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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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Keywords

Inflammation; Cytokines; Pneumovirus; Pattern-recognition receptors

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, high-lighting 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 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 α β 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 μ L of a 1:10,000 dilution (0.2 TCID₅₀ units for mice on a BALB/c background) or a 1:1000 dilution (2.0 TCID₅₀ 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 10⁴-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 2×10^9 or 2×10^{10} colony forming units (cfu)/mL in sterile pbs with 0.1% bovine serum albumin (bsa) for intranasal inoculation under isoflurane anesthesia using the OD₆₀₀ vs. cfu/mL determinations reported previously (Gabryszewski et al., 2011). Each mouse received 50 μ L of this dilution ($=10^8$ or 10^9 cells or cfu) or 50 μ L 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 10¹¹ 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.

2.8. DNA microarray target preparation and analysis

Eight-week old female BALB/c mice (all born on same day and shipped at same time from provider) were inoculated under isoflurane anesthesia with live *L. plantarum* (50 µL of 2×10^{10} cfu/mL in pbs/bsa) or diluent control on day-14 and again on day-7 and then inoculated with 0.2 TCID₅₀ units (1:10,000 dilution in 50 µL) of PVM on day 14 or vehicle control. Each step of the study, including all mouse inoculations, RNA harvests to DNA microarray target preparation was designed and performed in a manner so as to avoid batch processing effects in the data due to mouse and sample type. Mouse inoculations, tissue harvest, RNA extraction, DNA target preparation batches were balanced between treatment and time. Lung tissues were harvested on days 17, 18, 19 and 20 and were snap frozen in liquid nitrogen. Samples (total 24, 6 mice per group) from mice that received two inoculations of *L. plantarum* or two inoculations of pbs/bsa diluent prior to virus or vehicle only challenge and harvested on day 19 were processed further for DNA microarray analysis. RNA extraction and target preparation were performed as described (Mackey-Lawrence et al., 2013) for all samples except RNA was extracted using RNeasy 96 well kit (Qiagen, Valencia, CA). Hybridization, fluidics and scanning were performed according to standard Affymetrix protocols (<http://www.affymetrix.com>) with the whole mouse genome 430 2.0 chip within

the Genomics Unit of the Research Technologies Section (NIAID). Command Console (CC v3.1, <http://www.Affymetrix.com>) software was used to convert the image files to cell intensity data (cel files). All cel files, representing individual samples, were normalized by using the trimmed mean scaling method within expression console (EC v1.2, <http://www.Affymetrix.com>) to produce the analyzed cel files (chp files) along with the report files. The cel files were input into Partek Genomics Suite software (Partek, Inc. St. Louis, Mo., v6.6-6.12.0907) and quantile-normalized to produce the principal components analysis (PCA) graph and dendrogram. An ANOVA was performed within Partek to obtain multiple test corrected *p*-values using the false discovery rate method (FDR; (Klipper-Aurbach et al., 1995)) at the 0.05 significance level which were combined with fold change values for each comparison of interest. The array data presented in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO series accession number GSE66721.

2.9. PRR activation assay

Activation of Toll-like receptors (TLRs 2, 3, 4, 5, 7, 8 and 9), NOD-like receptors (NOD 1 and NOD 2), and C-type lectin receptors (CLRs dectin 1a and 1b) were evaluated via a commercial assay in which NF- κ B response via a single PRR expressed in HEK293 cells is measured via linked secretory alkaline phosphatase (SEAP; Invivogen). Upon introduction of 20 μ L heat-inactivated *L. plantarum* (10^8 cells/mL), diluent (negative) control, or TLR/NOD/CLR ligand (positive control) to 200 μ L of HEK293 cells ($50\text{--}75 \times 10^3$ in 96 well plate), activation was measured via production of secretory alkaline phosphatase from a reporter gene under control of a promoter element inducible by NF- κ B. Alkaline phosphatase produced is measured via detection of colorimetric products at OD = 650 nm. Positive ligands included: TLR2, heat-inactivated *Listeria monocytogenes* 10^8 cells/mL; TLR3, poly I:C 1 μ g/mL; TLR4, *Escherichia coli* LPS 1 μ g/mL; TLR5, *Salmonella typhimurium* flagellin 1 μ g/mL; TLR7/8, R848 10 μ g/mL; TLR9 CpG ODN 2006 1 μ g/mL; NOD1, C12-iE-DAP 10 μ g/mL; NOD2, L18-MDP 10 μ g/mL; Dectin 1a and 1b, beta-glucan from *Saccharomyces cerevisiae* at 10 ng/mL, curdlan at 100 μ g/mL and zymosan at 5 μ g/mL.

