



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



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ARTICLE INFO

Article history:

Received 17 July 2012

Revised 28 November 2012

Accepted 10 December 2012

Available online 26 December 2012

Keywords:

Cytokines

Neutrophils

Peptidoglycan

Genomic DNA

Heterologous immunity

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- α and IL-17A) but not Th1 cytokines. IFN γ 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.

Published by Elsevier B.V.

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

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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

strains of mice (Bem et al., 2011; Rosenberg and Domachowski, 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 Hsueh and colleagues (Goulding et al., 2007; Didierlaurent et al., 2007; Hsueh 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 (Δ PT *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 γ ^{−/−} 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 OD₆₀₀ 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 μ L PBS/BSA with 10^9 or 10^8 cfu live *L. plantarum* or *L. reuteri* in 50 μ L PBS/BSA, respectively, at time points indicated. In some experiments, mice were inoculated intranasally with purified Gram-positive peptidoglycan (100 μ g/50 μ L 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 μ L of PVM stock (2×10^5 PVM_{SH}/ μ L) 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 μ L 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

evaluated using the SMART-M12 ELISA as per manufacturer's instructions (Biotech Trading Partners).

2.6. Bacterial clearance

Lung tissues from mice primed with 10^8 cfu *L. reuteri* or PBS/BSA were collected 6, 12, and 24 h after intranasal inoculations. Lungs were blade-homogenized in 1 mL PBS/BSA and 100 μ L of undiluted, 1:50, 1:100 and 1:200 dilutions were plated on MRS agar; colonies were enumerated after overnight incubation at 37 °C.

2.7. Preparation of *Lactobacillus* genomic DNA

L. reuteri was grown overnight at 37 °C in Difco MRS Broth (BD Biosciences). Ten mL of bacteria was harvested by centrifugation for 10 min at 1500 rpm in a Sorvall RT6000B centrifuge, washed twice with sterile PBS, resuspended in 1 mL Gram-positive bacterial lysis buffer (20 mM Tris–HCl pH 8.0, 2 mM EDTA, 1.2% Triton X-100, 50 mg/mL lysozyme (Sigma Aldrich)), and incubated at 37 °C for 1 h. 100 mL of 10% SDS and 10 μ L of Purgene Proteinase K solution (Qiagen) was added followed by incubation at 37 °C for 3 h until the solution appeared clear. Remaining debris was removed by centrifugation at 25 °C 13,000 RPM for 5 min; 750 μ L supernatant harvested was extracted with 1 volume Ultrapure Phenol:Chloroform:Isoamyl Alcohol (Invitrogen). Upper aqueous phase was re-extracted twice with phenol:chloroform, followed by a final extraction with 1/10th by volume chloroform (J.T. Baker). DNA was precipitated from the upper aqueous phase with 1/10 volume of 3 M Sodium Acetate pH 5.2 and 1 volume of isopropanol followed by centrifugation at 4 °C at 13,000 rpm for 10 min. The pellet was washed with 75% ethanol, centrifuged at 4 °C at 7500 rpm for 10 min, the supernatant was decanted, and the remaining pellet was allowed to dry for 10 min. The final pellet was suspended in sterile dH₂O and the concentration of DNA was measured by Nanodrop Spectrophotometer ND-1000 (Thermo Scientific). This procedure yields 300–500 μ g genomic DNA per 10 mL *Lactobacillus* culture.

2.8. Detection of *Lactobacillus* genomic DNA in mouse lung tissue

Mice were inoculated intranasally with 10^8 cfu *L. reuteri* in 50 μ L PBS/BSA and lungs were harvested at the time points indicated and stored at –80 °C in RNAlater (Ambion). Approximately 7–10 mg of the lung tissue was washed in dH₂O, minced in a petri dish using a straight razor blade, and transferred to a 15 mL tube containing 2 mL Tissue Lysis Buffer (60 mM Tris–HCl pH 8.0, 100 mM EDTA, 20 mg/mL lysozyme). Lungs were stirred for 1 h at 37 °C, then 0.5% SDS and 10 μ L of Purgene Proteinase K (Qiagen) were added and samples were stirred for an additional 4 h at 55 °C until the solution was clear. Samples were then spun at 25 °C 4,000 RPM in a Sorvall RT6000B centrifuge for 5 min to remove any remaining debris and remaining supernatant was extracted and DNA precipitated as described above. This procedure yields 600–1500 ng genomic DNA from mouse lung tissue. *L. reuteri* genomic DNA was detected by quantitative PCR using primers and probe designed to detect 16S–23S intergenic region as described in (Haarman and Knol, 2006), including forward primer, 5'-ACC GAG AAC ACC GCG TTA TTT-3'; reverse primer, 5'-CAT AAC TTA ACC TAA ACA ATC AAA GAT TGT CT-3'; and probe, 5'-ATC GCT AAC TCA ATT AAT-3'-MGB (minor groove binding). 300 ng of extracted DNA from *L. reuteri* or control-inoculated mouse lung tissue was added to TaqMan universal PCR master mix and evaluated on an ABI 7500 Real Time PCR system according to manufacturer's instructions (Applied Biosystems). Results were calibrated via a standard curve, which included 1 to 10^8 copies of 1 kb LR 16–23S sequence (Genbank AF080100.1, generated by PCR using primers

LR16sF (5'-CGGATCAGCATGCTGCGGTGAAC-3') and LR16sR (5'-TGTTAGTCCCGTCCTTCATCGGCT-3') (Chagnaud et al., 2001) and cloned into the pCR 2.1 vector (Invitrogen) and confirmed by bidirectional sequencing.

