B Cells Are Not Essential for *Lactobacillus* -Mediated Protection against Lethal Pneumovirus Infection

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B Cells Are Not Essential for *Lactobacillus*-Mediated Protection against Lethal Pneumovirus Infection

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We have shown previously that priming of respiratory mucosa with live *Lactobacillus* species promotes robust and prolonged survival from an otherwise lethal infection with pneumonia virus of mice, a property known as heterologous immunity. *Lactobacillus* priming results in a moderate reduction in virus recovery and a dramatic reduction in virus-induced proinflammatory cytokine production; the precise mechanisms underlying these findings remain to be elucidated. Because B cells have been shown to promote heterologous immunity against respiratory virus pathogens under similar conditions, in this study we explore the role of B cells in *Lactobacillus*-mediated protection against acute pneumovirus infection. We found that *Lactobacillus*-primed mice feature elevated levels of away IgG, IgA, and IgM and lung tissues with dense, B cell (B220*)-enriched peribronchial and perivascular infiltrates with germinal centers consistent with descriptions of BALT. No B cells were detected in lung tissue of *Lactobacillus*-primed B cell deficient μMT mice or Jh mice, and *Lactobacillus*-primed μMT mice had no characteristic infiltrates or away lgs. Nonetheless, we observed diminished virus recovery and profound suppression of virus-induced proinflammatory cytokines CCL2, IFN-γ, and CXCL10 in both wild-type and *Lactobacillus*-primed μMT mice. Furthermore, *Lactobacillus plantarum*-primed, B cell–deficient μMT and Jh mice were fully protected from an otherwise lethal pneumonia virus of mice infection, as were their respective wild-types. We conclude that B cells are dispensable for *Lactobacillus*-mediated heterologous immunity and were not crucial for promoting survival in response to an otherwise lethal pneumovirus infection. The Journal of Immunology, 2014, 192: 000–000.

Inflammatory responses to the human pneumovirus pathogen, respiratory syncytial virus (RSV), can be strong and persistent, beyond the control of conventional antiviral and anti-inflammatory therapies, resulting in profound negative consequences for the host (reviewed in Ref. 1). Pneumonia virus of mice (PVM), a rodent pneumovirus pathogen closely related to RSV, serves as a model of severe respiratory virus infection and is an important platform for exploring immunomodulatory therapy and disease prevention (2, 3). No single mouse model can reproduce all features of human disease or represent the full spectrum of human experience; however, unlike RSV challenge–clearance models in mice, PVM undergoes robust replication in mouse lung tissue, elicits disease-relevant outcomes, and fulfills Koch’s postulates for a host/ pathogen relationship in rodent species.

In previous work, we described important immunomodulatory properties of Gram-positive *Lactobacillus* species at the respiratory mucosa (4, 5). Specifically, we found that priming of the respiratory tract of wild-type mice with *Lactobacillus plantarum* or *Lactobacillus reuteri* promoted both profound and sustained protection against an otherwise lethal PVM infection. These observations represent a robust example of heterologous immunity, also known as innate imprinting, or trained immunity, concepts that have been invoked to explain increased resistance (or in some cases, susceptibility) to an unrelated infection observed upon recovery from a primary innate immune or inflammatory response (6–9). There are numerous examples of these observations in the literature. Among them, several groups have reported that mice vaccinated against tuberculosis with bacillus Calmette–Güérin (BCG) were protected against infections with antigenically unrelated pathogens, including Gram-positive bacteria, the fungus *Candida albicans*, and the parasite *Schistosoma mansoni* (reviewed in Ref. 6); analogous findings have been reported in BCG-vaccinated children (10). Heterologous immunity has also been invoked to explain the increased susceptibility to infection with *Streptococcus pneumoniae* observed among patients recovering from severe influenza infection (reviewed in Ref. 11).

In an effort to elucidate the mechanisms underlying heterologous immunity to lethal respiratory virus infection, we have evaluated the molecular and cellular inflammatory responses generated in situ in lung tissue of *Lactobacillus*-primed mice. Among these findings, mice primed with *Lactobacillus* and subsequently challenged with PVM respond with moderate suppression of virus recovery together with diminished expression of an array of proinflammatory cytokines (4).

