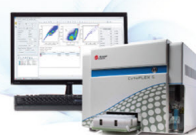


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Immunization with a Recombinant *Bacillus Calmette–Guérin* Strain Confers Protective Th1 Immunity against the Human Metapneumovirus

Christian E. Palavecino,* Pablo F. Céspedes,* Roberto S. Gómez,*[†] Alexis M. Kalergis,*^{†,‡} and Susan M. Bueno*[†]

Along with the human respiratory syncytial virus (hRSV), the human metapneumovirus (hMPV) is one of the leading causes of childhood hospitalization and a major health burden worldwide. Unfortunately, owing to an inefficient immunological memory, hMPV infection provides limited immune protection against reinfection. Furthermore, hMPV can induce an inadequate Th2 type immune response that causes severe lung inflammation, leading to airway obstruction. Similar to hRSV, it is likely that an effective clearance of hMPV would require a balanced Th1 type immunity by the host, involving the activation of IFN- γ -secreting T cells. A recognized inducer of Th1 immunity is *Mycobacterium bovis* bacillus Calmette–Guérin (BCG), which has been used in newborns for many decades and in several countries as a tuberculosis vaccine. We have previously shown that immunization with BCG strains expressing hRSV Ags can induce an efficient immune response that protects against this virus. In this study, we show that immunization with rBCG strains expressing the phosphoprotein from hMPV also can induce protective Th1 immunity. Mice immunized with rBCG were protected against weight loss, airway inflammation, and viral replication in the lungs after hMPV infection. Our rBCG vaccine also induced the activation of hMPV-specific T cells producing IFN- γ and IL-2, which could protect from hMPV infection when transferred to recipient mice. These data strongly support the notion that rBCG induces protective Th1 immunity and could be considered as an efficient vaccine against hMPV. *The Journal of Immunology*, 2014, 192: 214–223.

Human metapneumovirus (hMPV) was described for the first time in 2001 as a pathogen responsible for acute viral respiratory infections in children (1). This pathogen, classified into two main viral groups, A and B, is considered one of the most relevant agents of acute respiratory tract infections in children (1). hMPV has taken relevance as the second cause of bronchiolitis and pneumonia in young children, as well as an important emergent pathogen in both the elderly and immunocompromised patients (2). Global studies indicate that 100% of children become infected by the age of 5 (3), and that hMPV accounts for nearly 5–10% of hospitalizations in children <3 y of

age (4). Additionally, respiratory disease outbreaks of high morbidity caused by hMPV have been described in elderly patients (5). Clinical manifestations caused by hMPV are similar to other paramyxoviruses, including upper and lower respiratory tract illness. Accordingly, clinical signs of hMPV infections are not distinguishable from those caused by most closely related viruses, such as the human respiratory syncytial virus (hRSV) (4, 5).

Despite its epidemiological relevance, no vaccines are currently available for preventing hMPV-induced disease, and formalin-inactivated virus inoculations have proven to be deleterious for subsequent infections with live virus in both primates and cotton rats (6, 7). Other vaccine candidates eliciting a Th2-biased immunity have been inefficient at preventing hMPV-induced disease, despite the generation of hMPV-neutralizing Abs (8, 9). Moreover, therapies based on hMPV-neutralizing Abs do not prevent the virus infection and disease (10–12). Therefore, the generation of efficient and safe vaccines against hMPV is highly relevant. Among the potential targets for vaccine development, the attachment glycoprotein and the fusion protein, which locate in the virion surface and participate in the recognition and fusion to target cells, have been used previously to promote an Ab-based immunity (9). Also, the intracellular proteins nucleoprotein, matrix protein, the M2-1 protein, and phosphoprotein, which are well conserved between several strains of hMPV, can be good candidates to promote protective, T cell–based immunity against hMPV (13). Furthermore, the M2-1 protein, which participates in the regulation of viral transcription, has been shown to elicit a CD8⁺-restricted immunity (14–16) and therefore has been considered to be a good vaccine candidate (17).

Since its introduction in 1921, the *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) strain has been used in >1 billion humans for the prevention of tuberculosis. This vaccine induces a type 1 cell–mediated immune response (Th1) both in adults and newborns, which is characterized by the production of IFN- γ by

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Abbreviations used in this article: BAL, bronchoalveolar lavage; BCG, bacillus Calmette–Guérin; hMPV, human metapneumovirus; hRSV, human respiratory syncytial virus; N, nucleoprotein; P, phosphoprotein; qPCR, quantitative real-time PCR; RSV, respiratory syncytial virus; RT, room temperature; WT, wild-type.