2.9. Detection of peptidoglycan in the lung after *L. reuteri* inoculation

Lung tissue from mice receiving inoculations with 10^8 cfu *L. reuteri* or PBS/BSA (negative control) was collected 6, 24, 48, and 96 h thereafter. Lungs were blade-homogenized in 1 mL PBS/BSA passed through a 40 μ m strainer and stored at –80 °C. Peptidoglycan (PGN) was detected in whole lung homogenates using the silk-worm larvae plasma (SLP) reagent set from Wako (Richmond, VA), which measures the formation of melanin in response to the presence of PGN in a sample (Inada et al., 2003). Briefly lung homogenates (diluted 1:1000 in endotoxin-free water) or PGN standard dilution series (Wako) were dispensed into wells of a microplate followed by addition of an equal volume of SLP test solution. To measure the formation of melanin, a kinetic assay was run and absorbance at 650 nm was measured every 2 min for 60 min using a Bio-rad Benchmark plus reader. Concentrations were calculated from a standard curve generated by doing a linear regression of a logarithmic plot of concentrations of the standards vs. onset time (Suppl. Fig. 1). Onset time was defined as the time at which the standards or samples reached $A_{650} = 0.4$, a midpoint within the linear range.

2.10. Statistical analysis

Data were evaluated using *t* test, Mann–Whitney *u*-test or the log-rank test for survival curves as appropriate. Statistical outliers were detected using the Grubb's test. All statistical tests used are included in the GraphPad Prism 5 Software (GraphPad Software, La Jolla, CA). Bar graphs indicate mean \pm SEM.

3. Results

3.1. Priming of the respiratory mucosa protects mice from lethal respiratory virus infection

The standard experimental protocol and timeline is shown in Fig. 1A. Mice were inoculated intranasally on day –14 and again on day –7 with *L. plantarum*, *L. reuteri*, or PBS/BSA diluent control. On day 0 or at time points thereafter, mice were inoculated with pneumonia virus of mice (PVM). We reported previously that adult mice primed with *L. plantarum* or *L. reuteri* as per this protocol were fully protected from the lethal sequelae of subsequent PVM infection (Gabryszewski et al., 2011). Here we extend this observation, and show that 40% of the mice inoculated with 10^9 cfu *L. plantarum* on day –14 and again on day –7 survive lethal infection when challenged with PVM at day 153, or 5 months after initial *Lactobacillus* priming (Fig. 1B). *Lactobacillus*-priming elicited no anti-PVM IgG that could be detected in serum at day 0 (Gabryszewski et al., 2011). Likewise, no anti-PVM IgG was detected in serum at two months after initial priming (data not shown).

Although our priming protocol was designed to include two intranasal inoculations with *L. plantarum* or *L. reuteri*, we had not determined previously whether one or both inoculations were necessary to elicit full protection against lethal virus challenge. As shown in Fig. 1C, we found that at least two inoculations with *L. plantarum* were required to elicit protection via this protocol. Interestingly, although the single inoculations with *Lactobacillus* at either day –14 or day –7 had no impact on survival, they did have a significant impact on virus recovery (Fig. 1D). Virus recoveries from control-primed mice were ~3-fold higher than those from

