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Priming of the Respiratory Tract with Immunobiotic *Lactobacillus plantarum* Limits Infection of Alveolar Macrophages with Recombinant Pneumonia Virus of Mice (rK2-PVM)

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ABSTRACT

Pneumonia virus of mice (PVM) is a natural rodent pathogen that replicates in bronchial epithelial cells and reproduces many clinical and pathological features of the more severe forms of disease associated with human respiratory syncytial virus. In order to track virus–target cell interactions during acute infection *in vivo*, we developed rK2-PVM, bacterial artificial chromosome-based recombinant PVM strain J3666 that incorporates the fluorescent tag monomeric Katushka 2 (mKATE2). The rK2-PVM pathogen promotes lethal infection in BALB/c mice and elicits characteristic cytokine production and leukocyte recruitment to the lung parenchyma. Using recombinant virus, we demonstrate for the first time PVM infection of both dendritic cells (DCs; CD11c+/major histocompatibility complex class II+ and alveolar macrophages (AMs; CD11c+/sialic acid–binding immunoglobulin-like lectin F+) in vivo and likewise detect mKATE2+ DCs in mediastinal lymph nodes from infected mice. AMs support both active virus replication and production of infectious virions. Furthermore, we report that priming of the respiratory tract with immunobiotic *Lactobacillus plantarum*, a regimen that results in protection against the lethal inflammatory sequelae of acute respiratory virus infection, resulted in differential recruitment of neutrophils, DCs, and lymphocytes to the lungs in response to rK2-PVM and a reduction from ~40% to <10% mKATE2+ AMs in association with a 2-log drop in the release of infectious virions. In contrast, AMs from *L. plantarum*–primed mice challenged with virus *ex vivo* exhibited no differential susceptibility to rK2-PVM. Although the mechanisms underlying *Lactobacillus*–mediated viral suppression remain to be fully elucidated, this study provides insight into the cellular basis of this response.

IMPORTANCE

Pneumonia virus of mice (PVM) is a natural mouse pathogen that serves as a model for severe human respiratory syncytial virus disease. We have developed a fully functional recombinant PVM strain with a fluorescent reporter protein (rK2-PVM) that permits us to track infection of target cells *in vivo*. With rK2-PVM, we demonstrate infection of leukocytes in the lung, notably, dendritic cells and alveolar macrophages. Alveolar macrophages undergo productive infection and release infectious virions. We have shown previously that administration of immunobiotic *Lactobacillus* directly to the respiratory mucosa protects mice from the lethal sequelae of PVM infection in association with profound suppression of the virus–induced inflammatory response. We show here that *Lactobacillus* administration also limits infection of leukocytes *in vivo* and results in diminished release of infectious virions from alveolar macrophages. This is the first study to provide insight into the cellular basis of the antiviral impact of immunobiotic *L. plantarum*.

Human respiratory syncytial virus (hRSV, family Paramyxoviridae, genus Pneumovirus) is a major cause of morbidity and death among infants and children worldwide and is recognized as a significant health concern among the elderly (1). The anti-hRSV antibody palivizumab is highly effective when used as prophylaxis in infants identified as high risk (2), although there are many infants hospitalized with severe hRSV disease who are not identified in any of the high-risk cohorts (3). Treatment options for hRSV disease are primarily supportive. The antiviral agent ribavirin is currently recommended only for severely ill and immunocompromised children (4, 5). New antiviral agents that focus specifically on hRSV are in development (6–8), and the inflammatory responses characteristic of severe hRSV disease are also recognized as targets for therapeutic intervention (9, 10).

Pneumonia virus of mice (PVM) is a rodent pathogen of the same family and genus as hRSV. PVM infection in inbred strains of mice reproduces many of the clinical and pathological features of the more severe forms of hRSV disease, which has facilitated the exploration of new therapeutic strategies *in vivo* (11, 12). Similar to hRSV (13), PVM infects bronchial epithelial cells and promotes...
influx of granulocytes to the lung in association with the production of proinflammatory cytokines and chemokines. Blockade of proinflammatory signaling pathways, including those involving the chemokine receptor CCR1 and also chemerin R23, cysteiny1-leukotrienes, and sphingosine-1-phosphate (14–17), promotes improved outcomes by targeting the lethal inflammatory sequelae of PVM infection.