Interestingly, Harmsen and colleagues (12, 13) recently reported that protein cage nanoparticles, multisubunit immunostimulatory
molecules derived from the small heat shock protein of the thermophilic bacteria *Methanocaldococcus jannaschii*, promote B cell–dependent heterologous immunity in mice against a wide variety of respiratory virus pathogens, including PVM. Specifically, protein cage nanoparticles induced accelerated adaptive immunity to virus pathogens (13) in association with the formation of BALT, a tertiary lymphoid structure induced in response to microbial and/or inflammatory provocation that features germinal center B cells, follicular dendritic cells, and T cells (14–17).

In our earlier work, we found that *Lactobacillus*-mediated priming of the mouse respiratory tract also resulted in the induction of peribronchiolar and perivascular cuff-like infiltrates, consistent with histological descriptions of BALT (4). With this information, together with the larger understanding of B cells as immunomodulatory and antiviral mediators, including production of natural IgM (18), Ab-independent support of macrophages (19), and production of the anti-inflammatory cytokine IL-10 (20), our goal was to determine whether B cells played a crucial role in *Lactobacillus* priming and protection elicited against lethal respiratory virus infection.

**Materials and Methods**

**Mouse strains**

Wild-type C57BL/6 and BALB/c mice were purchased from the Division of Cancer Therapeutics, National Cancer Institute (Frederick, MD). B cell–deficient mouse strains used include µMT mice (The Jackson Laboratory, stock 2288; C57BL/6 background) (21) and Jh mice (Taconic; BALB/c background) (22). All mouse studies were approved by National Institute of Allergy and Infectious Diseases and carried out in accordance with National Institute of Allergy and Infectious Diseases Institutional Animal Care and Use Committee Guidelines.

**Lactobacillus**

Cultures of *L. plantarum* NCIMB 8826 (ATCC BAA-793) were grown overnight in MRS broth at 37°C in a shaker incubator. Bacteria were washed in sterile PBS and resuspended at $2 \times 10^{10}$ CFU/ml (4) in sterile PBS with 0.1% BSA for intranasal inoculation under isoflurane anesthesia. Each mouse received 50 µl of this dilution or 50 µl PBS with 0.1% BSA diluent control per inoculation, which reaches both upper and lower respiratory tracts (23) at days −14 and −7 of the protocol (see timeline in Fig. 1A). For the experiment involving heat-inactivated *L. plantarum*, live cultures grown and washed as above were resuspended in a minimal volume of PBS, heated to 95°C for 10 min in a thermocycler, and then frozen in a dry ice/ethanol bath; this was repeated for a total of three cycles. Heat-inactivated bacteria were then resuspended in PBS with 0.1% BSA at $10^{11}$ CFU/ml (determined as per original live titration) (4) and stored at −80°C prior to use at appropriate dilution. For experiments in which mice were provided with *L. plantarum* in drinking water, bacteria were grown overnight as above, washed, and resuspended at $10^{9}$ live CFU/ml in standard drinking water (250/ml/cage). Mice were provided with fresh drinking water (with freshly cultured live bacteria) every 3 d for 2 wk prior to PVM inoculation and for the remaining period thereafter. Mice were weighed every 3 d prior to PVM inoculation to ascertain appropriate water intake throughout.

**Virus**

Fifty percent tissue culture-infective dose assays (24) provided quantitative evaluation of mouse-passaged PVM J3666 stocks. Infections were established

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**FIGURE 1.** Priming of the respiratory tract of wild-type mice with live *L. plantarum* results in protection against subsequent lethal pneumovirus infection. (A) BALB/c mice inoculated intranasally with $10^{9}$ CFU *L. plantarum* on days −14 and −7 are fully protected from an otherwise lethal infection with PVM; n = 10 mice/group. ***, p < 0.001. (B) Virus recovery from lung tissue at day 5 after inoculation with PVM from mice primed with *L. plantarum* or PBS/BSA as in (A); n = 6 mice/group. *p < 0.05. (C) BALB/c mice inoculated intranasally with $10^{9}$ CFU heat-inactivated *L. plantarum* on days −14 and −7 are fully protected from a lethal infection with PVM, inoculation on day +28; shown are weights (g ± SD) at day −14 and days 28–40 (n = 5 mice/group) and at days 34 and 35 (n = 3 and 2 mice/group), respectively, in the group inoculated with PBS + 0.1% BSA, owing to lethal outcomes. (D) Mice received $10^{9}$ CFU *L. plantarum*/ml drinking water for 2 wk prior to inoculation with PVM on day 0; n = 15 mice/group.
in isoflurane-anesthetized mice via intranasal inoculation with 0.2–2 fifty percent tissue culture-infective dose units in 50 μl DMEM diluent.

