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Lactobacillus-Mediated Priming of the Respiratory Mucosa Protects against Lethal Pneumovirus Infection

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The Journal of Immunology

Respiratory virus infections pose a major burden to society, both with regard to clinical illness and health care costs. Respiratory syncytial virus (RSV), a pneumovirus of the family Paramyxoviridae, is a chief cause of hospitalization for infants and young children and, likewise, results in significant morbidity and mortality in the elderly (1, 2). In most infants and children, RSV bronchiolitis is self-limited, whereas in others, disease can progress to pneumonia and respiratory failure. Prematurity, multiple births, cardiovascular and pulmonary anomalies, and immunocompromised state are among the risk factors predisposing to severe RSV disease (3), although a recent prospective population-based surveillance study of acute RSV infection among children younger than 5 y of age indicated that most hospitalized children were previously healthy, with none of the documented risk factors (4). The lack of a safe and effective vaccine for RSV and the expense and limited availability of prophylactic Ab therapy (5) represent serious hurdles in managing this significant public health problem.

In an effort to improve our understanding of the pathogenesis of severe pneumovirus infection in vivo, our laboratory has developed a model featuring the natural rodent pathogen, pneumonia virus of mice (PVM) (6, 7). Although no one animal model can replicate all the complexities of human disease, PVM has the advantage of undergoing robust replication and eliciting symptomatic disease in most inbred strains of mice (8). With this model, we have documented molecular and cellular pathology, including granulocyte recruitment to the lung tissue and virus-induced production of proinflammatory cytokines; in doing so, we have highlighted parallels to the more severe forms of human RSV disease (9–11). Using the PVM infection model, we have shown that morbidity and mortality depend largely on augmented inflammation, which can persist even when virus replication has been brought under control (12–14). The importance of inflammation to the sequelae of severe respiratory virus infection has motivated our interest in immunomodulatory therapy (15) and, most recently, has led us to explore the immunotherapeutic potential of probiotic microbes and the expanding field of related microbial-derived biologics and biotherapies.

Probiotics are defined by the World Health Organization as “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (16, 17); recently, the term “pharmabiotic” has been introduced so as to include inactivated microorganisms, biologicals that influence live organisms, and therapeutically active metabolites (18). Among the most familiar of this target group are microorganisms of the genus Lactobacillus, which includes more than 100 species of Gram-positive bacteria that form lactic acid as a product of carbohydrate metabolism. Lactobacilli are best known as minor components of the commensal microflora of the mammalian gastrointestinal (GI) tract, and they have been popularized by the food industry for the...
purposes of acidification, flavor enhancement, and nutrition. Multiple human studies have demonstrated the immunotherapeutic properties of probiotic lactobacilli as nutritional supplements targeted to the GI tract, including prevention of acute diarrhea, alleviation of the symptoms of allergy, and therapy for inflammatory bowel disease (19, 20). In mouse model studies, colony-forming units of *L. plantarum* were administered orally prior to influenza virus challenge; however, all mice eventually succumbed to the lethal sequelae of infection (21). Likewise, a recent review of randomized controlled trials in which humans with respiratory tract infections were treated orally with lactobacilli indicates little to no effect on disease incidence, as well as conflicting data on the duration of symptoms (22).

Given that lactobacilli clearly modulate inflammation at the GI epithelium, it is conceivable that the oral route of administration is not optimal for regulating local responses to infection with respiratory pathogens. As such, we considered the possibility that targeting live lactobacilli to the respiratory mucosa might result in a more effective immunomodulatory response, similar to the benefits observed when the intestinal mucosa is exposed directly to these organisms. To address this question, we primed the respiratory mucosae of mice with either *Lactobacillus plantarum*, an ecologically flexible species first isolated from human saliva (23), or *Lactobacillus reuteri*, a common GI bacterium initially isolated from human feces (24), by direct intranasal inoculation with live microorganisms. We use the term “priming” to mean “providing a treatment that alters the capacity to respond to a second stimulus” (based on the definition in Ref. 25), a phenomenon we observed upon administering lactobacilli directly to the respiratory epithelia of mice. Specifically, we found that mice primed with either of the two clinically benign *Lactobacillus* species were completely protected from a subsequent lethal respiratory virus challenge. In this study, we explore the nature and characteristics of this response.