2.10. Statistical analysis

Findings were analyzed for statistical significance using 1-way analysis of variance (ANOVA), Mann-Whitney *U*-test, or Log-rank test, as appropriate. Data are presented as mean $\pm$ standard deviation.

3. Results

3.1. Priming with *L. plantarum* results in suppression of a specific cohort of virus-induced proinflammatory mediators

Using a modification of our standard priming protocol (Gabryszewski et al., 2011), BALB/c mice were inoculated intranasally on day-14 and again on day-7 with 10^9 cells of live *L. plantarum* in pbs/bsa (50 μ L of 2×10^{10} cells/mL) or pbs/bsa diluent control alone. In this experiment, mice are then challenged 21 days later (on day +14) with an otherwise lethal dose of PVM or vehicle only. RNA was isolated from whole lung tissue from 24 mice (four

protocols, one time point, six mice per group) and was subjected to whole genome microarray analysis; differential expression of thirty-one (31) soluble proinflammatory mediators identified in this experiment is featured in Table 1. As shown here, PVM infection alone results in increased expression of transcripts encoding numerous inflammatory mediators, including CC and CXC chemokines, including CCL2 and CXCL10. Likewise, PVM infection results in increased expression of acute phase reactants amyloid A1 and A3 (Sandri et al., 2008), granzyme B, a serine protease with antiviral and immunomodulatory properties generated by NK cells, T cells and mast cells (Wensink et al., 2015), lipocalin 2, an immunomodulatory and antimicrobial protein stored in and released from a neutrophil tertiary granules (Dittrich et al., 2013; Flo et al., 2004), and galectins 3 and 9, galactose-binding proteins mediate innate and acquired immunity with roles including targeting the survival of effector cytotoxic T lymphocytes (CTLs), T helper 1 (T_H1) cells and targeting regulatory T cells (Rabinovich and Toscano, 2009; Wu et al., 2014).

Interestingly, not all pro-inflammatory mediators were up-regulated in response to PVM infection. Specifically, we observed *diminished* expression of epithelial cytokines and alarmins, HMGB1 and IL-33 in response to PVM infection alone, and priming with *L. plantarum* counteracts this response to some degree. Release of IL-33 and HMGB1 from host cells and their role in augmenting virus-induced asthma has been considered previously in the literature (Yoo et al., 2013; Kumar et al., 2014), while more recent work (Holtzman et al., 2014) has identified a population of specialized epithelial cells that produces IL-33 as a response to recovery from virus infection. Interestingly, expression of the epithelial-derived chemokine CXCL15 (originally known as lungkine (Rossi et al., 1999; Chen et al., 2001)), is also diminished in response to PVM infection, a response that also is reversed by *L. plantarum* priming.

Most important with respect to the findings in this manuscript, the microarray findings document that priming of the respiratory tract with *L. plantarum* prior to virus infection results in a specific anti-inflammatory profile. Among those inflammatory mediators with expression most profoundly suppressed was virus-induced interleukin (IL)-6, with expression diminished 105-fold in response to *L. plantarum* priming. Other chemokines that undergo profound suppression include CCL2, CXCL10, CXCL2 and CXCL11, with 14, 11, 20 and 21-fold reduced expression, respectively. In contrast, other chemokines, including CCL12, CXCL13, CCL5, CCL9 and CXCL16, experience no significant differential expression in response to *L. plantarum* priming.

3.2. *L. plantarum* and *L. reuteri* administered to the respiratory mucosa after virus challenge also avert the lethal sequelae of infection

In our previous work, we focused on *L. plantarum* and *L. reuteri* and their role in preventing the lethal sequelae of respiratory virus infection when administered as a priming agent, prior to virus challenge. Here, BALB/c mice were inoculated with PVM at day 0, followed by intranasal administration *L. plantarum* or *L. reuteri* (10^7 to 10^9 cells/mouse) at one or more days after virus challenge as outlined in Fig. 1A. Mice that received *L. plantarum* (10^7 cells/mouse) on days 1 and 2 or on days 1, 2, and 3 responded with 40% survival. In contrast, if the first dose was delayed until day 2, and mice received 10^7 cells on days 2 and

3, no significant survival over background was observed (Fig. 1B). In contrast, mice that received a single inoculum of 10^8 cells on day 1 alone were fully protected (100% survival) against an otherwise lethal PVM infection (Fig. 1B); we have been able to extend the “window of opportunity” beyond the first 24 h with varying dosing strategies (data not shown). Similar to results obtained with *Lactobacillus* priming prior to virus infection (Gabryszewski et al., 2011), we show here that live *L. plantarum* is just as effective as the heat-inactivated in preventing the lethal sequelae of PVM infection when administered post-virus challenge (Fig. 1C). Furthermore, protection elicited post-virus challenge is not a unique property of a given *Lactobacillus* strain or species, as both live and heat-inactivated *L. reuteri* were also effective in this role (Fig. 1D).