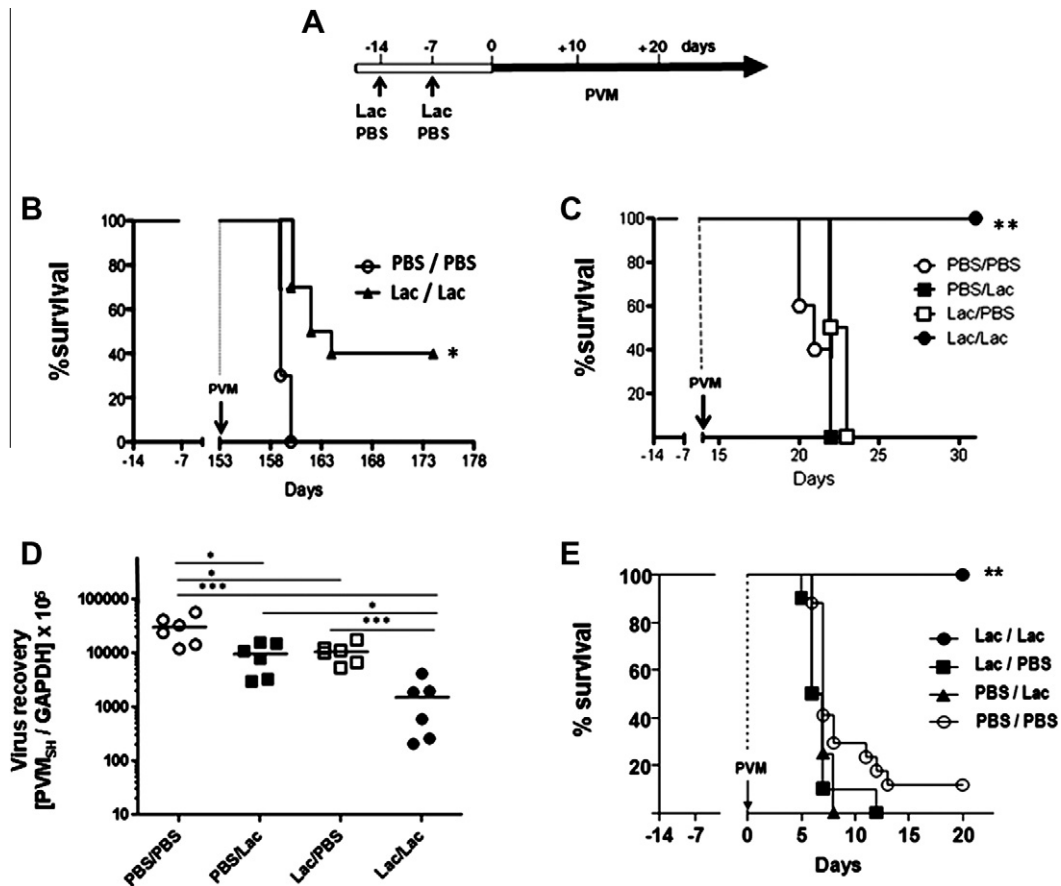


Fig. 1. Priming of the respiratory mucosa with live *Lactobacillus* species protects mice from lethal sequelae of subsequent virus challenge. (A) Standard experimental protocol and timeline. Mice are inoculated intranasally with *Lactobacillus* (Lac), either 10⁹ cfu live *Lactobacillus plantarum*, 10⁸ cfu live *Lactobacillus reuteri*, or PBS/BSA (PBS) control on day -14 and again on day -7. On day 0 or at a time point thereafter, mice are challenged with pneumonia virus of mice (PVM). (B) Survival of 8 week old BALB/c mice inoculated with *L. plantarum* as indicated in A., and challenged with PVM on day +153 (5 months), $n = 10$ mice per group. (C) Survival of 8 week old BALB/c mice primed on day -14 and day -7 with PBS/BSA, with PBS/BSA on day -14 and *L. plantarum* on day -7, with *L. plantarum* on day -14 and PBS/BSA on day -7, or with *L. plantarum* on day -7 and all inoculated with PVM on day +14, $n = 5$ mice/group. (D) Virus recovery on day +17 (PVM_{SH} / GAPDH) from lung tissue of mice primed and challenged with PVM as in (C). (E) Survival of 3 week old C57BL/6 mice primed on day -14 and day -7 with PBS/BSA only, with *L. reuteri* on day -14 and PBS/BSA on day -7, with PBS/BSA on day -14 and *L. reuteri* on day -7, or *L. reuteri* on day -7 and day -14; $n = 10$ mice/group; statistical significance, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

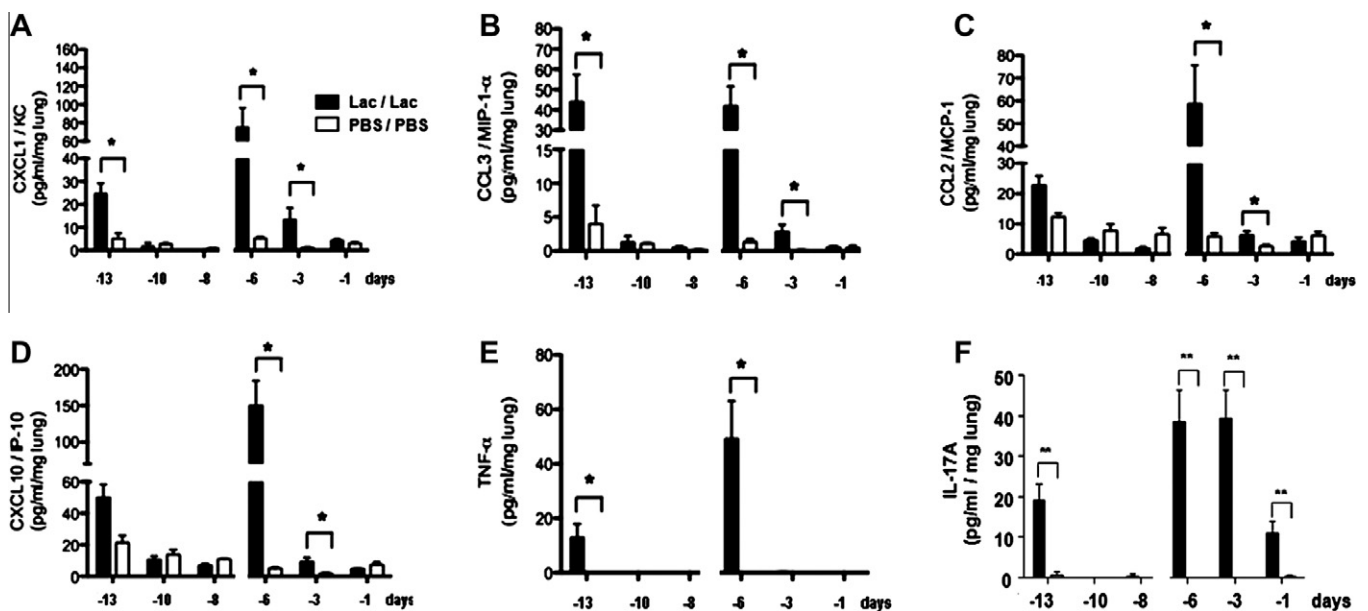


Fig. 2. Proinflammatory cytokines detected in response to *Lactobacillus* priming. (A) CXCL1, (B) CCL3, (C) CCL2, (D) CXCL10, (E) TNF-α and (F) IL-17A detected in whole lung homogenates (pg/mL/mg lung tissue) at three time points after the first (day -14) and second (day -7) intranasal inoculation with *L. reuteri* (Lac, filled bars) or PBS/BSA control (PBS, open bars); $n = 4-8$ mice/time point; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. No type I interferons were detected at any time point. Data compiled from 2 independent experiments.

mice primed only once with *L. plantarum* ($*p < 0.05$), while virus recoveries from control-primed mice were ~20-fold higher than those from mice primed twice with *Lactobacillus* ($*p < 0.005$). Overall, these findings are consistent with our previous observations, specifically: while *Lactobacillus* priming has a clear impact on virus

replication and clearance, virus recovery may be variable. While statistically significant, relatively small changes in virus recovery may not predict changes in survival.

Finally, similar to findings in adult mice, we show here that three-week old C57BL/6 mice receiving two intranasal inoculations,

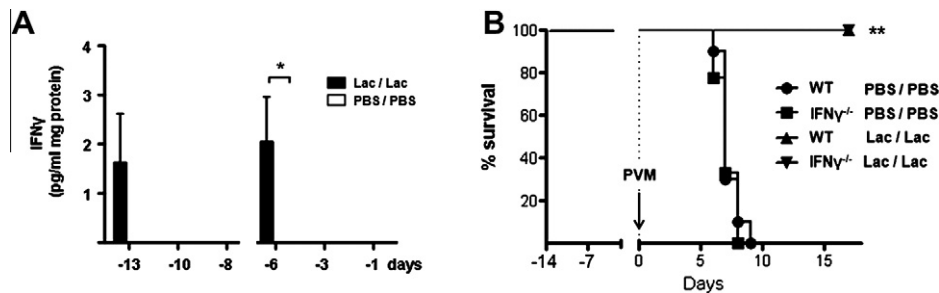


Fig. 3. *Lactobacillus*-priming and interferon- γ . (A) IFN γ detected in whole lung homogenates (pg/ml/mg lung tissue) at three time points after first (day -14) and second (day -7) intranasal inoculation with *L. reuteri* (Lac, filled bars) or PBS/BSA control (PBS, open bars); $n = 4-8$ mice/time point. (B) Survival of IFN γ ^{-/-} and wild-type mice inoculated with *L. plantarum* as per standard protocol (Fig. 1A) and challenged at day 0 with PVM; $*p < 0.05$; $**p < 0.01$.