## Materials and Methods

### Mice

Wild-type BALB/c and C57BL/6 mice were purchased from Taconic (Gaithersburg, MD). Homozygous *MyD88*^-/-^ mice on a C57BL/6 genetic background were used with permission from Dr. Shizuo Akira (26). All mouse experiments were performed in accordance with Animal Study Protocol LAD-8E, approved by the National Institute of Allergy and Infectious Diseases Animal Care and Use Committee.

### Bacteria preparation and quantification

*L. plantarum* NCIMB 8826 (ATCC BAA-793) and *L. reuteri* F275 (ATCC 23272) were cultured overnight at 37˚C in Difco Lactobacilli MRS Broth (BD Biosciences, Sparks, MD). *Listeria innocua* Seeliger (ATCC BAA-680) cultures were generated by overnight growth at 37˚C in Sheep Brain Heart infusion medium (Thomas Scientific). Serial dilutions were plated and counted to correlate the number of CFU per mL.
milliliter with the OD at 600 nm. Bacterial cultures were harvested by centrifugation (5 min, 1500 rpm), washed with PBS, and suspended in PBS with 1% BSA just prior to inoculation. Inactivated bacteria were prepared by washing in PBS and heating to 95°C for 30 min prior washing in PBS and resuspension in PBS with 1% BSA; inactivation was confirmed by overnight culture in appropriate culture broths.

**Virus preparation and quantification**

Virions of PVM strain J3666 passedag in vivo as described (27) were quantitated by dual standard curve quantitative RT-PCR targeting the PVM SH gene (28) to determine both virion copies per unit volume for inoculation (PVM<sub>mg/m</sub>/µl) and virus recovery from infected mouse lung tissue (PVM<sub>mg/m</sub>/GAPDH). Specifically, RNA was prepared from mouse lung tissue that had been immersed and stored in RNAlater (Ambion, Austin, TX) and subsequently isolated with RNAzol Bee (Tel-Test, Friendswood, TX). Isolated RNA was treated with DNase I to remove genomic DNA contaminants. Reverse transcription was performed using a first-strand cDNA synthesis kit (Roche; catalog no. 1,483,188) with random primers and no reverse transcriptase control. The quantitative PCR reactions were run in triplicate, with the ABI TaqMan reagent, primer-probe mixes, and cDNA or plasmid standard in a 25 µl triplicate, with the ABI 7500 absolute quantitation program (Applied Biosystems) supplied 50°C for 2 min (UNG incubation), 95°C for 10 min (AmpliTaq Gold activation), and 40 amplification cycles alternating 95°C for 15 s and 60°C for 1 min. Primer-probe mixes include 1) GAPDH-Vic (Applied Biosystems catalog no. 4308313) to target GAPDH cDNA and 2) PVM<sub>SH</sub>-Fam (custom design, primer 1, 5' GCC GTC ATC AAC ACA GTG TGT-3'; primer 2, 5' GCC TGA TGG GCG AAT GCT T-3'; probe 6FAM-CGC TGA TAA TGG CCT GCA GCA-TAMRA) to target the PVM SH gene to target the virus genome. GAPDH standard curve includes serial dilutions (10<sup>-7</sup>, 10<sup>-6</sup>, 10<sup>-5</sup>, 10<sup>-4</sup>, 10<sup>-3</sup> molecules/reaction) of mouse GAPDH coding sequence in pCMV Sport 6 (ATCC cat no. 10539385). PVM<sub>SH</sub> standard curve includes serial dilutions (10<sup>-7</sup>, 10<sup>-6</sup>, 10<sup>-5</sup>, 10<sup>-4</sup> molecules/reaction) of the full-length PVM SH gene, GenBank (http://www.ncbi.nlm.nih.gov/nuccore) accession number AY573815, in pBacPAK8. Experimental triplicate data points are interpolated to linear standard curves over the concentration ranges indicated. A sample calculation from data generated by this method is shown in Supplemental Fig. 1.

**Inoculations and sample collection**

In our standard experimental protocol (see Fig. 1A), mice were anesthetized in 2% halothane in mineral oil and inoculated intranasally on days −14 and −7 with 10<sup>9</sup> CFU L. plantarum, L. reuteri, or control (PBS plus 1% BSA) in a 50 µl volume. On day 0, Lactobacillus- or control-primed mice were inoculated with 50 µl PVM diluted 1:3000, to 2000 virion copies PVM<sub>SH</sub> per µl in IMDM. At selected time points, mice were sacrificed by cervical dislocation under anesthesia, and mice were subjected to tracheal lavage. Whole lung tissue was stored in RNAlater for RNA isolation, as detailed elsewhere (28) or bladder-homogenized in ice-cold IMDM, followed by centrifugation at 4°C for preparation of clarified tissue homogenates. Clarified supernatants were stored in aliquots at −80°C until use.