Virus recovery

Virus recovery was determined from cDNA generated from total RNA from mouse lung tissue via a dual standard curve quantitative RT-PCR method targeting the PVM SH gene and mouse GAPDH that generates absolute copy numbers per copy GAPDH (PVM\textsubscript{SH}/GAPDH) as previously described (4).

ELISAs

Cytokine ELISAs (R&D Systems, Minneapolis, MN) were performed on clarified homogenates of lung tissue and corrected for total protein by BCA assay. Ig ELISAs (Kamiya Biomedicals, Seattle, WA) were performed on bronchoalveolar lavage fluids. All kits were used as per manufacturers’ instructions.

Flow cytometry

Lung tissue was harvested and single-cell suspensions were prepared as previously described (4, 5). Live/Dead stain (Invitrogen) was added to the cells and Ab binding to Fc receptors was blocked with anti-mouse CD16/CD32. Cells were then stained with anti-CD3-FITC, anti-CD19-V450, and anti-GR1-allophycocyanin in PBS with 0.1% BSA at 4°C for 1 h and washed with this buffer prior to analysis. All Abs were purchased from BD Biosciences (Durham, NC). A minimum of 100,000 events were collected on an LSR II flow cytometer (BD Biosciences) and findings were analyzed in FlowJo 9.2. Cell numbers were calculated from the percentage live CD3\textsuperscript{+}CD19\textsuperscript{2} (T cells) and percentage live CD3\textsuperscript{2}CD19+ (B cells) within the lymphocyte gate (low forward/low side scatter), corrected for the fraction of total cells that were analyzed from each single-cell suspension.

Immunohistochemistry

Tissue sections prepared from 10% phosphate-buffered formalin-fixed lung tissue were stained with H&E. Unstained sections were probed with purified rabbit polyclonal anti-RGS13 Ab (25), rat anti-mouse B220 mAb (mAb1217; R&D Systems) or appropriate control Abs followed by peroxidase-conjugated anti-Ig and developing reagents (Histoserv, Germantown, MD).

Statistical analysis

Data were analyzed using Mann–Whitney U test and one-way ANOVA as appropriate.

Results

Primming of the respiratory tract with \textit{L. plantarum} results in protection from the lethal sequelae of PVM infection and generation of dense lymphocytic infiltrates in mouse lung tissue

As reported in our previous publications (4, 5), BALB/c mice primed via intranasal inoculation with live \textit{L. plantarum} (10\textsuperscript{9} CFU/mouse on days −14 and −7) are fully protected against the lethal sequelae of a subsequent PVM infection (Fig. 1A). \textit{Lactobacillus}-mediated protection is associated with diminished virus recovery from lung tissue (Fig. 1B), although we have shown previously that virus recovery is not a strong predictor of the degree of protection elicited by \textit{Lactobacillus} priming (4, 5). Heat-inactivated \textit{Lactobacillus} is also protective against the lethal sequelae of acute PVM infection (4). In this study, we show that heat-inactivated \textit{L. plantarum} can protect against the lethal sequelae of infection even when PVM inoculation is delayed for 4 wk, from day 0 to day 28. As shown in Fig. 1C, mice primed with vehicle control rapidly lose weight in response to PVM infection and ultimately succumb to infection. In contrast, mice primed with \textit{L. plantarum} (10\textsuperscript{6} CFU/mouse on
days −14 and −7) do not lose weight and survive long-term thereafter. In contrast to our findings with direct mucosal priming, we observed no protection against the lethal sequelae of PVM infection among mice receiving *L. plantarum* in drinking water (10⁹ CFU/ml for 2 wk prior to PVM inoculation on day 0; Fig. 1D).