Heat-inactivated (hi) *L. plantarum* administered post-virus challenge results in diminished virus recovery (Fig. 2A). Lung tissue from PVM-infected mice that received pbs/bsa diluent control displayed prominent alveolitis and congestion (Fig. 2B, i, at arrows), in contrast to lungs from PVM-infected mice that received hi-*L. plantarum* on days 1 and 2 after PVM challenge, which were comparatively clear (Fig. 2B, ii). Furthermore, analogous to what we have observed in response to *L. plantarum* priming (Table 1), survival in response to either heat-inactivated or live *L. plantarum* administered after PVM challenge resulted in profound suppression of virus-induced cytokines (Fig. 2C and D).

Administration of hi *L. plantarum* on days 1 and 2 after PVM challenge also limits neutrophil recruitment to the lungs (Fig. 3). Specifically, we detected 5–6-fold fewer neutrophils at day 5, a reduction from $14 \pm 1.5 \times 10^6$ to $2.6 \pm 0.5 \times 10^6$ cells in response to *L. plantarum* administered post-virus challenge ($p < 0.001$). We observed no significant differences in total CD4⁺ T cells, CD8⁺ T cells, B cells, NK or NKT cells at this time point, although *L. plantarum* administration did result in increased numbers of eosinophils and dendritic cells.

Mice on the C57BL/6 background respond similarly to *Lactobacillus* administered after virus challenge. As shown in Fig. 4A, mice are protected in response to 10^8 or 10^9 cells of hi *L. plantarum* administered on days 1 and 2, or 10^9 cells administered on day 1 alone; protection is also conferred by 10^9 cells of live *L. reuteri* (Fig. 4B). Similar to observations in BALB/c mice, protection in response to *L. plantarum* in C57BL/6 mice is associated with profound suppression of virus-induced neutrophil recruitment (Fig. 4C), diminished virus recovery (Fig. 4D), and suppression of virus-induced proinflammatory cytokines (Fig. 4E and F).

3.3. Protection afforded by *L. plantarum* is not mediated by IL-10

The cytokine IL-10 is produced by monocytes, Th2 cells and regulatory T cells (Saraiva and O’Garra, 2010) and its role in modulating the anti-inflammatory impact of probiotic bacteria, including *Lactobacillus*, in the gastrointestinal tract has been explored extensively (Levast et al., 2015; Mengheri, 2008). Interestingly, gene-deletion of IL-10 has no impact on *Lactobacillus*-mediated protection at the respiratory tract; administration of *L. plantarum* to the respiratory tract (10^9 cells/mouse on days 1 and 2 after virus challenge) results in full protection from the lethal sequelae of PVM infection in both wild-type and IL-10^{-/-} mice (Fig. 5A). Administration of *L. plantarum* to PVM-infected IL-10^{-/-} mice results in

diminished virus recovery from lung tissue (Fig. 5B) and prominent suppression of cytokines IL-6 and CCL2 (Fig. 5C).

3.4. *L. plantarum* interacts in vitro with pattern recognition receptors TLR2 and NOD2

In order to define clearly the specific interactions of *Lactobacillus* with mucosal immune cells, hi *L. plantarum* was evaluated in assays designed to identify responses of individual pattern recognition receptors (PRRs) via an NF- κ B-linked colorimetric readout (see Materials and Methods). As shown in Fig. 6A, *L. plantarum* elicits responses that would be anticipated from a gram-positive microorganism, as it activates via both toll-like receptor 2 (TLR2) and nucleotide-binding oligomerization domain-containing protein 2 (NOD2), at 20-fold and 6-fold over diluent control, respectively. We observed no response from any other TLR, from NOD1, or of from C-type lectin-receptors Dectin1a or Dectin1b (data not shown), although positive ligands were uniformly reactive (see Materials and Methods).