**Histopathology**

On day 7 of our treatment protocol, lungs of sacrificed mice were inoculated trachealily using 250 µl 10% phosphate-buffered formalin. The lungs and heart were removed and fixed overnight in 10% phosphate-buffered formalin at 4°C. Samples were paraffin-embedded, sectioned, and stained with H&E (Histoserv, Germantown, MD).

**Single-cell suspensions**

Single-cell suspensions were generated from mouse lung tissue as described (29). Lungs were perfused with 5–10 ml PBS plus 10 mM EDTA and placed in digestion medium, consisting of RPMI 1640 medium (Invitrogen) supplemented with 5% FBS (BioWhittaker, Walkersville, MD), 20 µg/ml grade II DNase I (Roche Diagnostics, Mannheim, Germany), and 40 µg/ml collagenase D (Roche Diagnostics). The tissue was cut into ∼3-mm<sup>3</sup> pieces and incubated in digestion medium (37°C, 90 min, with rocking). Upon adding cold EDTA (10 mM) to the cell suspension, the cells were passed through a 40-µm nylon cell strainer (BD Biosciences, Durham, NC) and washed with PBS supplemented with 0.3% BSA, 5 mM EDTA, and 20 µg/ml DNase I. Cells were centrifuged (5 min, 1500 rpm), RBCs were eliminated with 1 in ACK lysing buffer (BioWhittaker) for 2 min as needed, and the remaining

**Flow cytometry**

Prior to fixation, lung cells (∼10<sup>6</sup> cells per condition) were blocked with anti-mouse CD16/CD32 mAb (BD Biosciences) and, as appropriate, stained with mouse CD16/CD32 mAb (BD Biosciences) and, as appropriate, stained with

**FIGURE 2.** Priming of the respiratory mucosa with heat-inactivated lactobacilli and other Gram-positive bacteria. Standard experimental protocol is as per Fig. 1A unless otherwise noted. A. Survival of mice inoculated with L. plantarum (LP), heat-inactivated (hi) L. plantarum (10<sup>9</sup> CFU-equivalents), or PBS prior to virus infection. B. Survival of mice inoculated with 10<sup>9</sup> CFU Listeria innocua (LI), 10<sup>9</sup> CFU equivalents heat-inactivated L. innocua (li), or PBS prior to virus infection. C. Survival of naive BALB/c mice inoculated with PVM on day 0, then inoculated with lactobacilli (10<sup>9</sup> CFU L. plantarum, 10<sup>9</sup> CFU equivalents heat-inactivated [hi] L. plantarum, or PBS plus BSA) on day 3. Each group includes 10 or more mice per condition, representative of at least two independent experiments. ***p < 0.001.

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Table 1. Survival of L. plantarum-primed BALB/c mice

<table>
<thead>
<tr>
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<th>Day 0</th>
<th>Day 7</th>
<th>Day 21</th>
<th>Day 91</th>
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<tr>
<td>Survival</td>
<td>8/8 (100%)</td>
<td>7/8 (88%)</td>
<td>8/8 (100%)</td>
<td>6/10 (60%)</td>
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Survival of L. plantarum-primed BALB/c mice inoculated with PVM on day 0 as per standard protocol (Fig. 1A) or delayed 7, 21, or 91 d thereafter. Survival was determined at t = 3 d after each virus inoculation. All surviving mice (29 of a total 34) underwent seroconversion to PVM Ags (SMART-M12; see Materials and Methods).

The single death among the mice inoculated on day 7 occurred 8 d after PVM inoculation. The full survival analysis of the mice inoculated 91 d after Lactobacillus priming is shown in Fig. 1D.
LIVE/DEAD violet-fluorescent reactive dye (Invitrogen, Eugene, OR) and/or DX-5 PE (BD Pharmingen; applied prior to fixation) in PBS plus 1% BSA. Ab-fluorochrome conjugates included B220 Alexa Fluor 488, CD3 PE-Cy5, CD4 PerCP-Cy5.5, CD8 allophycocyanin-Cy7, and GR1 allophycocyanin (BD Pharmingen). Cells were stained for 30 min at 4°C, washed with PBS plus 1% BSA, and analyzed using an LSR II flow cytometer in conjunction with FACSDiva software (BD Biosciences), with a minimum of 100,000 events collected per sample.