As part of our ongoing interest in the host antiviral inflammatory response, we have explored the immunomodulatory potential of various *Lactobacillus* species. While the impact of oral administration of probiotics, including *Lactobacillus*, in improving outcomes secondary to respiratory virus infection remains uncertain (18, 19), we have shown that direct priming of the respiratory tract with live or heat-inactivated *Lactobacillus plantarum* results in robust and sustained protection against a subsequent lethal PVM infection in association with profound suppression of virus-induced proinflammatory cytokines (20–22). This is a unique example of heterologous immunity, a response of the innate immune system that offers cross-protection from unrelated pathogens after a primary inflammatory or infectious event; this is known in other contexts as trained immunity, innate imprinting, or innate memory (23–25). Among several relevant examples of this concept, Wiley and colleagues (26) found that inhalation of *Methanococcus*-derived nanoparticles protected mice against several different acute respiratory virus infections. Likewise, Easton and colleagues (27) found that intranasal administration of defective interfering influenza virus particles is protective against a subsequent lethal PVM infection challenge, and Schnoeller and colleagues (28) recently reported that an attenuated preparation of *Bordetella pertussis* protects mice against symptoms related to a subsequent challenge with hRSV. Remaining unclear in all of these examples and likewise in response to priming with *L. plantarum* is the fate of the respiratory virus, specifically, whether the priming agent alters not only virus clearance but also the way in which the virus interacts with innate immune target cells in the respiratory tract.

In order to address these questions, we have generated a recombinant virus featuring PVM strain J3666 that incorporates the far-red fluorescent protein monomeric Katushka 2 (mKATE2) (29) by using a bacterial artificial chromosome (BAC)-based methodology developed by Hotard and colleagues (30). Using mKATE2 fluorescence to detect PVM-infected cells, we focused on interactions of the virus with resident leukocytes (e.g., alveolar macrophages [AMs]), as well as with cells that are recruited to the respiratory tract in response to acute infection.

### MATERIALS AND METHODS

**Mice.** BALB/c mice (6- to 8-week-old females) were from the Charles River Laboratories, Frederick, MD, facility. All mouse studies were approved by NIAID and carried out in accordance with Animal Care and Use Committee guidelines.

*Lactobacillus*. *L. plantarum* BAA-793 was grown in Mann-Rogosa-Sharpe medium; the ratio of the optical density at 600 nm (OD$_{600}$) to the CFU count was determined experimentally (20). Bacterial cells were washed, inactivated by serial freezing-thawing (20), and stored at −80°C at 10$^{11}$/ml.

**Generation of PVM minigenome.** The PVM minigenome reporter pGEM-PVM-Luc was constructed by replacing the RSV leader and trailer sequences derived from PVM strain J3666. The PVM leader sequences (PVM 5’ untranslated region [UTR]), GenBank accession no. NC_006579 bp 1 to 42), the PVM N gene start (bp 1036 to 1044 [32]), the PVM N noncoding region (bp 1045 to 1066) with flanking NotI and BamHI sites, the PVM trailer (PVM L noncoding region, GenBank accession no. NC_006579 bp 14653 to 14657), the PVM L gene end (bp 14781 to 14794 [32]), and the PVM 3’ UTR (bp 14795 to 14885) with flanking Xhol and HindIII sites were inserted to replace the RSV sequences. All DNA fragments were synthesized by GenArt (Invitrogen); sequences were confirmed prior to transfer in order to generate pGEM-PVM-Luc. The pGEM-PVM-Luc plasmid was transfected into BSR T7/5 cells (33) as described below by using 0.8 μg of pGEM-PVM-Luc. Luciferase activity was monitored at 24 h by using the dual-luciferase reagent in accordance with the manufacturer’s protocol (Promega).

**Generation of the recombinant pSynK-PVMJ3666 antigenome in a BAC.** The antigenome of PVM strain J3666 (GenBank accession no. NC_006579.1) was synthesized in three fragments that were provided to us in the pMA-T cloning vector (GeneArt; Invitrogen). To facilitate sequential cloning of the three fragments, Zral and Spili restriction sites were introduced between the BstB1 and MluI sites already present in BAC vector pKBS3 via a 47-bp linker, generating pKBS3-LINKER. Among the unique features of this construct, the gene encoding the red fluorescent protein mKATE2 (29) was introduced between the PVM N and L noncoding regions prior to the NS1 gene to allow tracking of PVM-infected cells by fluorescence as described previously for recombinant RSV line 19F (30). These and other features of the antigenome construct, to which we refer as pSynk-PVMJ3666, are provided in Table 1.

**Isolation of pSynk-PVMJ3666 BAC DNA.** NEB10β competent *Escherichia coli* (New England BioLabs) was transformed with isolated DNA in accordance with the manufacturer’s directions with the following modifications. Bacteria were permitted to recover from heat shock for 2 h at 32°C with aeration at 250 rpm. After 2 h, the transformed bacteria were collected by centrifugation and resuspended in 250 μl of super optimal broth with glucose; 20 or 200 μl was then plated on Luria-Bertani agar plates containing 20 ng/ml chloramphenicol (Sigma) and grown overnight at 32°C. Colonies from these plates were grown at 32°C at 250 rpm in medium containing 20 ng/ml chloramphenicol. BAC DNA was isolated with a Qiagen midi kit and a user-optimized protocol (QP01; Qiagen, Hilden, Germany). Approximately 2 μg of DNA was recovered from a 100-ml culture.