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primed T cells, at least during 1 y after immunization (18–20). The recent description of a prototypical hRSV vaccine composed by RSV-recombinant strains of BCG, which confers full protection against hRSV-induced disease (21, 22), has opened new opportunities for the design of new safe and efficient vaccines for respiratory viruses, such as hMPV. In this study we have evaluated whether BCG strains expressing hMPV Ags promote a protective and safe immune response against hMPV infection in mice. Our data show that vaccination with a rBCG strain expressing the hMPV phosphoprotein (rBCG-P) was efficient at inducing protective immunity against viral infection. The BCG-based vaccine was able to prevent weight loss in infected mice, reducing hMPV RNA loads in lungs. Furthermore, this vaccine was able to prevent lung pathology as evidenced by reduced polymorphonuclear leukocyte infiltration in both the airways and lung parenchyma. Finally, we observed that protection was mediated by an hMPV-specific, Th1 T cell response, which was sufficient to prevent hMPV lung disease when transferred to recipient mice. Our data suggest that a BCG-based vaccine could be considered as an efficient prophylactic tool to protect against hMPV infection.

Materials and Methods

hMPV preparation

LLC-MK2 cells (American Type Culture Collection) were used to propagate hMPV serogroup A, clinical isolate CZ0107 (clinical isolate obtained from the Laboratorio de Infectología y Virología of the Hospital Clínico de la Pontificia Universidad Católica de Chile) as described previously (23, 24). Briefly, LLC-MK2 monolayers were grown in T75 flasks with RPMI 1640 medium, 10% FBS (Life Technologies Invitrogen, Carlsbad, CA). At 70–80% confluence, T75 flask of LLC-MK2 cells were inoculated with 2×10^5 PFU hMPV in 5 ml Opti-MEM I media supplemented with CaCl_2 (100 $\mu\text{g}/\text{ml}$), left for 1 h for virus absorption, followed by medium change, and finally cultured at 37°C either for 72 h or until cellular detachment was observed. During virus collection, cells were scrapped and the infectious media were pooled and centrifuged at $500 \times g$ for 5 min to remove cell debris. Viral titers in supernatants were quantified in 96-well plates with 4×10^4 LLC-MK2 cells per well, as described previously (24, 25). hMPV inocula were routinely evaluated for LPS and by PCR for species of Mycoplasma contamination.

Recombinant BCG generation

Total RNA from LLC-MK2 cells infected with hMPV was retrotranscribed using random primers to generate cDNA. The phosphoprotein and M2-1 genes of hMPV were PCR amplified using the following primers: for the hMPV-P gene, the primers were 5'-GAATTCATGTCATTCCCTGAAGGAAA-3' (forward) and 5'-GAATTCCTACATAATTAAGTGGTAAA-3' (reverse); for the M2-1 gene, the primers were 5'-GAATTCATGTCATTCCCTGAAGGAAA-3' (forward) and 5'-GAATTCCTACATAATTAAGTGGTAAA-3' (reverse). The PCR reaction was performed using the high-fidelity Pfx polymerase enzyme (Life Technologies) in an Axigen thermocycler. The PCR products were ligated into the cloning vector TOPO TA (Life Technologies). All primers had the EcoRI sequence (underlined) to facilitate the subcloning into the integrative mycobacterial vector pMV361 (26). This vector has the attachment sequence to integrate itself into the mycobacterial genome, and it also has a strong mycobacterial promoter where the viral genes were inserted in-frame to constitutively express the viral protein by BCG. Transformation conditions used were previously described (21, 22). Screening for expression of phosphoprotein and M2-1 protein was performed by Western blot using specific mAbs (see Fig. 1).

Lung histopathology and immunofluorescence assays

Lungs obtained from mice 6 d after hMPV infection were fixed in 4% paraformaldehyde and included in paraffin or frozen in OCT compound at -80°C . For histopathology, sections of 5 μm from paraffin-included tissue were obtained using a Thermo Scientific Microm HM 325 microtome and stained with H&E as previously described (27). For histopathology scores, five images of each animal were analyzed in a double-blinded manner and scores were recorded using the following criteria: 0, no cellular infiltration; 1, minimal cellular infiltration; 2, slight cellular infiltration; 3, moderate cellular infiltration; 4, severe cellular infiltration.