3.5. Immunomodulation and protection elicited by *L. plantarum* in mice with single gene-deletions of TLR2 or NOD2

Wild-type C57BL/6, TLR2^{-/-}, or NOD2^{-/-} mice that were inoculated with virus on day 0 and with *L. plantarum* (10⁹ cells/mouse) on days 1 and 2 were fully protected from the lethal sequelae of infection compared to their counterparts that received pbs/bsa only (Fig. 6B). Differential virus recovery was not a prominent component of the response to *L. plantarum* in NOD2^{-/-} mice, results which are similar to those obtained in experiments performed previously with *L. plantarum*-primed MyD88^{-/-} mice (Gabryszewski et al., 2011; Fig. 6C). However, *L. plantarum* administration did result in suppression of virus-induced cytokines IL-6 and CCL2 (Fig. 6D and E). Similarly, mice devoid of type I interferon ($\alpha\beta$) receptors also respond to *L. plantarum*, and are fully protected from the lethal sequelae of PVM infection under the same experimental conditions (Fig. 6F).

4. Discussion

We demonstrate here for the first time that *L. plantarum* is effective at promoting survival when administered to the respiratory tract *after* virus challenge. While we and others have explored the priming of the respiratory mucosa with *L. plantarum* as a means to prevent the negative sequelae of subsequent infection with various viruses (Gabryszewski et al., 2011; Garcia-Crespo et al., 2013; Percopo et al., 2014; Hori et al., 2001; Harata et al., 2010; Izumo et al., 2010; Park et al., 2013; Tomasada et al., 2013; see also Supplemental Fig. 1), this is the first example of a the use of this methodology for definitive intervention after virus exposure has taken place. The fact that we identified a significant “window of opportunity” for complete (100%) post-exposure protection is particularly notable given that, in earlier work, we observed no protection whatsoever in response to live or heat-inactivated *L. plantarum* administered shortly thereafter, at day 3 post-virus challenge (Gabryszewski et al., 2011). We continue to explore the mechanisms underlying susceptibility (and resistance) to *Lactobacillus*-mediated suppression.

Similar to what we have observed in response to priming prior to PVM infection, administration of *L. plantarum* after virus challenge is associated with diminished virus

recovery, and with suppression of virus-induced neutrophil recruitment and production of cytokine mediators, including IL-6, CCL2 and CXCL10. Each of these mediators has been identified in clinical studies in association with disease severity [Tabarani et al., 2013; Sow et al., 2011; Lee et al., 2011; Ichikawa et al., 2013; Doyle et al., 2010].

The clinical implications of our findings regarding *L. plantarum* and its efficacy post-virus challenge, and our interest in its unique antiviral mechanism at the respiratory mucosa, are clear and significant. One is often fully aware of acute exposure to respiratory virus infection, for example, in crowded venues such as public transportation and in office or classroom settings. A 24 h window of opportunity after primary exposure, such as that revealed in this study, is a reasonable time frame in which to administer a preventive agent, particularly one that can be provided simply and efficiently via an intranasal route. This is in contrast to an agent based on mechanisms defined by *Lactobacillus* priming, which would be designed to provide generalized protection against respiratory virus infection prior to exposure, and might be directed against pathogens for which vaccine strategies have limited efficacy and/or are unavailable.

There are only a few agents currently available for use in preventing the negative sequelae of respiratory virus infection after an initial exposure has taken place. Among these agents are the neuraminidase inhibitors, which are used for both prophylaxis and treatment of influenza; when administered within 48 h of the onset of symptoms, these antivirals may limit duration of disease and ensuing complications (reviewed in Nguyen-Van-Tam et al., 2015) although the overall risk vs. benefits of these drugs have been questioned (Jefferson et al., 2014). With respect to respiratory syncytial virus (RSV), the monoclonal antibody palivizumab directed against the virus F (fusion) protein is highly effective at preventing severe disease among those infants identified at high risk (reviewed in Andabaka et al., 2013) although this does not represent the full measure of the disease burden (Hall et al., 2009). At the same time, treatment options for RSV disease are limited. The antiviral ribavirin is available for only the most severely ill and immunocompromised children (AAP Committee on Infectious Diseases, 1996; Chu and Englund, 2013). A number of drugs are currently in development for the treatment of RSV disease, including antivirals based on siRNA (DeVincenzo et al., 2010) and the cell fusion inhibitor GS-5806 (DeVincenzo et al., 2014). Just recently, Plant et al. (2015) have described a high-throughput screening assay designed to identify agents that inhibit RSV replication. Our groups have focused on the role of the inflammation and its contribution to acute respiratory virus infection, and we have documented the efficacy of combined antiviral/anti-inflammatory approaches in studies carried out using *in vivo* models (Rosenberg and Domachowske, 2012; Bonville et al., 2003; Bonville et al., 2004).