**Generation and isolation of helper plasmids expressing PVMJ3666 L, M2-1, N, and P proteins.** The DNA sequences for these plasmids were also based on the sequence of PVM strain J3666. The gene sequences encoding the PVM nucleoprotein (N), phosphoprotein (P), transcription factor M2-1, and polymerase (L) were converted to human codon preference, provided with a Kozak consensus sequence (34) at the ATG initiation codon, and inserted within the HindIII and EcoRI cloning sites of the pcDNA3.1 expression vector (GeneArt; Invitrogen). The nucleotide sequences of all support plasmids were confirmed directly. Bacteria transformed with these plasmids were grown at 32°C; plasmids were isolated with the Marligen maxiprep kit in accordance with the manufacturer’s instructions.

**Generation of recombinant mKATE2-PVM (rK2-PVM).** BSR T7/5 cells (33) were maintained in 10% fetal bovine serum (FBS) in Glasgow’s minimum essential medium (GMEM; Invitrogen) and passed into six-well plates so that the cells would be confluent on the day of transfection. With a 3:1 ratio of Lipofectamine 2000 (microtainers) to DNA (micrograms), the plasmids were transfected into these cells in the following quantities: 0.8 μg of pSynk-PVM J3666; 0.8 μg of pKBS3-LINKER (negative control); 0.4 μg each of pcDNA3.1 helper plasmids encoding the PVM J3666 M2-1, N, and P proteins; and 0.2 μg of plasmid pcDNA3.1 encoding the PVM J3666 L protein in a total volume of 500 μl of Opti-MEM (Invitrogen). After a 2-h incubation at room temperature on a plate shaker rotating at 300 rpm, an additional 500 μl of Opti-MEM was added to each well and the cells were incubated at 37°C in a 5% CO$_2$ incubator for 24 h. Opti-MEM was then replaced with 2 ml of 3% FBS in GMEM, and the cultures were moved to 32°C. The cells were monitored daily by
fluorescence microscopy for the expression of mKATE2. On day 3 post-transfection, the supernatant was collected and stored at −80°C prior to use in exploring the infectivity of rK2-PVM produced by these cells in naive cell monolayers in vitro. The remaining cells from the monolayer were then treated with trypsin and transferred into a T75 flask containing 10^6 parental BHK21 cells (CCL-10; ATCC). These cells were maintained until 50% were mKATE2 positive by fluorescence microscopy, and then the cells were passaged two more times onto fresh monolayers of BHK-21

### TABLE 1

<table>
<thead>
<tr>
<th>Feature</th>
<th>Location(s)</th>
<th>Size (nt)</th>
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<td>T7 promoter</td>
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<td>GGG</td>
<td>41–43</td>
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<td>Miscellaneous</td>
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<tr>
<td>AAA to AAG</td>
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<td>Elimination of MluI site</td>
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<td>Leader</td>
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<td>5' UTR</td>
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<td>P gene</td>
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<td>Insertion of G and GA to make SaII site</td>
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<td>L CDS, 2,020 aa</td>
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<td>Insertion to make BstWI site</td>
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<td>Hepatitis delta virus ribonuclease</td>
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<td>Chloramphenicol resistance CDS</td>
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<td>CDS for plasmid partitioning to daughter cells during division and stable BAC maintenance</td>
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### Notes:

- The PVmJ3666 genome sequence (GenBank accession no. NC_006579) was used to construct this plasmid.
- nt, nucleotides.
- GS, gene start.
- NC, noncoding.
- CDS, coding sequence.
- GE, gene end.
- IG, intergenic.
- aa, amino acids.
Infection of RAW 264.7 cells. Two milliliters of supernatant from BSR T7/5 cells transfected with either pSynK-PVMJ3666 or pKBS3-LINKER (negative control) were used to infect a subconfluent monolayer of RAW 264.7 cells (ATCC TIB-71) in an 80-cm² flask. Cells inoculated as described above were incubated for 2 h at room temperature on a plate shaker at 300 rpm and then moved to 37°C at 5% CO₂, for 24 h. After 24 h, the supernatant was replaced with 10 ml of RPMI 1640 medium with 2% FBS and the cells were moved to 32°C. The cells were monitored daily by fluorescence microscopy for the expression of mKATE2.

TCFD50 analysis. Virus stocks were quantified by a variation of the standard 50% tissue culture infective dose assay performed essentially as described previously (36) by detection of mKATE2* fluorescent cells as the endpoint. Serial 10-fold dilutions of virus were prepared in 2% FBS in RPMI medium with glutaCine, penicillin, and streptomycin and evaluated on RAW 264.7 or A2 (ATCC CRL1542) cells in 96-well plates incubated at 32°C. The cells were monitored daily for mKATE2* cells, and the 50% endpoint based on fluorescence (TCFD50/ml) was calculated at 5 to 6 days postinfection (37). By this method, the calculated TCFD50 of the mouse-passaged virus stocks ranged from 2.1 to 4.6 x 10⁴ U/ml.