For detection of viral proteins by immunofluorescence staining, tissue sections of 7 μm from OCT frozen samples were obtained using a Leica CM1510S cryostat. The slides were fixed and permeabilized in 70% ethanol overnight at -20°C . Then, tissue sections were transferred to 100% ethanol for 30 min and dried for another 30 min at room temperature (RT). Before staining, lung sections were hydrated, starting with 95% ethanol for 30 min and transferred to 75% ethanol for 5 min, then permeabilized with 0.4% Triton X-100 in PBS for 5 min and finally rinsed twice in PBS. Sections were incubated in blocking solution (4% BSA in $1 \times$ PBS) for at least 30 min at RT. Then, slides were incubated with purified mouse monoclonal anti-nucleoprotein hMPV Ab (clone XD10.C7, provided by Dr. Pierre Pothier at the Laboratoire de Virologie, Université de Dijon). Abs were diluted 1:100 in blocking solution and incubated overnight at 4°C . Sections were then incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (1:200; Molecular Probes/Invitrogen) for 1 h at RT and washed twice with PBS. Nuclei were stained with Hoechst 33342 (5 $\mu\text{g}/\text{ml}$) for 30 min. Coverslips were mounted using DABCO immunofluorescence mounting media and examined in a Nikon C2 Si confocal microscope.

Mouse immunization and hMPV challenge

BALB/cJ mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained at the pathogen-free animal facility at the Pontificia Universidad Católica de Chile (Santiago, Chile). All animal work was performed according to institutional guidelines and supervised by a veterinarian. Doses of rBCG strains expressing the phosphoprotein or M2-1 protein from hMPV were prepared as described previously (21). Five- to 6-wk-old BALB/c mice (six to eight animals per group) received an s.c. injection in the right dorsal flank with 1×10^8 CFU BCG-wild-type (WT) or rBCG strains expressing hMPV phosphoprotein (rBCG-P-MPV) or M2-1 protein (rBCG-M2-1-hMPV). After 14 d, as indicated above, mice were boosted with the respective BCG strain. Twenty-one days after immunization, mice were anesthetized with a mixture of ketamine (20 mg/kg)/xylazine (1 mg/kg) and challenged by intranasal instillation with hMPV (1×10^6 PFU). Following vaccination and infection, body weight was recorded daily for all groups.

Flow cytometry analyses of bronchoalveolar lavages and lung homogenates

Six days after infection, mice were terminally anesthetized by i.p. injection of a mixture of ketamine (110 mg/kg)/xylazine (5 mg/kg). Bronchoalveolar lavages (BALs) were collected as previously described (21). Cells from BALs were counted in a hematology chamber and stained with anti-CD11b-FITC (BD Pharmingen, clone CBRM1.5), anti-CD11c-PE (BD Pharmingen, clone HL3), and anti-Gr-1 (Ly6G/Ly6C)-allophycocyanin (BD Pharmingen, clone RB6-8C5) Abs. For pulmonary T cell studies, the superior lobe, middle lobe, and caval lobe were rinsed in a plate with ACK buffer. Later, tissue samples were cut into small pieces and placed in a collagenase-PBS (1 mg/ml) and DNase (50 $\mu\text{g}/\text{ml}$) solution and incubated in a shaker (300 rpm) at 37°C for 15 min. Then, tissues were incubated in EDTA-PBS (5 mM) to inactivate the collagenase. Tissue samples were homogenized using two sterile slides and filtered using a 40- μm cell strainer. Cellular suspensions were centrifuged at $300 \times g$ for 5 min and pellets were washed, counted in a hematology chamber, and stained with anti-CD4 (allophycocyanin) (BD Pharmingen, clone GK1.5), anti-CD8 (PE) (BD Pharmingen, clone 53-6.7), and anti-CD3 (PerCp) Abs. Data were acquired on a FACSCanto II flow cytometer (BD Biosciences) and analyzed using FCS Express 4 software (De Novo Software).