Among the intriguing questions is the mechanism via which *L. plantarum* at the respiratory tract limits virus replication *in vivo* (see Figs. 2A and 4D). Among several possibilities, several groups (Uehara et al., 2007; Birchler et al., 2001; Wang et al., 2003) have reported that epithelial cells respond to gram-positive bacteria and their components by producing beta-defensins, antimicrobial peptide with significant antiviral activity (Lehrer, 2004; Gong et al., 2010). Furthermore, in recent work with a novel recombinant PVM strain J3666 tagged with the red fluorescent protein, mKATE2, we have identified alveolar macrophages

(CD45⁺ SiglecF⁺ CD11c⁺) as the major leukocyte population that supports virus replication *in vivo*. *Lactobacillus*-priming of the respiratory tract results in a significant reduction in mKATE2⁺ alveolar macrophages (from 30% to 40% to fewer than 10%) in association with 2-log drop in release of infectious virions (Dyer et al., ms. in revision).

Also clear from these studies, the interactions of *Lactobacillus* with the local immune environment of the gastrointestinal mucosa are functionally distinct from what we observe in the respiratory tract. Among the most prominent differences, *Lactobacillus*-mediated protection from inflammatory sequelae has been attributed in large part to the actions of the anti-inflammatory cytokine, IL-10 (reviewed in Claes et al., 2011). For example, Macho Fernandez et al. (2011) showed that peptidoglycan derived *Lactobacillus salivarius* strain Ls33 served to protect mice from the inflammatory sequelae of chemical colitis via mechanisms that correlated with local production of IL-10. Similarly, Chen et al. (2005) found that inoculation of young mice with *Lactobacillus acidophilus* stimulated IL-10 expression in conjunction with protection against colitis induced by the bacterial pathogen, *Citrobacter rodentium*. Recently, Bosch et al. (2012) characterized novel probiotic strains of *L. plantarum* based on their capacity to induce IL-10 production *in vitro* from human monocyte THP-1 cells when co-incubated with lipopolysaccharide. In contrast, as we show in Fig. 5, inflammatory suppression and full protection against the lethal sequelae mediated by *L. plantarum* at the respiratory tract takes place in mice devoid of IL-10, as it does in their wild-type counterparts.

We also explored responses elicited by *L. plantarum* in mice devoid of pattern recognition receptors, TLR2 and NOD2. While *Lactobacillus* interacts with TLR2 and NOD2 alone *in vitro* signaling assays (Fig. 6), we found that mice with individual gene deletions were protected in both priming (data not shown) and post-virus challenge protocols. The survival responses using priming protocols in TLR2^{-/-} mice (data not shown) may have been anticipated to some extent given our earliest findings with priming in MyD88^{-/-} mice (Gabryszewski et al., 2011); however we had not previously explored the responses of either strain in the post-virus challenge protocol. Likewise, we have also shown that mice devoid of type I IFN receptors (ie, thus devoid of downstream signaling from IFNs resulting from TLR2 activation) are similarly protected by *L. plantarum*. Among several interpretations, it is conceivable that there may be substantial cross-talk between TLR2 and NOD2 pathways in this setting (Wu et al., 2015; Zeuthen et al., 2008; Borm et al., 2008; Netea et al., 2005; Pavot et al., 2014; Watanabe et al., 2006), a possibility that requires further consideration.

Among other possibilities, *Lactobacillus* species may elicit protection via interactions with another, fully independent group of PRRs, the peptidoglycan recognition proteins (PGLYRPs), a family of evolutionarily ancient secretory/bactericidal proteins that interact with bacterial cell wall peptidoglycan (Royet et al., 2011). Of particular note is PGLYRP-1, which is expressed primarily in neutrophil granules, and was recently identified by Read et al. (2015) as a ligand for the orphan neutrophil and monocyte/macrophage Ig superfamily receptor TREM-1, which augments inflammatory and innate immune responses triggered by exogenous pathogens by stimulating release of pro-inflammatory mediators (Ford and McVicar, 2009). Of particular interest, PGLYRP-1 acts either independently or in combination with PGN to promote signaling at this receptor. In our previous work (Garcia-

Crespo et al., 2013), we found that delivery of high concentrations of peptidoglycan resulted in prolonged survival ($t_{1/2} = 9.0$ vs. 10.5 days, $^*p < 0.05$) upon subsequent challenge with PVM, although not 100% protection that we readily observe in response to *Lactobacillus*. As such, PGLYRPs are unlikely to function alone to promote *Lactobacillus*-mediated protection. However, the fact that we observe even a minor response to PGN suggests that we cannot rule out the possibility of some contributions from these receptors.