Amplification and evaluation of high-titer virus stocks. rK2-PVM generated by the transfection of BSR T7/5 cells was amplified in mice. BALB/c mice received 100-μl inocula of the supernatant from the third-passage supernatant collected from the parental BHK21 cells. Lung tissue was collected 7 days after inoculation, and virus stocks were prepared as previously described (38). Briefly, lungs were collected under aseptic conditions and placed in 1 ml of ice-cold 10% FBS in Dulbecco’s modified Eagle medium. The lung tissue was blade homogenized, and cellular debris was removed by two rounds of centrifugation. The clarified supernatants were stored at −80°C or liquid nitrogen and used for subsequent passages. Mouse-passaged rK2-PVM was used for all of the in vivo studies described in this report, unless otherwise noted.

Infection of BALB/c mice with native and recombinant PVM and priming with L. plantarum. BALB/c mice under isoflurane anesthesia were inoculated intranasally with 50 μl of PVM strain J3666 (0.2 TCFD₅₀) or rK2-PVM (2.1 to 210 TCFD₅₀) diluted in Iscove’s modified Dulbecco’s medium. In the experiments indicated, BALB/c mice were first inoculated intranasally with heat-inactivated L. plantarum (50 μl, 2 x 10¹⁰ cells/ml) on day −14 and again on day −7 prior to a virus challenge on day 0, as previously described (20). Anti-PVM IgG1 in mouse serum was detected by enzyme-linked immunosorbent assay (ELISA; catalog no. SMART-M12; BioTech Trading Partners, El Cerrito, CA).

Isolation of airway macrophages from L. plantarum−primed, rK2-PVM-infected mice. Bronchoalveolar lavage (BAL) fluid was collected from control and L. plantarum-primed, PVM-infected mice in a total of 4 ml of sterile phosphate-buffered saline with 0.1% bovine serum albumin (PBS-BSA). Cells were centrifuged at 2,000 x g for 10 min at 4°C and resuspended in RPMI medium containing 10% FBS, 2 mM glutamine, 100 IU of penicillin, and 10 μg/ml streptomycin (complete RPMI). Equal numbers of cells from all of the BAL fluid samples were plated. After 2 h, the nonadherent cells were removed and the adherent macrophages remaining were cultured for an additional 48 h. After 48 h, the culture supernatant was collected and dialyzed against 10³ volumes of complete RPMI with 2% instead of 10% FBS across a 50-kDa membrane to remove low-molecular-weight cytokines prior to evaluation in a TCFD₅₀ assay as described in the next section. In some experiments, airway macrophages were isolated from mice that were control or L. plantarum primed as described above but not virus infected.

Detection of rK2-PVM-infected cells by flow cytometry. Single-cell suspensions from mouse lung tissue were prepared as previously described (20). Mediastinal lymph nodes from either side of the thymus dorsal to the heart (39) were dissected, and single-cell suspensions were prepared in RPMI 1640 by passing tissue through a 40-μm cell strainer. Cells were incubated with Live-Dead Fixable Aqua Dead Cell stain (Life Technologies) for 30 min prior to washing with PBS-BSA and then stained with fluorochrome-conjugated antibodies against CD16/CD32, CD8a, CD19, silic acid-binding immunoglobulin-like lectin F (Siglec-F), and Gr1 (BD Biosciences); CD45, CD3, CD4, CD11c, and major histocompatibility complex class II (MHCIll-A)-/A-K (eBioscence); and CD11b, CD24, and CD64 (BioLegend). After incubation with antibodies for 30 min at 4°C, the cells were washed with 3 ml of PBS-BSA and then fixed in 4% paraformaldehyde. The samples were stored at 4°C in the dark until they were analyzed. A minimum of 100,000 events was collected on an LSRII flow cytometer (BD Biosciences), and data were analyzed in FlowJo (Tree Star, Inc.).

Identification of mRNA and determination of virus titers. RNA was isolated from cultured cells and supernatants of virus stocks with the RNeasy mini kit in accordance with the manufacturer’s instructions (Qiagen). Virus titers in lung tissue were determined as described previously (20, 40), except that the sequence 5′-AAG CAT TGC TAC ATC AGG C-3′, targeting the PVM SH gene, replaced the reverse primer.

Cytokine analysis. ELISAs (R&D Systems) of clarified homogenates of lung tissue were performed and corrected for total protein by BCA assay (Pierce) as previously described (20).

Statistical analysis. Findings presented as the mean ± the standard error of the mean were analyzed for statistical significance by two-way analysis of variance (ANOVA), Mann-Whitney U test, or log rank test, as indicated, in GraphPad PRISM 6.