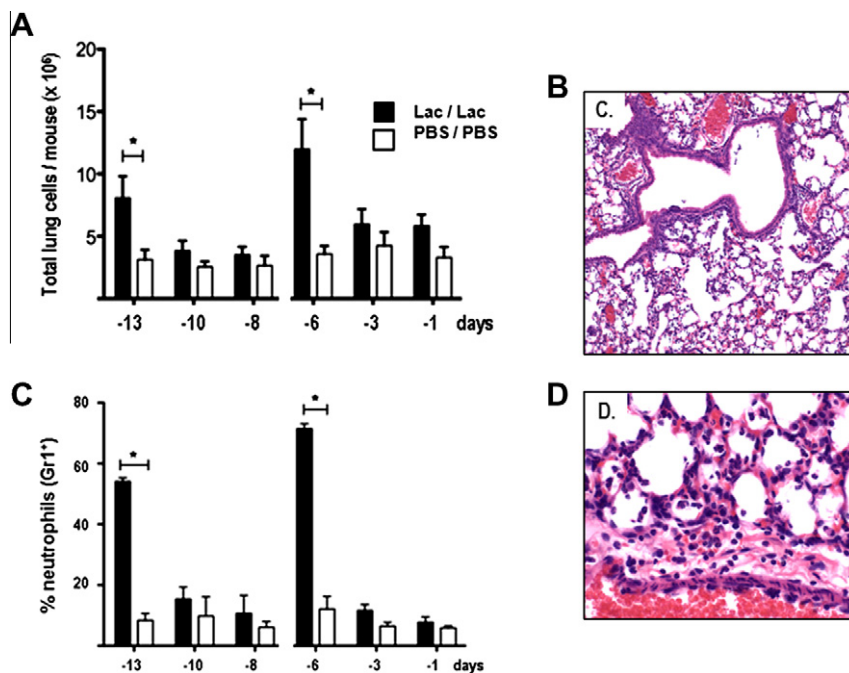


Fig. 4. Neutrophils are recruited to the lungs in response to *Lactobacillus* priming. Cells from whole lung tissue evaluated by flow cytometry at three time points after the first (at day -14) and second (at day -7) inoculations with *L. reuteri* (Lac; filled bars) or PBS/BSA (PBS; open bars); (A) Total lung cells (B) Percent Gr1⁺ (neutrophils); $n = 3$ mice pooled for each time point, data compiled from 3–5 independent experiments; $*p < 0.05$; (C & D) Formalin-fixed, hematoxylin and eosin (H&E)-stained lung tissue from day -6, after second *L. reuteri* inoculation (see Fig. 1A); original magnifications, 10X and 40X, respectively.

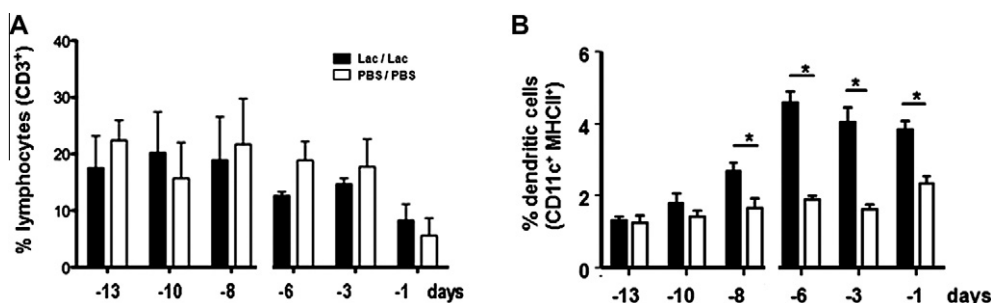


Fig. 5. Differential leukocyte recruitment in response to *Lactobacillus* priming. Cells from whole lung tissue evaluated by flow cytometry as in Fig. 4. (A) Percent CD3⁺ lymphocytes (B) percent CD11c⁺MHCII^{hi} dendritic cells; $p < 0.05$.