Cytokine expression

RNA from mouse lung tissue was isolated, pooled (12 μg total RNA per condition), and purified using the RT² qPCR-grade RNA isolation kit (SABiosciences, Frederick, MD). First-strand cDNA was generated using the RT² first-strand kit (SABiosciences) and, in conjunction with the RT² SYBR Green/ROX qPCR Master Mix (SABiosciences), analyzed for differential gene expression using the RT² Profiler mouse inflammatory cytokines and receptors PCR array (SABiosciences). ELISA analysis was performed to quantify immunoreactive proteins using Quantikine kits from R&D Systems (Minneapolis, MN) as per the manufacturer’s instructions. Data points generated using lung tissue homogenates were normalized to total protein per sample, which was determined via BCA protein assay (Pierce, Rockford, IL) with BSA standards.

Microarray data

Our microarray dataset (M430-2 chip) documents the normalized expression profiles of 39,000 transcripts expressed in mouse lung response to PVM infection over time (30). Profiles of specific chemokine transcripts were determined via algorithms within Genespring GX7.3 (Agilent Technologies).

Detection of granulocytes

Granulocytes were evaluated in BAL fluid at days indicated using modified Giemsa staining (Diff-Quik; Fisher Scientific, Pittsburgh, PA). To prepare cells for staining, BAL fluids were subjected to centrifugation and the cell pellets were resuspended in PBS plus 1% BSA. Cells (10⁵) were centrifuged onto slides using a Shandon Cytospin apparatus (Thermo Electron, Pittsburgh, PA). Following staining and mounting of cells, 10 high-power fields were visually inspected by light microscopy.

Seroconversion

Mouse sera were isolated from tail bleeds via standard procedures and assessed for seroconversion (immunoreactivity to PVM Ags) using the FIGURE 3. Virus recovery from lung tissue of Lactobacillus- and control-primed mice. A, Virus recovery from lung tissue at days 3, 5, and 7 after inoculation with PVM from mice treated with either Lactobacillus spp. or PBS plus BSA control, as determined by quantitative RT-PCR with two standard curves (Supplemental Fig. 1); n = 5–7 mice/time point/condition. *p < 0.05; **p < 0.01; n.s., not significant. B and C, Survival of and virus recovery from Lactobacillus (LP) and PBS plus BSA-primed mice. The virus (PVM) inoculum received by 10 PBS plus BSA-primed mice was reduced to 600 copies/μl so that peak recovery at day 5 would be comparable to that detected in the 10 L. plantarum-primed mice. Virus recovery was evaluated in five mice selected randomly on day 5 from each group as shown, and percentage survival of five mice remaining is as shown. **p < 0.01. LR, L. reuteri.
SMART-M12 PVM kit (Biotech Trading Partners, Encinitas, CA) per the manufacturer’s guidelines.

**Lactobacillus clearance**

Mice were inoculated with $10^9$ CFU *L. plantarum* or *L. reuteri* and sacrificed at 1, 2, 3, and 7 d thereafter; lungs were blade-homogenized in 1 ml sterile PBS plus 1% BSA. Homogenates (100 µl) were plated at 1:1, 1:20, or 1:100 on MRS agar and incubated overnight at 37°C. Homogenates from PBS plus BSA-treated mice were included as controls.

**Statistical analysis**

Data were evaluated using Welch’s t test, Mann–Whitney *U* test, ANOVA with Bonferroni’s multiple comparison test, or the log-rank test for survival curves, as appropriate. All statistical tests were included in the GraphPad Prism 5 software package (GraphPad Software, La Jolla, CA). Significant outliers were identified using the Grubbs test. All bar graphs indicate the mean ± SEM.