RESULTS

Recombinant mKATE2-PVM (rK2-PVM): production and recovery of infectious recombinant virions. We first evaluated the effectiveness and specificity of the four individual helper plasmids, each with human codon-optimized sequences of the virus genes for P, N, M2-1, and L, at promoting the activity of a PVM minigene reporter (pGEM-PVM-Luc) in BSR T7/5 cells (33) (Fig. 1A). As shown, significant luciferase activity from the pGEM-PVM-Luc reporter was detected only with the cotransfection of all four helper plasmids.

The antigenome of PVM strain J3666 preceded by the sequence encoding the far-red fluorescent protein mKATE2 (29) and flanked by PVM leader and trailer sequences was cloned into the Bac vector pKBS-LINKER, a modified version of vector pKBS3 that was altered to include Zral, Sphl, and MluI restriction enzyme sites in the multicloning site (pSynK-PVMJ3666; Fig. 1B). BSR T7/5 cells were transfected with pSynK-PVMJ3666 and the aforementioned four helper plasmids. Expression of mKATE2 in BSR T7/5 cells was detected from day 3 posttransfection (Fig. 1C). Supernatant from the transfected BSR T7/5 cells was used as a source of recombinant virus (rK2-PVM) to infect naïve target cells of the RAW 264.7 mouse macrophage line. Expression of mKATE2 in the naïve target macrophage cells was detected as early as 24 h after infection (Fig. 1D). No fluorescence was detected in cells infected with supernatants from BSR T7/5 cells transfected with helper plasmids and control plasmid pKBS3-LINKER (data not shown).

Infection with rK2-PVM is lethal in mice and elicits the characteristic inflammatory response. BALB/c mice were inoculated intranasally with mouse-passaged rK2-PVM. As shown in Fig. 2A, 210 TCFD₅₀ in a 50-μl inoculation volume resulted in a fully lethal infection with a median survival time (t₅₀) of 9 days. In contrast, a 60% mortality rate was observed in response to 21 TCFD₅₀/50 μl. No deaths were observed in response to 2.1 TCFD₅₀ in 50 μl, although seroconversion was observed (40% of mice at day 21). As shown in Fig. 2B, the recombinant virus is stable, with minimal
variation (virion copy number versus TFCD_{50}) observed over three passages in vivo. Although rK2-PVM is significantly (~300 times) less virulent than parent PVM strain J3666 (Fig. 2C), fluorescence units (TCFD_{50}) correlated directly with the standard measure of absolute virus copy number (absolute number of copies of virus SH per absolute copy of virus glyceraldehyde 3-phosphate dehydrogenase [GAPDH]) over a 10^4 range of virus concentrations (replication and clearance, days 3 to 11; Fig. 2D). As observed previously in response to infection with PVM strain J3666, proinflammatory cytokines interleukin-6 (IL-6) (Fig. 2E), CCL2 (Fig. 2F), and CXCL10 (Fig. 2G) were detected in lung tissue in response to infection with rK2-PVM. Furthermore, infection with rK2-PVM results in significant leukocyte (CD45+ cell) recruitment to the lung (Fig. 2H).

Differential detection of mKate2 among myeloid cells in rK2-PVM-infected mice. Both mKate2+ leukocytes (CD45+) and mKate2− nonleukocytes (CD45−) were detected at day 7 after inoculation. Of the leukocyte subsets examined, we detected significant fractions of mKate2+ AMs, dendritic cells (DCs), and neutrophils. The remaining leukocyte subsets, including eosinophils, NK cells, B cells, CD4+ T cells, and CD8+ T cells, had no substantial mKate2+ populations at that time point (<1%).

Priming with L. plantarum averts the lethal sequelae of rK2-PVM infection and limits PVM infection of CD45− cells. We have shown previously that intranasal inoculation or priming of the respiratory tract with live or heat-inactivated preparations of L. plantarum results in robust and sustained protection from the lethal sequelae of severe pneumovirus infection in association with suppression of virus-induced inflammation (20–22). Our standard L. plantarum priming protocol is shown in Fig. 3A. Mice received an intranasal inoculation of heat-inactivated L. plantarum (10^9 cells in 50 μl) or diluent alone (PBS-BSA) on day −14 and again on day −7, followed by rK2-PVM (210 TFCD_{50} in 50 μl) on day 0. Similar to our previous findings with the parent strain, PVM J3666 (20, 21), mice that received diluent did not survive (t_{1/2} = 8 days) while those that were primed with L. plan-
tum were fully protected (100% survival) (Fig. 3B), with all undergoing seroconversion by day 21. As shown in Fig. 3C, L. plantarum-primed, rK2-PVM-infected wild-type mice cleared the virus from their lung tissue; minimal residual virus was detected at day 20 after inoculation.