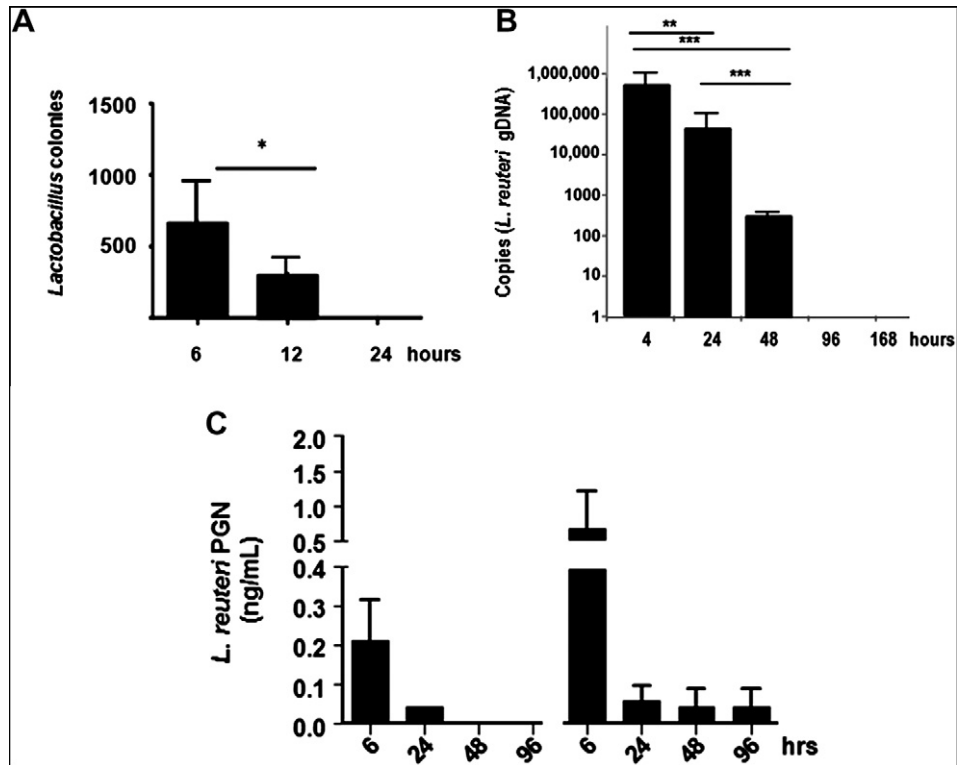


Fig. 6. *Lactobacillus* clearance from lung tissue. (A) Live *L. reuteri* detected in lung tissue at time points after inoculation with 10^8 cfu; $n = 3$ mice/time point. (B) *L. reuteri* genomic DNA (gDNA) detected by qPCR targeting 16S–23S intergenic region, normalized to a standard curve. (C) *L. reuteri* PGN detected by kinetic melanin-generating spectrophotometric assay (see Suppl. Fig. 1) normalized to *S. aureus* standards; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$.

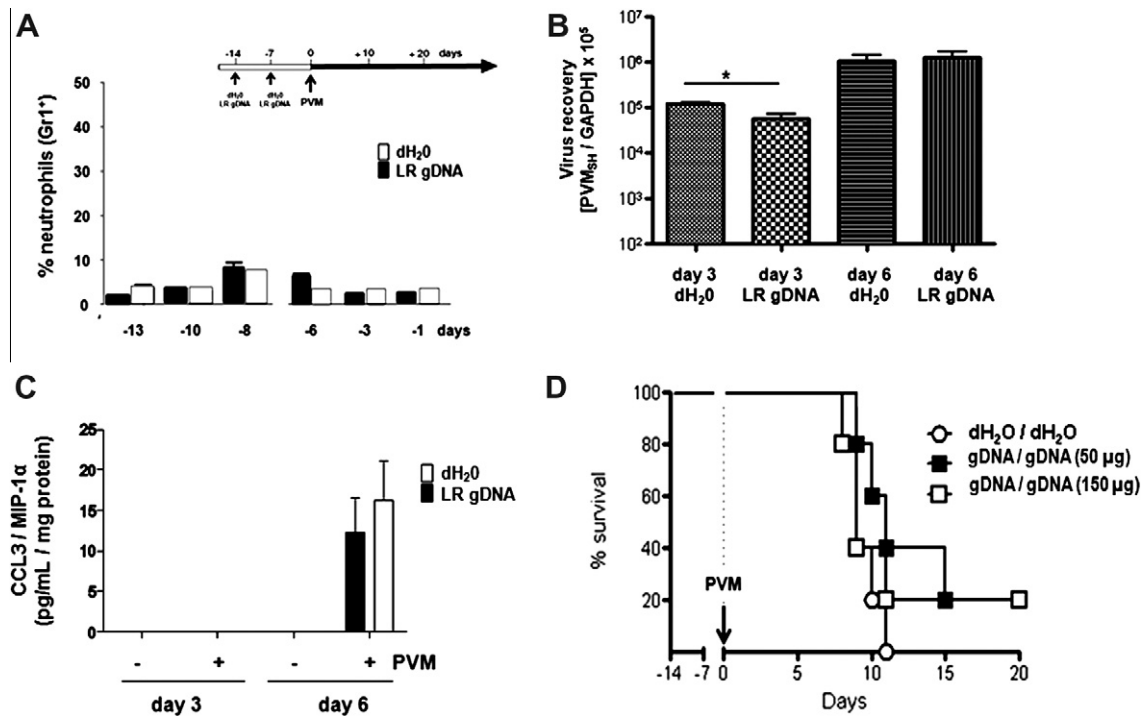


Fig. 7. Priming with *L. reuteri* genomic DNA does not elicit protection from the lethal sequelae of PVM infection. (A) Cells from whole lung tissue evaluated by flow cytometry after a first (day -14) and second (day -7) inoculation with 150 μ g *L. reuteri* gDNA or diluent (dH₂O). Percent neutrophils (Gr1⁺ cells) at each time point are as shown; $n = 3$ mice per time point per condition, data compiled from 3 experiments. (B) Virus recovery ($PVM_{SH}/GAPDH$) from mice primed with 50 μ g *L. reuteri* genomic DNA or diluent (dH₂O) at days +3 and +6 after PVM challenge on day 0 (C) Detection of CCL3/MIP-1 α in whole lung homogenates from mice primed and PVM-challenged as in B. (D) Survival of mice inoculated with *L. reuteri* gDNA (50 or 150 μ g at days -7 and -14) or dH₂O diluent control, and challenged with PVM on day 0, $n = 5$ mice per group.

each at 10^8 cfu *L. reuteri*, were also fully protected from the lethal sequelae of subsequent PVM infection (Fig. 1E). Mice that received only one intranasal *Lactobacillus* inoculation either at one week (day -7) or two weeks (day -14) prior to virus challenge were not protected, and exhibited survival rates that were indistinguishable from the controls.