**Results**

**Priming of the respiratory mucosa with lactobacilli results in protection from lethal pneumovirus infection**

The first question motivating our study was whether direct stimulation or priming of the respiratory mucosa with live lactobacilli would alter the outcome of a lethal pneumovirus infection. To explore this question, we devised a standard protocol whereby mice were primed with two intranasal inocula (on days 214 and 27, respectively), each containing $10^9$ CFU live *L. plantarum* or *L. reuteri*; these inocula were followed by intranasal challenge with PVM on day 0 (Fig. 1A). We found that BALB/c mice primed in this fashion were fully protected from an otherwise lethal respiratory virus infection (Fig. 1B). BALB/c mice responded to *Lactobacillus* priming with transient granulocyte recruitment (Fig. 1C). *Lactobacillus* priming alone resulted in no acute mortality, and we observed complete clearance of live lactobacilli from the lung tissue within 7 d after inoculation (data not shown). Direct *Lactobacillus* priming of the respiratory mucosa resulted in protection from lethal sequelae of virus infection even when challenge was delayed from day 0 until day 7 or day 21 (Table I), and we observed prolonged survival and significant long-term protection even when virus challenge was delayed until 91 d (13 wk) after initial priming (Fig. 1D). Priming with heat-inactivated *L. plantarum* ($10^9$ CFU equivalents) also generated protection against lethal virus challenge (Fig. 2A), as did priming with live or heat-inactivated Gram-positive *Listeria innocua* (Fig. 2B), indicating that these findings are not unique to *Lactobacillus* species. Of note, neither live nor heat-inactivated *L. plantarum* was effective when administered therapeutically, 3 d after virus inoculation, rather than as a priming agent (Fig. 2C).

**Virus recovery from lungs of Lactobacillus- and PBS-primed mice**

Virus recovery from whole lung tissue was determined on days 3, 5, and 7 after PVM challenge (Fig. 3A). Virus replication was detected in *Lactobacillus*-primed as well as PBS-primed mice, and seroconversion to PVM Ags was detected in all mice surviving...
filtrates, consistent with descriptions of induced BALT (Fig. 4) featured compact, peribronchiolar, and perivascular cuff-like infusions. Primed, virus-infected mice exhibited minimal inflammation peripherally and widespread, diffuse granulocyte recruitment and early onset edema. In agreement with our previous studies (12, 13), lungs of control-primed mice survived virus infection (100%), whereas 95% of the PBS-primed mice did not (Fig. 1B). These results suggest that Lactobacillus-mediated alteration of virus kinetics (replication and clearance) may not provide a sufficient explanation for differential survival. To evaluate the relationship between virus recovery and survival directly, 10 L. plantarum- and 10 PBS (PBS plus 1% BSA)-primed mice were inoculated on day 0 with PVM (standard protocol, Fig. 1A), with the virus inoculum received by the control (PBS-primed) mice reduced to 600/μL, so that peak virus recoveries (day 5) would be comparable. Five mice were selected randomly from each group for determination of virus recovery. As anticipated, differences in peak virus recoveries were insignificant (Fig. 3B), yet none of the remaining PBS-primed, virus-infected mice survived beyond day 9, whereas all of the remaining L. plantarum-primed, virus-infected mice survived long-term (Fig. 3C; **p < 0.01). From these findings we conclude that, although Lactobacillus priming modulates virus replication and clearance, virus recovery alone cannot predict disease outcome or long-term survival.

**Lung histopathology and differential leukocyte recruitment**

In agreement with our previous studies (12, 13), lungs of control-primed, PVM-infected mice revealed a profound alveolitis, with widespread, diffuse granulocyte recruitment and early onset edema (Fig. 4A–4C). In contrast, the lungs of L. plantarum-primed, PVM-infected mice exhibited minimal inflammation peripherally and featured compact, peribronchiolar, and perivascular cuff-like infiltrates, consistent with descriptions of induced BALT (Fig. 4D–4F) (31, 32).

Consistent with these histopathologic findings, priming with L. plantarum prior to PVM infection resulted in a 5-fold reduction in the fraction of GR1⁺ granulocytes (Fig. 5A, 5B) and a concomitant 2-fold increase in the fraction of lymphocytes in whole-lung single-cell suspensions compared with PBS-primed, PVM-infected controls (Fig. 5B). Additional analysis of the lymphocyte populations revealed that Lactobacillus priming prior to PVM infection had no impact on the relative proportions of CD4⁺ T cells (CD3⁺CD4⁺CD8⁻), CD8⁺ T cells (CD3⁺CD4⁻CD8⁺), or B cells (B220⁺), whereas the fraction of NK cells (CD3⁻DX5⁺) was diminished (Fig. 5C).