An example of gating, comparing lung cell suspensions prepared from naïve, PVM strain J3666-infected, and rK2-PVM-infected mice is shown in Fig. 3D. As shown in Fig. 3E, L. plantarum priming had no impact on the number of CD45<sup>+</sup> cells (nonleukocytes, predominantly type I pneumocytes [41]) detected in lung tissue. However, L. plantarum priming did suppress the infection of these cells, reducing the mKATE2<sup>+</sup> population by approximately one-third (Fig. 3F, P < 0.001).

**Priming with L. plantarum augments recruitment while suppressing infection of CD11c<sup>+</sup> DCs.** Given our earlier findings, we focused on AMs (CD11c<sup>+</sup> Siglec-F<sup>+</sup>), DCs (CD11c<sup>+</sup> MHCII<sup>+</sup>), and neutrophils (CD11c<sup>−</sup> Gr1<sup>+</sup>) as the significant leukocyte targets of L. plantarum priming. Consistent with our previous work (20), L. plantarum priming prior to virus infection resulted in diminished neutrophil recruitment (Fig. 4A; P = 0.01), although it had no impact on the relatively small fraction of neutrophils (>5%) that were mKATE2<sup>+</sup> (Fig. 4B). In contrast, while PVM infection has been associated previously with the recruitment of CD11c<sup>+</sup> plasmacytoid DCs (42), we show here that L. plantarum priming resulted in augmented recruitment of CD11c<sup>+</sup> MHC-II<sup>+</sup> DCs (Fig. 4C; P < 0.0001) while at the same time reducing the fraction of infected mKATE2<sup>+</sup> DCs by half (Fig. 4D; P < 0.01). Interestingly, although CD4<sup>+</sup> T, CD8<sup>+</sup> T, and B lymphocytes are not major targets of rK2-PVM infection and B cells are not critical for virus clearance or L. plantarum-mediated protection (22), priming with L. plantarum resulted in recruitment of these cells to the lungs at levels that exceed those observed in response to rK2-PVM infection alone (Fig. 5A to D).

**Priming with L. plantarum targets AMs.** Priming resulted in a significant difference in the number of CD11c<sup>+</sup> Siglec-F<sup>+</sup> AMs detected, which also diminished over the course of infection (Fig. 6A; P < 0.001). AMs are long-lived cells that are capable of self-renewal and are not typically replenished from circulating progenitors under homeostatic conditions; however, exogenous perturbation, such as virus infection (and perhaps L. plantarum priming) may result in recruitment of monocytes from the periphery that will differentiate into AMs (reviewed in reference 43). L. plantarum priming also had a substantial impact on the fraction of mKATE2<sup>+</sup> AMs (Fig. 6B and C; P < 0.0001). Specifically, at the peak (day 5), 30 to 40% of the AMs from control-primed mice were mKATE2<sup>+</sup>, while only 5 to 7% of the AMs from L. plantarum-primed mice were mKATE2<sup>+</sup>. These results are consistent whether we use the immunophenotyping protocol described by Guilliams and colleagues (44), in which AMs are reported as CD4<sup>+</sup> CD11c<sup>+</sup> Gr1<sup>−</sup> MHCI<sup>II</sup><sup>+</sup> Siglec-F<sup>+</sup> (utilizing Siglec-F, the only unique marker for AMs, when comparing them to other lung monocyte/macrophage and DC populations) (45), or the protocol of Misharin and colleagues (46), in which AMs are identified as CD4<sup>+</sup> CD11c<sup>+</sup> CD11b<sup>−</sup> CD64<sup>+</sup>. Exploring this observation further, we note that L. plantarum priming did not result in diminished viability of total AMs preferentially in rK2-PVM-infected mice; however, we did observe significant loss of AMs in both control and L. plantarum-primed, rK2-PVM-infected mice over time (P < 0.001, Fig. 6D), a finding that may account for some (or nearly all) of the decline in total AMs shown in Fig. 5A. Likewise, L. plantarum priming promotes no differential viability of the infected (mKATE2<sup>+</sup>) subpopulation of these cells (Fig. 6E). We also...
performed experiments to determine whether \textit{L. plantarum} priming might promote the migration of rK2-PVM-infected cells to draining lymph nodes. Total and mKATE2\textsuperscript{+} cells were isolated from the mediastinal lymph nodes of control and \textit{L. plantarum}-primed mice at 5 days after rK2-PVM inoculation. As shown in Fig. 6F, 25\% \pm 13\% of the total CD11c\textsuperscript{+} cells, including 29\% \pm 15\% of the CD45\textsuperscript{+} CD11c\textsuperscript{+} CD64\textsuperscript{+} DCs (45) identified in mediastinal lymph nodes from control-primed, rK2-PVM-infected mice, were mKATE2\textsuperscript{+}. In contrast, few (<0.1\%) to no mKATE2\textsuperscript{+}CD11c\textsuperscript{+} cells were detected in single-cell suspensions from mediastinal lymph nodes isolated from mice that were \textit{L. plantarum} primed prior to rK2-PVM infection. Overall, our findings suggest that \textit{L. plantarum} priming limits rather than promotes the transit of rK2-PVM and/or rK2-PVM-infected leukocytes to draining lymph nodes.