5. Conclusions

In summary, *L. plantarum* is a versatile and flexible agent for prevention of lethal sequelae of respiratory virus infection via its actions at the respiratory mucosa. These interactions are distinct from those elicited by *Lactobacillus* species at the gastrointestinal mucosa, and do not rely on signaling mediated by IL-10. We demonstrated previously that priming of the respiratory mucosa with *L. plantarum* results in robust and sustained protection against lethal infection with PVM; we demonstrate here that administration of heat-inactivated *L. plantarum* within 24 h after virus challenge also results in full survival from an otherwise lethal virus challenge, a finding of significant clinical potential. *In vitro* signaling assays indicate that *L. plantarum* interacts with PRRs NOD2 and TLR2, yet mice devoid of each of these individual PRRs remain fully protected against lethal PVM infection by *L. plantarum*. Taken together, these results speak to the possibility of (a) significant redundancy of these critical responses and/or (b) mechanisms that complement, bypass or supersede PRR responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PVM	pneumonia virus of mice
<i>Lp</i>	<i>Lactobacillus plantarum</i>
<i>Lr</i>	<i>Lactobacillus reuteri</i>

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.antiviral.2015.07.001>.

Fig. 1.

L. plantarum or *L. reuteri* introduced *after* virus challenge promote survival in response to an otherwise lethal infection in BALB/c mice. (A) Experimental protocol: BALB/c mice are inoculated with PVM at day 0 followed by intranasal inoculations with live or heat-inactivated (hi) *L. plantarum* (*Lp*) or *L. reuteri* (*Lr*) at 2×10^8 cells/mL (10^7 cells/mouse), 2×10^9 cells/mL (10^8 cells/mouse), 2×10^{10} cells/mL (10^9 cells/mouse), or diluent control (pbs/bsa) in a 50 μ L volume at day (d)1, d2, and/or d3 as indicated. (B) Survival of mice inoculated with hi *Lp* or pbs/bsa diluent control as in (A); *** $p < 0.001$, ** $p < 0.01$ vs. pbs/bsa, $n = 5$ –10 mice per group. (C) Survival of mice inoculated with live or hi *Lp* or pbs/bsa diluent control 10^8 cells/mouse or pbs/bsa diluent control on d1 and d2 (see (A)); *** $p < 0.001$, $n = 5$ mice per group. (D) Survival of mice inoculated with live or hi *Lr* at 10^9 cells/mouse or pbs/bsa diluent control on d1; $n = 5$ mice per group (see (A)), *** $p < 0.001$.

Fig. 2.

L. plantarum introduced after virus challenge limits virus recovery and virus-induced inflammation. (A) Virus titer in lung tissue on day 5 after inoculation with PVM from mice that received intranasal inoculations of hi *Lp*, 10⁸ cells/mouse on d1 or on d1 and d2; **p* < 0.05; ***p* < 0.01 vs. pbs/bsa. (B) Formalin-fixed, hematoxylin and eosin-stained lung tissue from mice inoculated on d1 and d2 as described in the legend to (A); arrows in panel i. point to areas of inflammation. (C) Proinflammatory cytokines IL-6, CCL2, and CXCL10 in lung tissue of mice inoculated as in the legend to (A); **p* < 0.05; ***p* < 0.01. (D) Proinflammatory cytokines IL-6 and CCL2 in lung tissue on day 5 after inoculation with PVM from mice that received intranasal inoculations of live *Lp*, 10⁸ cells/mouse on d1 and d2; ***p* < 0.01.

Fig. 3.