3.2. Inflammatory cytokine production and leukocyte recruitment

In order to understand the nature of the antiviral state, we proceeded to examine the inflammatory responses to *Lactobacillus*-mediated priming, including pro-inflammatory cytokine production and leukocyte recruitment to lung tissue. Elevated levels of leukocyte chemoattractants CXCL1, CCL3, and IL-17A and the pro-inflammatory mediator TNF- α were detected in lung tissue within 24 h after the first intranasal inoculation with *L. reuteri* (day -13, timeline as in Fig. 1A). These responses were transient; levels of these mediators fell to baseline by day -10 (Fig. 2). In response to the second *L. reuteri* inoculation, cytokine production was more robust and sustained. In addition to the aforementioned mediators, we also detected significantly elevated levels of CCL2 and CXCL10 at 24 h after the second inoculation (day -6), with levels of all but TNF- α remaining above baseline control (PBS/PBS) levels at the day -3 time point. In parallel experiments, levels of CXCL1 and CXCL10

were elevated in serum at days -13 and -6, within 24 h after *Lactobacillus*-priming, and total IgG levels remained unchanged (Suppl. Fig. 2). No immunoreactive IFN- α or IFN- β was detected in response to priming with *L. reuteri* at any time points (data not shown). Similarly, *Lactobacillus*-priming elicited only minimal production of IFN- γ (Fig. 3A); no biologically-active IL-12p70 was detected over baseline (data not shown). *Lactobacillus*-priming protects IFN- γ gene-deleted mice just as effectively as wild-type mice (Fig. 3B), overall suggesting no direct role for Th1 cytokines in this response.

Similar to the pattern of cytokine production, total cell counts in single cell lung suspensions rose within 24 h of each intranasal inoculation with *L. reuteri* and returned to baseline levels immediately thereafter (Fig. 4A). Neutrophils comprised ~60–70% of total cells detected at these time points (Fig. 4B). Neutrophils were prominent in lung tissue sections (Fig. 4C and 4D), and were detected throughout the parenchyma, within the alveoli and were dispersed throughout the surrounding tissue. There were no localized inflammatory infiltrates and no evidence of edema.

Neither T lymphocyte (Fig. 5A), B lymphocyte, NK cell (Suppl. Fig. 3) or regulatory T cell (data not shown) recruitment was detected at any of the time points indicated. Recruitment of CD11c⁺ dendritic cells was first detected on day -8, nearly a full week after the first *Lactobacillus* inoculation (Fig. 5B). In contrast to neutrophil