Lung tissues from *L. plantarum*–primed mice feature dense mononuclear cell infiltrates at the airways and blood vessels (Fig. 2A–C), consistent with descriptions of BALT (14). The infiltrates are enriched with B cells (B220⁺; Fig. 2D) and also can also be detected with RGS13, a marker for germinal center B cells (Fig. 2E, 2F). Likewise, elevated levels of total IgG (Fig. 3A), IgA (Fig. 3B), and IgM (Fig. 3B) were detected in the airways of *L. plantarum*–primed mice.

**Lactobacillus priming and PVM infection in B cell–deficient mice**

To explore the possibility that B cells play a role in heterologous immunity promoted by *Lactobacillus* priming, we examined the results of *L. plantarum* priming and PVM infection two B cell–deficient mouse strains, including mMT (C57BL/6 background) in which targeting of the Cμ locus results in arrested development at the pre–B cell stage (21) and Jh (BALB/c background) in which deletion of four JH segments from the H chain locus (22). As anticipated, no B cells (CD3⁻CD19⁺) were detected in lung cell suspensions from either control or *L. plantarum*–primed, PVM-infected mice (Fig. 4A, 4B); T cell recruitment (CD3⁺CD19⁻) persisted despite the B cell deficiency (Fig. 4C). Similarly, no B cells were detected in lung tissue from *L. plantarum*–primed, PVM-infected B cell–deficient Jh mice (Fig. 4D–F).

Similar to what we observed in BALB/c mice, C57BL/6 mice also respond to *Lactobacillus* priming and PVM infection by developing prominent infiltrates (Fig. 5A) enriched with (B220⁺) B cells (Fig. 5B). No infiltrates are detected in *L. plantarum*–primed, PVM-infected B cell–deficient mMT mice (Fig. 5C, 5D). Likewise, analogous to what we observed in *L. plantarum*–primed BALB/c mice (Fig. 3A), elevated levels of total IgG were detected in the airways of *L. plantarum*–primed C57BL/6 mice; no IgG was detected in the airways of B cell–deficient mMT mice (Fig. 5E). Similar results were obtained for total IgM and IgA (data not shown).

**Virus recovery and survival of *L. plantarum*–primed, PVM-infected wild-type and B cell–deficient mice**

We observed no statistically significant differences in virus recovery when comparing wild-type and B cell–deficient (mMT) mice; *Lactobacillus* priming resulted in 0.5–0.8 log reductions in virus recovery in both wild-type and B cell–deficient strains (Fig. 6A). Similarly, as shown in Fig. 6B, B cell deficiency has no impact on *Lactobacillus*-promoted survival from an otherwise lethal respiratory virus infection. All control (PBS/BSA) primed PVM-infected mice died between days 7 and 11, and all *L. plantarum*–primed PVM-infected mice survived throughout (*p < 0.001).

**Cytokine responses of B cell–deficient mice**

Although virus recovery and survival responses of B cell–deficient mice are indistinguishable from those of their wild-type counterparts, patterns of cytokine suppression are not fully superimposable. Specifically, *L. plantarum* priming prior to virus infection results in profound suppression of both CCL2 (Fig. 7A) and CXCL10 (Fig. 7B) in both wild-type and mMT mice. Suppression of IFN-γ is also observed in both strains, although significantly more of this cytokine is produced in response to PVM infection in B cell–deficient mice (Fig. 7C); we have shown previously that IFN-γ is not a crucial mediator of *L. plantarum*–mediated protection against PVM infection (5). In contrast, CXCL1, which undergoes substantial suppression in *Lactobacillus*-primed, PVM-infected mice, is not a major feature of the response to PVM infection in the mMT strain (Fig. 7D). Finally, IL-10, a prominent anti-inflammatory cytokine produced by regulatory B cells (20) as well as by monocytes, is not detected in response to *Lactobacillus* priming in wild-type or B cell–deficient mMT mice (Fig. 7E).

**Discussion**

From these studies, we conclude that B cells are dispensable for *Lactobacillus*-mediated heterologous immunity and have no role in promoting survival in response to an otherwise lethal respiratory virus infection.