**Airway macrophages support active replication of rK2-PVM and produce replication-competent virions.** In order to evaluate the possibility of productive infection and the impact of \textit{L. plantarum} priming on this process, we isolated macrophages from the airways of rK2-PVM-infected mice primed with \textit{L. plantarum} or diluent alone (Fig. 7A). There was no difference in the AMs (defined as CD45\textsuperscript{+} CD11c\textsuperscript{+} Gr1\textsuperscript{−} MHC-II\textsuperscript{−} Siglec-F\textsuperscript{−}) as a percentage of the total leukocytes (%CD45\textsuperscript{+} cells) isolated from the airways of \textit{L. plantarum}-primed versus control-primed, rK2-PVM-infected mice (Fig. 7B). However, as might be anticipated from the results in Fig. 5A, we recovered significantly more CD11c\textsuperscript{+} Siglec-F\textsuperscript{−} AMs from the airways of \textit{L. plantarum}-primed mice than from those of control mice (5.8 \times 10^3 \pm 15 \times 10^3 versus 8.5 \times 10^2 \pm 1.8 \times 10^2 per mouse; \textit{P} < 0.01; Fig. 7C). As shown in Fig. 5B, although we isolated more CD45\textsuperscript{+} CD11c\textsuperscript{+} Gr1\textsuperscript{−} MHC-II\textsuperscript{−} Siglec-F\textsuperscript{−} AMs from the airways of \textit{L. plantarum}-primed mice, the fraction of these AMs that were also mKATE2\textsuperscript{+} was substantially smaller (0.5\% \pm 0.1\% mKATE2\textsuperscript{+} cells from \textit{L. plantarum}-primed, rK2-PVM-infected mice versus 38\% \pm 7\% mKATE2\textsuperscript{+} cells from control-primed, rK2-PVM-infected mice \textit{P} < 0.01; Fig. 7D). To evaluate productive infection, equivalent numbers of air-
way macrophages from each group were evaluated ex vivo (100,000/well); no differential survival was observed after 48 h in culture (Fig. 7E), although there were significantly fewer mKATE2+/H11001 fluorescent foci among the airway macrophages from L. plantarum-primed, rK2-PVM-infected mice (34% ± 10% versus 2% ± 1%, P < 0.05; Fig. 7F). Supernatants from both cultures were evaluated in TCFD50 assays; those from control mice demonstrated a 100-fold (2-log) higher titer (TCFD50/ml) than those from L. plantarum-primed mice (Fig. 7G; P < 0.01). These results indicate that airway macrophages undergo productive infection when challenged with rK2-PVM and that L. plantarum priming of the respiratory tract limits not only virus replication but productive infection from these cells. It is most intriguing that when airway macrophages are isolated from L. plantarum-primed mice, introduced into tissue culture as described above, and challenged with rK2-PVM ex vivo, virus recovery is indistinguishable from that from control-primed counterparts (Fig. 7H). The implications of this finding with respect to L. plantarum priming and its mechanism of antiviral action are discussed below.

DISCUSSION
In this study, we explored the interactions of PVM with leukocytes in the respiratory tract, identified both DCs and AMs as significant targets of infection in vivo, and explored the antiviral actions of immunobiotic L. plantarum administered to the respiratory tract.

As a crucial component of this study, we present a novel reverse genetics system for the production of mKATE2-tagged recombinant PVM strain J3666 (rK2-PVM). This method is based on a reverse genetics system developed by Hotard and colleagues (30) for the production of recombinant RSV A-line 19F. There are several features that distinguish our system and that of Hotard and colleagues from others that have generated recombinant pneumoviruses (35, 47). Among the most prominent differences is the use of a BAC to support the virus antigenome. Similar methodologies have been used to generate recombinant Coronaviridae and Flaviviridae (reviewed in reference 48); among the advantages of this approach is the ease with which mutations can be introduced into the virus backbone via methods developed for the genetic engineering of E. coli. Another distinguishing feature of our systems is the conversion of the sequences encoding the helper plasmids to human codon preference in an effort to optimize protein expression. Lastly, the sequence encoding the fluorescent protein mKATE2 was added at the 3’ end of the PVM genome to facilitate virus detection both in vitro and in vivo. This 3’-end insertion may be responsible for (all or part of) the lower virulence of rK2-PVM.
than the parent PVM J3666 strain (Fig. 2B); as genes at the 3′ end of the virus genome are transcribed with greater frequency than those at the 5′ end (49), this insertion may lead to an overall reduction in the transcription of more distal PVM genes. However, we found that addition of mKATE2 had no impact on virus stability; there was no significant change in virus recovery, as determined by fluorescence intensity or by virion copy number, over several passages in mouse lung tissue (Fig. 2C).