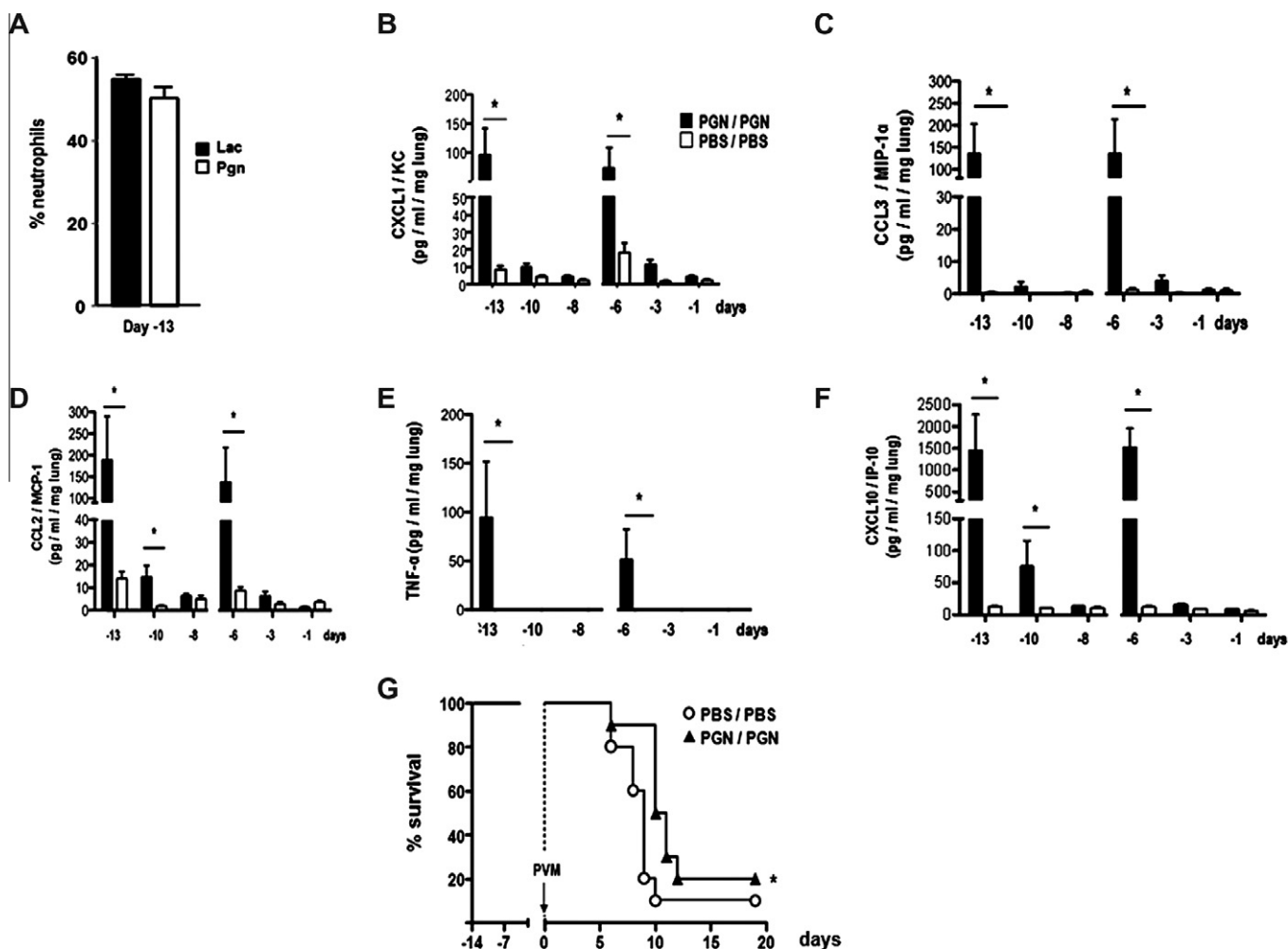


Fig. 8. Priming with peptidoglycan results in neutrophil recruitment and proinflammatory cytokine production but does not protect mice against the lethal sequelae of PVM infection. (A) Percentage of GR1⁺ neutrophils detected in single cell suspensions from whole lung from mice inoculated with 100 μ g PGN compared to 10^8 cfu *L. reuteri* (Lac). (B–F) Proinflammatory cytokines CXCL1, CCL3, CCL2, TNF- α , and CXCL10 detected in whole lung homogenates after first (day -14) and second (day -7) intranasal inoculations with 100 μ g PGN (filled symbols) or PBS/BSA control (open symbols); (G) Survival of mice primed with PGN (100 μ g/mouse; filled symbols) or PBS/BSA (PBS; open symbols) prior to inoculation with PVM on day 0; $n = 10$ mice/group; statistical significance, * $p < 0.05$.

recruitment which was profound but transient, dendritic cell recruitment was less prominent but persistent, notably after the second *Lactobacillus* inoculation.

3.3. *Lactobacillus* clearance from lung tissue

Consistent with the aforementioned pattern of neutrophil recruitment and production of proinflammatory mediators, we found that live *L. reuteri* were cleared rapidly from lung tissue; there was no evidence of bacterial replication or colonization. Live *L. reuteri* were isolated from lung tissue homogenates at 6 and 12 h after inoculation, but no live *L. reuteri* was detected in lung tissue at 24 h after the first (Fig. 6A) or second (data not shown) *L. reuteri* inoculations. Peak levels of *L. reuteri* genomic DNA (gDNA) were similarly detected at the first time point (4 h) after inoculation; prominent detection continued 24 and 48 h after inoculation (Fig. 6B). Similarly, *L. reuteri* peptidoglycan was detected in lung tissue for 1–days after complete clearance of live *L. reuteri* (Fig. 6C).

3.4. The impact of genomic DNA on inflammation and survival in response to PVM infection

In contrast to results obtained upon priming with live *L. reuteri*, priming with *L. reuteri* genomic DNA (gDNA) in amounts ~100–1000 fold greater than found in the bacterial inoculum did not promote neutrophil recruitment to lung tissue (Fig. 7A). Likewise, priming with *L. reuteri* gDNA had only minimal and transient impact on virus recovery (Fig. 7B). *L. reuteri* gDNA also had no impact on induction of the proinflammatory mediator, CCL3 (Fig. 7C), or on survival in response to PVM infection (Fig. 7D).

3.5. The impact of peptidoglycan on inflammation and survival in response to PVM infection

In our previous study (Gabryszewski et al., 2011), we found that priming with both live and heat-inactivated *L. plantarum* and *L. reuteri*, as well as an unrelated live and heat-inactivated Gram-positive *Listeria innocua*, elicited full protection against a subsequent lethal PVM infection. Prominent neutrophil recruitment was observed in response to priming with Gram-positive PGN (50 µg/mouse), at levels indistinguishable from those observed in response to live *L. reuteri* (Fig. 8A). The cytokine expression patterns detected in response to PGN alone were also similar to those observed in response to priming with *L. reuteri* (Fig. 8B–F). However, although priming with a large inoculum of PGN (100 µg/mouse/inoculation, roughly equivalent to a PGN inoculum from 10⁹ Gram-positive bacteria) resulted in delayed mortality (median survival, $t_{1/2}$ = 9.0 vs. 10.5 days, * p < 0.05 (Fig. 8G), it did not confer sustained survival such as that observed in response to priming with live *L. reuteri*. No protection against lethal PVM challenge was observed in response to priming with 10 or 50 µg PGN/mouse/inoculation (data not shown).

4. Discussion

In our previous work, we showed that lactobacilli targeted to the respiratory tract were highly effective at suppressing virus-induced inflammation and protecting against lethal disease. Specifically, we found that wild-type adult mice primed via intranasal inoculation with live or heat-inactivated *Lactobacillus* strains were completely protected against lethal infection with pneumonia virus of mice (PVM), and that significant protection against subsequent virus challenge persisted for at least three months after initial priming (Gabryszewski et al., 2011). Several other groups have

reported similar results with influenza A, albeit findings limited to short-term protection only in response to intranasal inoculation with various *Lactobacillus* species (Harata et al., 2010; Izumo et al., 2010; Hori et al., 2001; Young et al., 2012).

Here, we have extended these findings and found that *Lactobacillus*-mediated protection can be sustained for as long as five months, can be elicited in younger mice, and that at least two intranasal inoculations with *L. plantarum* or *L. reuteri* were required to elicit protection against a lethal virus challenge. Although priming with *L. reuteri* results in suppression of inflammation upon virus challenge (Gabryszewski et al., 2011), we show here that priming itself elicits a robust but transient inflammatory response, including production of proinflammatory cytokines and leukocyte chemoattractants but interestingly, no type I interferons. Parker and Prince (2011) recently reviewed the literature on type I interferons and extracellular bacteria in the airways, which include specific responses to Gram-positive *Staphylococcus aureus* and *Streptococcus pneumoniae* and Gram-negative *Pseudomonas aeruginosa*. It is not immediately clear why these microorganisms (and others, as discussed below) elicit prominent type I interferon responses in lung tissue while inoculation with *L. reuteri* does not. It is also not immediately clear what the impact of this finding is with respect to the outcome of a subsequent infection, particularly with an unrelated virus pathogen.

