cells were characterized by staining with anti-CD45-eF450 (eBioscience), anti-CD3-AF700 (BD), and anti-CD49/DX5-PE (BD). A minimum of 100,000 events were collected on an LSR II flow cytometer (BD Biosciences) and findings were analyzed in FlowJo 9.2.