As PVM is a natural rodent pathogen that promotes acute symptomatic respiratory disease in mice, our initial goal was to utilize rK2-PVM to identify and to characterize interactions of the virus with target cells in the respiratory tract. In previous studies, we demonstrated that PVM replicates in mouse macrophage cell lines and also in primary peritoneal macrophages isolated from mice and challenged ex vivo (50, 51). However, we demonstrate here for the first time that PVM infects both CD11c+ MHC-II+ DCs and CD11c+ Siglec-F+ AMs in vivo and that AMs support both active replication and production of infectious virions.

DCs have been characterized extensively as targets of respiratory virus infection, although infection is typically not productive of infectious virions (reviewed in references 43 and 52 to 55). hRSV specifically infects culture-derived monocyte-derived DCs (56, 57), as well as primary myeloid (CD11c+) and plasmacytoid (CD11c–) DCs, the former with greater efficiency (58). Human and mouse myeloid DCs are also susceptible to infection with influenza virus; infection is likewise not productive of infectious virions (59, 60) but can result in impaired antigen presentation (61).

Our findings, that recombinant PVM infects AMs in vivo and that AMs are a primary target of infection, are consistent with results of influenza virus infection studies (53, 54). In studies featuring pneumoviruses, isolated AMs from neonatal lambs support the replication of bovine RSV (62), as do AMs from hRSV-infected children (63). Adult human AMs can also be infected with hRSV ex vivo (64); this infection is productive and can generate virions in culture, although virion productivity has been described as short-lived (65).

These reports lead directly to our final observations. As shown in our earlier studies (20–22), priming of the respiratory tract with L. plantarum promotes heterologous immunity and results in full protection from the lethal sequelae of a subsequent acute respiratory virus infection in association with both profound suppression of the antiviral inflammatory response and diminished virus recovery from lung tissue. It is not immediately clear how priming with L. plantarum might alter virus kinetics (e.g., reduce the susceptibility of target cells and/or augment clearance via exogenous or endogenous means). We showed clearly that L. plantarum priming does not promote the trafficking of infected CD11c+ leukocytes to draining lymph nodes (Fig. 6F) or promote the differ-
ential survival of AMs in vivo (Fig. 6D). Considering first the case for endogenous mechanisms, several groups have shown that AMs are a major source of type I interferons (IFNs) in virus-challenged mice (66, 67). In our microarray analysis of whole lung tissue, we noted that L. plantarum versus control priming, followed by PVM infection, resulted in the differential regulation of numerous IFN-regulated genes, notably, IFN-induced GTP binding proteins Mx1 and Mx2, Stat3, and IFN regulatory proteins Irf1 and Irf7 (see Gene Expression Omnibus file GSE66721 and additional details in reference 68). Furthermore, L. plantarum priming resulted in the differential expression of transcripts encoding IFN-abR1, IFN-b1, Oas1b, and Isg15, among others, in AMs from rK2-PVM-infected mice (K. D. Dyer and H. F. Rosenberg, unpublished results). The role of type I IFNs in the limitation of virus replication in response to L. plantarum priming is currently under study. Interestingly, and despite our understanding of IFNs as potent antiviral regulators (69), L. plantarum-primed mice with deletions of genes for type I (or II) IFN receptors remain fully protected from the lethal sequelae of PVM infection (21, 69); indeed, the entire issue of distinct M1/M2 states of macrophage activation has recently undergone substantial reconsideration (72). Interestingly, human cytomegalovirus and dengue virus replicate more (as opposed to less) efficiently in alternatively activated macrophages (73, 74).

A final point to consider is the impact of L. plantarum priming on lymphocyte recruitment. We have shown previously that L. plantarum priming augments the recruitment of lymphocytes to the lungs in response to PVM infection (20); we have added to these findings, and we show here that L. plantarum priming prior to rK2-PVM infection results specifically in greater recruitment of B cells, CD4 T cells, and CD8 T cells than that observed in response to rK2-PVM infection alone (Fig. 5). An examination of this finding is particularly important, as augmented levels of T cell-mediated virus clearance might explain the differences observed between virus replication in macrophages in vivo (in the presence of T cells) and that observed when primed AMs were challenged in tissue culture (i.e., Fig. 7G and H). We have already ruled out any substantial contributions from B cells alone; specif-
mKATE2 found that simultaneous depletion of both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells during acute infection will permit a more comprehensive evaluation of this potential antiviral mechanism overall, future experiments with plantarum mediated virus clearance may not play a prominent role in the establishment findings. Although these results suggest that T-cell mediated virus clearance may not play a prominent role in the L. plantarum antiviral mechanism overall, future experiments with rK2-PVM in Rag1<sup>-/-</sup> mice that focus on virus replication in AMs and/or virus clearance.

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