As noted earlier, heterologous immunity, also known in other contexts as innate imprinting, and trained immunity (6–9) are concepts that explain increased (or decreased) resistance to unrelated infections that result from primary innate immune or inflammatory response. Netea et al. (7) introduced the term “trained immunity” as a means to explain and to explore nonspecific cross-protection to unrelated microbes. Among these findings, as noted earlier, were observations documenting systemic exposure to attenuated *Mycobacterium tuberculosis* (BCG vaccination) that resulted in nonspecific systemic immunity to several unrelated microbes.
pathogens (reviewed in Ref. 6). BCG-mediated macrophage activation and antimicrobial peroxide production was initially implicated in this mechanism (26). Kleinnijenhuis et al. (27) recently reported that nonspecific protection elicited by BCG vaccination in human subjects was directly dependent on persistent, NOD2-dependent epigenetic alterations in circulating monocytes, specifically altered histone methylation patterns, and that BCG vaccination likewise elicited induction of proinflammatory cytokines IFN-γ, TNF-α, and IL-1β.

Lactobacillus priming of the respiratory mucosa and protection generated against PVM infection is another dramatic example of this concept, as we observe not only robust but ongoing and sustained protection against an unrelated respiratory pathogen (4, 5). Specifically, we have shown that significant protection against a lethal inoculum of PVM can be sustained for 5 mo or longer after only two intranasal inoculations of L. plantarum. Interestingly, as we compare our findings to the aforementioned results of Kleinnijenhuis et al. (27), we find that Lactobacillus

![FIGURE 4](image1.png)

**FIGURE 4.** Flow cytometric detection of lymphocytes in lungs of L. plantarum–primed, PVM-infected wild-type and B cell–deficient mice. (A) Total (B) B (CD3−CD19+) cells and (C) T (CD3+CD19−) cells in single-cell suspensions of lungs of L. plantarum or control-primed (days −14 and −7), PVM-infected wild-type (C57BL/6), and B cell–deficient (µMT) mice; n = 3 mice/group. *p < 0.05, **p < 0.01. (D) Total (E) B (CD3−CD19+) and (F) T (CD3+CD19−) cells in L. plantarum–primed, PVM-infected wild-type BALB/c and B cell–deficient Jh mice; n = 4 mice/group. *p < 0.05. Total B and T cells determined from percentage of total lymphocytes of live cells with forward scatterlow/side scatterlow.

![FIGURE 5](image2.png)

**FIGURE 5.** No infiltrates in lung tissue of Lactobacillus–primed, PVM-infected B cell–deficient µMT mice. (A) H&E-stained lung tissue from L. plantarum–primed, PVM-infected wild-type C57BL/6. Original magnification ×10. (B) Lung tissue in (A), probed with rat monoclonal anti-mouse B220. Original magnification ×40. (C) H&E-stained lung tissue from L. plantarum–primed, PVM-infected B cell–deficient µMT mice. Original magnification ×10. (D) Lung tissue from L. plantarum–primed, PVM-infected µMT mice probed with rat monoclonal anti-mouse B220. (E) Total IgG detected in bronchoalveolar lavage fluid from L. plantarum–primed (days −14 and −7) C57BL/6 wild-type mice and B cell–deficient µMT mice (day +3). ***p < 0.001.
priming of the respiratory mucosa is similarly associated with induction of proinflammatory cytokines IFN-\(\gamma\) and TNF-\(\alpha\), although we found in our earlier work that IFN-\(\gamma\) was dispensable for heterologous immunity (i.e., survival in response to subsequent PVM challenge) (5). Interestingly, and in contrast to findings with BCG vaccination (27), preliminary results from our laboratory indicate that heterologous immunity elicited in response to \textit{Lactobacillus} priming of the respiratory mucosa is not directly dependent on the presence of the pattern recognition receptor NOD2 (H.F. Rosenberg, unpublished results).