**Suppression of virus-induced proinflammatory cytokines**

Morbidity and mortality in response to severe respiratory virus infection can result in large part from uncontrolled amplification of proinflammatory signaling networks (33). We observed that Lactobacillus priming of the respiratory mucosa resulted in suppression of multiple cytokines associated with the inflammatory pathology of respiratory virus infection. Specifically, priming with lactobacilli resulted in marked suppression of IFN-inducible protein-10 (CXCL10), MCP-1 (CCL2), neutrophil-activating protein-3 (CXCL1), MIP-1γ (CCL9), TNF, and eotaxin-2 (CCL24) (Fig. 6A). CCR1 is a prominent CCR detected on granulocytes (34); differential detection of this transcript is consistent with the flow cytometric results shown in Fig. 4A and 4B. Of note, Lactobacillus-mediated suppression of CXCL10 and CCL2 was evident as early as day 4 after virus inoculation (Supplemental Fig. 2). Cytokine mediators determined as not differentially expressed in response to Lactobacillus-priming included CCL3, CCL5, CXCL12, and TGF-β1. CXCL10, CCL2, and CXCL1 are expressed prominently in mouse lung tissue in response to PVM infection. These proinflammatory mediators are among those with transcriptional profiles that parallel respiratory dysfunction as detected in our PVM gene expression microarray database (30), which documents the full spectrum of transcriptional responses to the PVM pathogen in otherwise unmanipulated wild-type mice (Supplemental Fig. 3). Immune-active CXCL10, CCL2, CXCL1, and TNF were detected in BAL fluid of PBS-primed, PVM-infected mice; expression was suppressed in mice primed with lactobacilli, most notably expression of CCL2 (~20-fold suppression) (Fig. 6B). Interestingly, we detected expression of both IFN-β and IL-10 in response to PVM infection, but neither of these cytokines, implicated in Lactobacillus-mediated immunomodulation in a variety of other settings.

**FIGURE 5.** Flow cytometric analysis of leukocyte subsets. A. Flow cytometric analysis of whole-lung single-cell suspensions generated from control- (PBS plus BSA) or L. plantarum (LP)-primed, PVM-infected mice (day 7, n = 5–6 mice/condition). Total lung cells were gated for side light scatter (SSC) and expression of the cell surface granulocyte marker GR1, shown with no Ab and isotype-matched Ab controls. Data shown are representative of four independent experiments. B. Percentage of total viable lung cells identified as granulocytes (GR1⁺) or lymphocytes (identified by characteristic forward/side light scatter) from PBS plus BSA-primed, PVM-infected or L. plantarum-primed, PVM-infected mice. C. Percentage of total viable lymphocytes (in B), with CD4⁺ T cell (CD3⁺CD4⁺CD8⁻), CD8⁺ T cell (CD3⁺CD4⁻CD8⁺), NK cell (CD3⁻DX5⁺), or B lymphocyte (B220⁺) immunophenotype. Data shown are compiled from four independent experiments. *p < 0.05; **p < 0.01.
(35–38), was detected as differentially expressed in response to Lactobacillus priming in BALB/c mice (Fig. 6C).

**MyD88-dependent signaling, virus recovery, and differential survival**

Previous studies published by several groups have identified Lactobacillus-mediated immunomodulatory mechanisms that are dependent on TLRs, primarily TLR2 and TLR4 (39). As an initial exploration of the TLR dependence of Lactobacillus-mediated protection against virus infection, we carried out our inoculation protocol with mice devoid of MyD88 (MyD88^-/-), the (near) universal TLR adapter protein. These mice were available to us on the C57BL/6 background. Mice of this background are less susceptible to the lethal sequelae of Lactobacillus infection than are BALB/c mice at the same virus inoculum (8). Lactobacillus-mediated priming was effective at preventing lethal sequelae of PVM infection in the C57BL/6 background (Fig. 7A). Although some mortality was observed among the L. plantarum-primed MyD88^-/- mice at this virus inoculum, priming of the respiratory mucosa resulted in prolonged survival and significant long-term protection against the lethal sequelae of virus infection for a substantial fraction of mice (Fig. 7B).

Analysis of the differential survival (Supplemental Fig. 4) documents that TLR–MyD88 signaling did not contribute substantially to Lactobacillus-mediated protection. Similar to what was observed in experiments performed with control- and L. plantarum-primed wild-type BALB/c mice (Fig. 3A), Lactobacillus priming of C57BL/6 mice resulted in diminished virus recovery from lung tissue (∼5-fold; Fig. 7C). Virus recovery from lungs of control-primed MyD88^-/- mice was not significantly different from that measured in the control-primed wild-type C57BL/6 mice. Likewise, virus recovery from lungs of L. plantarum-primed MyD88^-/- mice was indistinguishable from that of the control-primed MyD88^-/- mice, despite clear evidence of differential survival within this genotype (Fig. 7B). These results are consistent with those shown in Fig. 3 and stand in support of our earlier conclusion that although Lactobacillus priming has a significant impact on virus recovery, virus titer in lung tissue does not serve as a predictor of disease outcome or long-term survival.