Although lactobacilli are cleared rapidly, both peptidoglycan and genomic DNA from *L. reuteri* are detected for several days after live bacteria have cleared. Several groups have explored the immunomodulatory potential of *Lactobacillus* genomic DNA. Kim et al. (2012) reported that pretreatment of monocytic THP-1 cell line with *L. plantarum* gDNA inhibited production of TNF α in response to LPS. Likewise, Bouladoux et al. (2012) have identified unique immunosuppressive motifs enriched in the DNA of *Lactobacillus* species that inhibit activation of dendritic cells in the gastrointestinal tract. Despite prominent recruitment of dendritic cells to the respiratory tract in response to whole *Lactobacillus*-priming, we find that *L. reuteri* genomic DNA administered in amounts far exceeding that received with bacterial inoculation had no impact on any parameters associated with protection against lethal virus infection.

Pattern recognition receptors (PRRs) that recognize gram-positive bacteria and their components are among the factors that might be considered important in *Lactobacillus*-mediated priming in the lung. The nucleotide-binding oligomerization (NOD) protein, NOD2 is a cytosolic PRR expressed in epithelial and antigen presenting cells (dendritic cells and macrophages) that detects muramyl-dipeptides from both gram-positive and gram-negative bacteria and activates inflammatory gene transcription via NF- κ B activation pathways (reviewed in (Strober et al., 2006; Sorbara and Philpott, 2011; Biswas et al., 2012)). At present, there is a small literature on the interactions between *Lactobacillus* strains and the NOD2 receptor. Among the most interesting, Macho Fernandez et al. (2011) found that peptidoglycan from the specific *L. salivarius* strain Ls33 protected mice from chemical colitis in a NOD2-dependent, MyD88-independent fashion. Although the main focus has been the gastrointestinal mucosa, NOD2 is expressed in cells recruited to lung tissue, and NOD2 gene-deleted mice display aberrant inflammatory responses to both bacterial and viral respiratory pathogens as well as to pathogen-derived antigens (Theivanthiran et al., 2012; Coulombe et al., 2012; Divangahi et al., 2008; Frutuoso et al., 2010; Davis et al. 2011).

Further experiments were designed to explore the relationship between transient neutrophil recruitment and protection against lethal virus infection. Recruitment paralleled *Lactobacillus*-mediated induction of numerous neutrophil chemoattractants, including CXCL1 and IL-17A (Pappu et al., 2012). Various strategies that result in depletion of neutrophils from blood and bronchoalveolar

lavage (BAL) fluid (Tate et al., 2011; Daley et al., 2008; McFarlane et al., 2008; Hahn et al., 2011) yielded only partial (~50%) depletion of neutrophils from whole lung tissue (Garcia-Crespo et al., unpublished results). Interestingly, these results (clearance from airways and blood, but not lung tissue) are similar to those observed clinically in response to anti-IL-5 monoclonal antibody therapy (mepolizumab) designed to eliminate eosinophils from the human asthmatic lung (Leckie et al., 2000). However, to address this point from another perspective, we found that priming with PGN elicited neutrophil recruitment but not full protection against respiratory virus infection. Thus, we conclude that neutrophil recruitment, while a prominent response to *Lactobacillus*-mediated priming, may not be sufficient to elicit protection against a subsequent virus challenge.

Lactobacillus-mediated protection against respiratory virus infection via the protocol featured here is a unique and particularly robust form of heterologous immunity. As such, our findings can be compared and contrasted with findings reported by Tuvim, Evans, Dickey and colleagues (Evans et al., 2010; Evans et al., 2011; Tuvim et al., 2009; Clement et al., 2008; Duggan et al., 2011) who likewise examined questions related to this issue. Their model focuses specifically on pathogen resistance in lung tissue in response to multiple infusions of a sterile lysate from the Gram-negative microorganism, non-typeable *Haemophilus influenza* (NTHi; reviewed in (Erwin and Smith, 2007; Foxwell et al., 1998)). Among the specific similarities, these researchers have found that one or more intranasal infusions with NTHi result in protection from the lethal sequelae of infection with mouse-passaged H3N2 influenza A; likewise, NTHi infusion elicits production of inflammatory cytokines (including TNF- α) and neutrophil recruitment. However, among the marked differences between our datasets, protection elicited by the sterile NTHi infusion is short-lived. In experiments examining protection against a heterologous pathogen, the protective impact of NTHi infusion peaked within 4 h of the intranasal infusion, with effectiveness waning at 48 h (Clement et al., 2008). In contrast, *Lactobacillus*-mediated priming elicits full protection against PVM when the virus pathogen is introduced one to three weeks after the final *Lactobacillus* inoculation, with significant protection persisting for up to 5 months. Furthermore, NTHi-mediated protection is dependent on MyD88-signaling (Duggan et al., 2011), while *Lactobacillus*-mediated protection against PVM can be elicited in MyD88 gene-deleted mice (Gabryszewski et al., 2011). Another significant difference, sterile NTHi infusion results in profound and immediate induction of IFN- α , IFN- α 2, and type I interferon-related transcripts (Tuvim et al., 2009); in contrast, no immunoreactive IFN- α or IFN- β was detected in whole lung lysates in response to *Lactobacillus*-priming. Thus, although both NTHi and *Lactobacillus*-priming may proceed to a shared outcome - protection against respiratory pathogens - the cellular and biochemical mechanisms promoting these responses are clearly unique and stimulus-specific.

Among our immediate goals is the ongoing exploration of the mechanisms promoting protection in response to *Lactobacillus* priming. These findings will ultimately lead us in new directions and toward novel immunomodulatory modalities.

Acknowledgments

The authors are grateful for the assistance of Dr. Alfonso Gonzalo and the staff of National Institute of Allergy and Infectious Diseases 14BS animal facility for care of mice used in these studies, Mr. Ricardo Dreyfuss of Medical Arts who prepared the photomicrographs for publication, and Dr. Stephanie Glineur for performing the studies to detect serum antibodies to PVM. HFR would also like to thank Dr. Peter J. Openshaw who brought the concept of innate imprinting to our attention. This work is funded by NIAID Division

of Intramural Research (DIR) AI000943 to HFR and Children's Miracle Network of New York to JBD.

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.2012.12.022>.

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