There are several intriguing examples of heterologous immunity elicited by microbial agents via direct priming at the respiratory mucosa that likewise appear to proceed via distinct mechanisms. For example, Clement, Tuvim, Evans, and colleagues (28–31) have examined immunity generated in mice subjected to multiple instillations of a sterile lysate from the Gram-negative microorganism nontypeable \textit{Haemophilus influenzae} (NTHi). Similar to our findings, they have reported that intranasal NTHi results in protection from the lethal sequelae of influenza A infection. However, in contrast to immunity elicited by \textit{Lactobacillus} priming against PVM (4, 5), protection elicited by the NTHi infusions is short-lived, results in a prominent type I IFN response, and is dependent on epithelial cell-specific MyD88 signaling. Thus, although direct priming with either NTHi or \textit{Lactobacillus} serves to promote protection against acute respiratory virus infection, the cellular and biochemical mechanisms promoting these responses are clearly distinct and stimulus-specific.

Likewise, several groups have shown that intranasal administration of \textit{Lactobacillus} species, using a variety of priming strategies, also protects mice against acute influenza infection (32, 33), similar to our findings with PVM (4, 5). Interestingly, oral administration of at least three distinct \textit{Lactobacillus} strains has also been reported as effective against the lethal sequelae of influenza (34–36). These results are significantly at odds with our findings with PVM, in which we document that prolonged oral administration of \textit{L. plantarum} has no impact whatsoever on survival (Fig. 1C). It would be intriguing to determine the source of these different results and what the underlying mechanisms might be.

In this study, we focused on a role for B cells in promoting heterologous immunity. Apart from their role in adaptive immunity, specific B cell subsets have also been implicated in antiviral host defense. For example, Choi and Baumgarth (37) and Jayasekera et al. (38) demonstrated that B1a cells play important roles in
inmate immunity to influenza infection by production of virus-neutralizing nonspecific IgM. Similarly, as reported by Moseman et al. (19), B cell–derived lymphocytotoxin αβ2 was necessary to support subcapsular sinus macrophages within draining lymph nodes, thus preventing fatal infection with vesicular stomatitis virus. B cells are also among the crucial components of BALT, which are tertiary lymphoid structures that develop in mouse lung tissue in response to inflammatory and/or microbial provocation (14–17). Classic BALT has a structured organization with distinct T and B cell areas, with the latter including follicular dendritic cells and proliferative germinal centers, although variants with primarily B cells (39) or intermixed zones (14) have been described. We have shown in the present study that the lymphoid clusters induced by L. plantarum include both B220-positive mature B cells as well as RGS13-positive cells, shown by Shi et al. (40) to be a specific marker for germinal center B cells.

As noted earlier, Harmsen and colleagues (12, 13) found that otherwise unmanipulated mice primed with protein cage nanoparticles, multisubunit particles derived from bacterial heat-shock proteins, developed BALT; furthermore, they reported that B cell–mediated immunomodulatory mechanisms, including accelerated generation of Igs in situ, were crucial to bacterial/protein-mediated protection against respiratory virus pathogens. Interestingly, we find that although Lactobacillus priming induces the formation of B cell–enriched infiltrates with germinal centers, Lactobacillus-mediated protection proceeds via mechanisms that are B cell independent. In contrast to results obtained with protein cage nanoparticle priming and influenza (12), in which diminished virus recovery was B cell dependent, we determined that virus recovery after PVM infection is diminished in both L. plantarum–primed B cell–deficient and wild-type strains. Furthermore, survival of Lactobacillus–primed, PVM infected B cell–deficient mice was indistinguishable from that of their wild-type counterparts. It is again intriguing to find that such fundamentally different mechanisms promote what are otherwise similar outcomes.

In summary, we have shown that mice primed via intranasal inoculation with L. plantarum are protected against subsequent PVM infection. We show in the present study that L. plantarum priming elicits formation infiltrates enriched with B lymphocytes and elevated levels of Igs in the airways. Although B cells have been implicated in promoting heterologous immunity against PVM and other respiratory virus infections (12), L. plantarum–primed, PVM-infected B cell–deficient mice respond with diminished virus recovery, indistinguishable from their wild-type counterparts. Furthermore, L. plantarum–primed, B cell–deficient mice are fully protected from the lethal sequelae of PVM infection. We conclude that B cell deficiency has no impact on these important outcomes underlying heterologous immunity promoted uniquely via Lactobacillus priming at the respiratory mucosa.

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Disclosures

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