Leukocytes identified in lung tissue of PVM-infected mice that treated with *L. plantarum* vs. diluent control. Total leukocytes (all CD45⁺) from single cell suspensions prepared from lungs of PVM-infected (PVM on day 0) BALB/c mice on day 5 after intranasal inoculation of heat-inactivated *Lp* (10⁸ cells/mouse/inoculum) or diluent (pbs/bsa) on d1 and d2. (A) Alv MΦs, alveolar macrophages (CD11c⁺SiglecF⁺); DCs, dendritic cells (CD11c⁺MHCII⁺); mono-mΦs, monocyte/macrophages (CD11c⁺MHCII⁻); Eos, eosinophils, (SiglecF⁺CD11c⁻); PMNs, neutrophils, (Gr1⁺). (B) NK (natural killer) cells, (CD49b⁺); NKT cells (CD3⁺CD49b⁺). (C) CD4⁺ T cells; CD8⁺ T cells; B cells (CD19⁺); *n* = 5 per group; * *p* < 0.05, ** *p* < 0.01.

Fig. 4.

L. plantarum introduced after virus challenge promotes survival in association with suppression of virus-induced inflammation in C57BL/6 mice. (A) Survival of mice inoculated with hi *L. plantarum* (*Lp*) as in Fig. 1A; *** $p < 0.001$ vs. pbs/bsa; $n = 5-10$ mice per group. (B) Survival of mice inoculated with live *L. reuteri* (*Lr*) as in Fig. 1A; *** $p < 0.001$ vs. pbs/bsa; $n = 5$ mice per group. (C) Leukocytes in bronchoalveolar lavage (BAL) fluid from mice challenged with PVM on day 0 and pbs/bsa or hi *Lp* (10⁹ cells) on d1; * $p < 0.05$; mΦs, macrophages; eos, eosinophils; pmns, neutrophils. (D) Virus titer, mice inoculated as described in the legend to (C), * $p < 0.05$. (E) IL-6 and (F) CCL2 in lung tissue from mice inoculated as described in the legend to (C), * $p < 0.05$, $n = 5$ mice per group.

Fig. 5.

The anti-inflammatory cytokine interleukin-10 (IL-10) does not mediate survival promoted by hi *L. plantarum* at the respiratory mucosa. (A) Survival of wild-type (wt) BALB/c and IL-10 gene-deleted (IL-10^{-/-}) mice inoculated with PVM at day 0 followed by hi *L. plantarum* (10⁸ cells/mouse) or diluent control at days (d1) and d2 as shown; *** *p* < 0.001 vs. mice receiving pbs/bsa, *n* = 5–15 mice per group. (B) Virus titer in lung tissue of IL-10^{-/-} mice inoculated as in (A) evaluated at d5; *** *p* < 0.001. (C) Proinflammatory cytokines IL-6 and CCL2 in lung tissue of mice inoculated as in (A) and evaluated on d5; ** *p* < 0.01 *n* = 5 mice per group.

Fig. 6.

TLR2 (TLR2^{-/-}) and NOD2 (NOD2^{-/-}) gene-deleted mice respond to *L. plantarum* and are protected from the lethal sequelae of PVM infection. (A) Activation of PRRs expressed by HEK293 transfectants; detection via products of secretory alkaline phosphatase (A650) in an NF- κ B-linked activation assay (Invivogen). Shown are results of triplicates of independent assays of PRRs challenged with 10^8 cells/mL hi *L. plantarum* (black filled bars) or control diluent alone (open bars); ** p < 0.01 vs. control. (B) Survival of wild-type, TLR2^{-/-} and NOD2^{-/-} mice inoculated with PVM at day 0 followed by hi *L. plantarum* (10^9 cells/mouse) at d1 and d2 as in Fig. 1A; *** p < 0.001, *** p < 0.001; ** p < 0.02 vs. mice of the same strain receiving diluent control, n = 5–10 mice per group. (C) Virus titer in lung tissue of NOD2^{-/-} mice inoculated with diluent only (–) or with hi *L. plantarum* (+) as in (B) and evaluated on day 7. (D and E) Expression proinflammatory cytokines IL-6 and CCL2 in lung tissue of NOD2^{-/-} mice inoculated with diluent (–) or with hi *L. plantarum* (+) as in (B) and evaluated on day 7; * p < 0.05, ** p < 0.01; n = 4–5 per group. (F) Survival of wild-type and IFN α β R^{-/-} mice inoculated with PVM at day 0 followed by hi *L. plantarum* (10^9 cells/mouse) on d1 and d2 as in Fig. 1A; *** p < 0.001 vs. mice of the same strain receiving diluent control, n = 5–10 mice per group.