**MyD88-dependent signaling, virus infection, and proinflammatory cytokines**

Initial identification of specific proinflammatory mediators that were differentially expressed in Lactobacillus-primed, virus-infected versus control-primed, virus-infected lung tissue from BALB/c mice was determined by PCR array of 84 potential transcripts and confirmed by ELISA (Fig. 6; see Materials and Methods). As shown in Fig. 8, differential cytokine expression in L. plantarum-primed versus control-primed, virus-infected wild-type C57BL/6 mice is comparable to what was determined in wild-type BALB/c mice. Specifically, we observed profound suppression of CCL2 and CXCL10 (Fig. 8A, 8B) and moderate suppression of CXCL1 and TNF (Fig. 8C, 8D). Of note, significant suppression of IL-10 was observed in L. plantarum-primed, virus-infected C57BL/6 mice (Fig. 8E); this was not observed in BALB/c mice. All of these cytokines were detected in lung tissue of PVM-infected MyD88^-/- mice, albeit at significantly reduced
concentrations as compared with PVM-infected C57BL/6 wild-type mice (except for TNF, which is detected at levels that are not statistically different from control-primed, PVM-infected wild-type C57BL/6 mice). Interestingly, Lactobacillus-mediated cytokine suppression was not detected in MyD88−/− mice. Thus, differential cytokine expression is not a crucial feature of Lactobacillus-mediated protection in the absence of TLR–MyD88 receptor signaling.

Discussion
In this study we demonstrate that priming of the respiratory mucosa with nonpathogenic Gram-positive microorganisms results in full protection from the otherwise lethal sequelae of severe pneumovirus infection. Protection is observed in response to both live and heat-killed Lactobacillus strains (L. plantarum and L. reuteri) and live and heat-killed Gram-positive Listeria innocua, which, although not pathogenic, had not been considered previously as a potential probiotic or pharabiotic microorganism. Administration of live lactobacilli directly to the respiratory mucosa resulted in diminished virus recovery at multiple time points and prominent suppression of virus-induced proinflammatory mediators.

Although virus recovery is diminished in response to Lactobacillus priming, similar to results obtained by several other groups in experiments with influenza virus (40–42), in this study we build on these findings, as we examine the unique responses to priming and the specific relationship to virus recovery and cytokine production. First, we have determined clearly that virus recovery alone cannot predict outcome of disease, and that the virus-induced inflammatory response can lead to lethal sequelae even after initial replication is blunted with effective antiviral therapy (12, 13). Given our current understanding of the immunomodulatory capacity of lactobacilli at the GI mucosa, including modulation of cytokine production by target epithelial cells (16, 17, 19, 43), our primary hypothesis was that the major protective role of lactobacilli at the respiratory mucosa was directly related to their ability to suppress local virus-induced inflammatory responses. Upon further analysis, we found the relationship between virus-induced inflammation and Lactobacillus-mediated protection to be more complex than previously anticipated. We observed that mice devoid of the gene encoding the MyD88–TLR signaling adaptor were also protected from the lethal sequelae of PVM infection after priming with L. plantarum. Clearly, this does not rule out potential roles played by TLR-mediated signaling pathways that circumvent MyD88 or other pattern-recognition receptors that may play a role in sensing Lactobacillus species (44, 45); TLR desensitization has been observed as a mechanism that explains reduced inflammatory responses observed after recuperation from influenza virus (46). The data on TLR signaling in response to lactobacilli is complex and, to date, no consensus findings or aligning principles have emerged. For example, Hisbergues et al. (47) reported that mouse bone marrow-derived dendritic cells produce both IL-10 and IL-12 in response to L. plantarum via TLR2/MyD88-dependent and TLR4/MyD88-independent pathways. At the same time, Ichikawa et al. (48) reported that cells from mouse spleen and mesenteric lymph node produce IL-12 in response to L. paracasei via pathways that are MyD88-dependent, but are completely independent of TLR2, TLR4, or TLR9. In
contrast, Chung et al. (49) found that *L. casei*-mediated protection in the mouse model of dextran sulfate sodium-induced colitis was primarily dependent on signaling via TLR4. At this time, it is not clear whether responses are defined by an individual *Lactobacillus* species or strain, unique to a given organ or tissue, or whether they are specified by mouse genotype or functional overlap of specific signaling pathways.