Table 1

Significant (0.05, except where noted *) differential gene expression (1.5-fold) of virus-induced soluble proinflammatory mediators in response to priming with *L. plantarum* (*Lp*). BALB/c mice were inoculated intranasally with *L. plantarum* (10^9 cells) or diluent control (pbs/bsa; PBS) on days-14 and -7 (see Gabryszewski et al., 2011), followed by inoculation with pneumonia virus of mice (PVM; 0.2 TCID₅₀ units/50 μ L) or vehicle (VEH) control on day +14. Featured is the differential expression of 31 soluble proinflammatory mediators, a subset of the 839 differentially expressed transcripts detected by whole genome microarray from lung tissue evaluated on day 19, 5 days after inoculation of PVM.

Gene symbol	Entrez gene	Description	PBS/PVM ^a vs. VEH	<i>Lp</i> /PVM ^b vs. VEH	<i>Lp</i> /PVM ^c vs. PVM
Gzmb	14939	Granzyme B	309.0	83.2	
Il6	16193	Interleukin 6	231.5	2.2*	-105.0
Ccl2	20296	Chemokine (C-C motif) ligand 2	225.3	16.6*	-13.6
Cxcl10	15945	Chemokine (C-X-C motif) ligand 10	75.2	6.8	-11.0
Lcn2	16819	Lipocalin 2	68.0	20.5	-3.3
Cxcl9	17329	Chemokine (C-X-C motif) ligand 9	60.8	8.1*	
Cxcl2	20310	Chemokine (C-X-C motif) ligand 2	56.7	2.8*	-20.0
Saa3	20210	Serum amyloid A 3	50.3	25.1	
Saa1	20208	Serum amyloid A 1	50.1	-1.7*	-86.7
Ccl7	20306	Chemokine (C-C motif) ligand 7	47.2	3.4*	-13.8
Cxcl1	14825	Chemokine (C-X-C motif) ligand 1	47.1	14.7	
Cxcl11	56066	Chemokine (C-X-C motif) ligand 11	32.8	1.6*	-21.1
Ccl2	20293	Chemokine (C-C motif) ligand 12	13.1	8.6*	
Il1m	16181	Interleukin 1 receptor antagonist	9.8	4.3*	
Lgals3 bp	19039	Lectin, galactoside-binding, soluble, 3 binding pro	9.3	8.3	
Thbs1	21825	Thrombospondin 1	8.9	1.6*	-5.5
S100a8	20201	S100 calcium binding protein A8 (calgranulin A)	7.3	-1.0*	
Csf1	12977	Colony stimulating factor 1 (macrophage)	7.2	3.6*	
S100a9	20202	S100 calcium binding protein A9 (calgranulin B)	6.6	-1.7*	-11.1
Lgals9	16859	Lectin, galactose binding, soluble 9	6.2	3.0*	
Cxcl13	55985	Chemokine (C-X-C motif) ligand 13	4.0	3.9*	

Gene symbol	Entrez gene	Description	PBS/PVM ^a vs. VEH	<i>Lp</i> /PVM ^b vs. VEH	<i>Lp</i> /PVM ^c vs. PVM
Ccl5	20304	Chemokine (C-C motif) ligand 5	3.1	2.9*	
Ccl9	20308	Chemokine (C-C motif) ligand 9	3.0	4.6	
Cxcl16	66102	Chemokine (C-X-C motif) ligand 16	3.0	1.7*	
Lgals3	16854	Lectin, galactose binding, soluble 3	2.6	2.4	
Hmgbl	15289	High mobility group protein B1-like	-2.2	-1.1*	2.0
Cxcl15	20309	Chemokine (C-X-C motif) ligand 15/IL8	-2.2	1.3*	2.8
Il33	77125	Interleukin 33	-2.9	1.9*	5.4
Cxcl12	20315	Chemokine (C-X-C motif) ligand 12	-5.9	-2.1*	2.8
Ccl8	20307	Chemokine (C-C motif) ligand 8	3.4*	16.7	5.0
Ccl6	20305	Chemokine (C-C motif) ligand 6	-1.3*	3.8	4.9

^a Mice primed with diluent (PBS) and inoculated with PVM vs. mice primed with diluent and inoculated with vehicle (VEH).

^b Mice primed with *L. plantarum* (*Lp*) and inoculated with PVM vs. mice primed with diluent (PBS) and inoculated with vehicle (VEH).

^c Mice primed with *L. plantarum* (*Lp*) and inoculated with PVM vs. mice primed with diluent (PBS) and inoculated with PVM; only statistically significant differences are shown.

* Values not significant (>0.05) over PBS/VEH.