Of note, to the best of our knowledge, this is the also the first documented evaluation of PVM infection in vivo in *MyD88* gene-deleted mice. Virus recovery from *MyD88*^−/−^ mouse lung tissue was similar to that reported by Bhoj et al (50) in their study of RSV clearance. However, in contrast to these findings, we observed accelerated replication of PVM in *MyD88*^−/−^ eosinophilic granulocytes from bone marrow cultures (51). The role of individual TLRs in mediating responses to pneumovirus infection remains incompletely explored (reviewed in Ref. 52); TLR2/6, TLR4, and TLR7 have all been implicated in augmenting immune responses in the RSV challenge mouse model, whereas closely related mouse pathogens Sendai virus (parainfluenza virus 1) and PVM do not engage TLR4 (53, 54).

Similar to findings described for wild-type mice, *Lactobacillus* priming promoted survival in PVM-infected *MyD88*^−/−^ mice, but, in contrast, it promoted no differential expression of proinflammatory mediators, CCL2, CXCL1, CXCL10, and TNF. These cytokines are all mediators that are prominently suppressed in association with *Lactobacillus*-mediated protection in wild-type mice. Although there are no unique roles attributed to any of these mediators in the pathogenesis of pneumovirus disease, collectively they have been implicated in promoting disease severity in a variety of settings. For example, the chemokine CCL2 has been detected in BAL fluid from RSV-infected infants (9, 55) and in RSV-challenged mice (56), and levels of this mediator correlate with respiratory dysfunction in the PVM infection model (30). Similarly, although CXCL10 has not received as much attention vis-à-vis the pathogenesis of pneumovirus-induced bronchiolitis (57), McNamara et al. (9) examined a series of BAL samples from infants intubated secondary to RSV bronchiolitis and detected CXCL10 as one of the two predominant chemokines. TNF has likewise been associated with severe RSV disease in several human studies (58, 59). Overall, our findings introduce an interesting and important conundrum, as it is clear that *Lactobacillus*-mediated protection is associated with diminished levels of multiple proinflammatory cytokines, but, given the fact that protection can be elicited in *MyD88*^−/−^ mice without evidence for this suppression, it is clear that this may be only one aspect of the overall protective mechanism. Among the possibilities, *Lactobacillus* priming may elicit multiple parallel, potentially degenerate responses that may compensate for one another (e.g., *MyD88*-independent pathways eliciting protection in equal force, duplicating the efforts of the derailed *MyD88*-dependent pathways). Given the variety and

**FIGURE 8.** *Lactobacillus*-priming of *MyD88*^−/−^ mice has no impact on virus-mediated production of inflammatory cytokines. Immunoreactive (A) CCL2, (B) CXCL1, (C) CXCL10, (D) IL-10, and (E) TNF detected in lung tissue homogenates from C57BL/6 and *MyD88*^−/−^ mice at day 7 after virus infection; *n* = 5–8 mice/time point/condition. Data shown are compiled from two independent experiments. *p < 0.05; **p < 0.01.
complexity of potential immunostimulants presented by unfractio-
nated Gram-positive organisms (i.e., peptidoglycan, lipoteichoic
acid, membrane proteins), this is a reasonable possibility that will
require careful mechanistic dissection.

In summary, we have observed that priming of the respiratory
mucosa with clinically benign Gram-positive Lactobacillus species
results in markedly diminished inflammatory responses upon chal-
lenge with a pneumovirus pathogen, and Lactobacillus–primed
hosts are fully protected from the otherwise lethal sequelae of
a severe respiratory virus infection. We demonstrate that virus
recovery from lung tissue is not a predictor of disease outcome.
Our goal is to characterize the protective responses at the mo-
lecular level and at the same time to determine a means by which
lactobacilli might be used as a broad-spectrum respiratory mu-
cosal immunomodulatory agent. These lactobacilli, or components
thereof, may serve as an effective innate immune shield to provide
critical protection against pneumovirus, and perhaps other severe
respiratory virus infections, prior to the development of safe,
specific, and reliable vaccines.

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