Determination of viral load by quantitative real-time PCR

Determination of viral RNA from lung tissue was performed similarly as described before for hRSV (22). Briefly, total RNA was isolated using TRIzol reagent (Life Technologies) and 500 ng RNA was reverse transcribed to cDNA using the ImProm-II reverse transcription system kit (Promega, Madison, WI), according to the manufacturer's instructions. Primers used for hMPV-nucleoprotein (N) gene detection in quantitative real-time PCR (qPCR) reactions were 5'-ACAGCAGATTCTAAGAAA-CTCAGG-3' (forward) and 5'-TCTTTGTCTATCTCTCCACCC-3' (reverse) with an amplicon length of 153 bp. Detection of mouse β -actin was used as a housekeeping reference gene using primers 5'-AGG CAT CCT GAC CCT GAA GTA C-3' (forward) and 5'-TCT TCA TGA GGT AGT CTG TCA G-3' (reverse) with an amplicon length of 384 bp. The products were detected using Fast qPCR Master Mix (Applied Biosystem) in a StepOne Plus thermocycler (Applied Biosystems). Relative gene expression data analyses were performed using the comparative C_T method (ΔC_T method). Standard curves for qPCR were generated from increasing concentrations of the templates pTOPO-N-hMPV and pTOPO- β -actin. The C_T results were entered in the standard curve with the quantity log.

Cytokine expression by RT-qPCR

Cytokine expression in lungs of infected and uninfected mice was quantified by RT-qPCR from total RNA for IFN- γ (Mm01168134_m1), TNF- α (Mm00443260_g1), TGF- β 1 (Mm01178820_m1), IL-4 (Mm00445259_m1), IL-5 (Mm00439646_m1), and IL-10 (Mm00439614_m1) using a TaqMan gene expression assay (Applied Biosystems, Foster City, CA). The PCR reaction was prepared in a volume of 10 μ l 1 $\times$ final probe and master mix of RNA-to-Ct one-step kit. Temperature cycling and real-time fluorescence measurements were carried out with the OneStep thermocycler (Applied Biosystems) using the following cycling conditions: 1 cycle of 48°C for 15 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The relative quantitation of gene expression was done using the comparative C_T ($\Delta\Delta C_T$) method normalizing with β_2 -microglobulin (Mm00437762_m1) gene expression relative to gene expression of unimmunized, uninfected mice. Three independent biological samples were assayed in duplicate and the C_T values were averaged. The $\Delta\Delta C_T$ was calculated as the difference of the corrected C_T values (ΔC_T) of the samples and normalized using β_2 -microglobulin gene: $\Delta\Delta C_T = \Delta C_{T \text{ sample}} - \Delta C_{T \beta_2 \text{ m}}$.

Cytokine detection by ELISA

Secretion of IFN- γ , IL-4, and IL-10 was detected in both supernatant from BAL and homogenized lung from immunized and unimmunized mice using the respective ELISA kits BD OptEIA (BD Biosciences Pharmingen). The lower limit of detection was 15.6 pg/ml. To evaluate the cytokine patterns secreted by splenocytes of immunized mice, four animals were immunized with rBCG-P-hMPV or BCG-WT and 21 d after immunization the spleens were recovered and homogenized and splenocytes were incubated for 7 d with 10 μ g/ml rP-hMPV protein or with PBS as a negative control. At days 4 and 7 of incubation, the supernatants of stimulated and unstimulated cells were assayed to quantify the IFN- γ , IL-2, IL-5, and IL-4 secretion using BD OptEIA ELISA kits. Each BD OptEIA kit provides the respective recombinant proteins that allow the generation of a standard curve to quantitatively measure each cytokine.

Immunoglobulin detection by ELISA

The titers of specific anti-P-hMPV IgG were detected by ELISA in sera from immunized and unimmunized mice before and 6 d after viral challenge. A 96-well plate was activated overnight with 200 ng/well specific recombinant protein in carbonate buffer (0.1 sodium carbonate [pH 9.5]) and blocked for 1 h at RT with PBS-3% BSA. Sera from infected mice were applied to each well in serial dilutions from 1:2 to 1:512 and incubated for 1 h at RT. Several washes were performed and the specific anti-P Ig was detected with a horseradish-linked goat anti-mouse IgG. The titers in ELISA were expressed as the $\log_2$ of the reciprocal of the final dilution.

Ig titers of total IgG, IgG1, and IgG2a were measured in serum of immunized and unimmunized mice. Mice sera were collected previous to immunization (preimmune sera) at day 21 before the infection and at day 27. The isotype of Igs in sera were measured using a mouse Ig isotyping ELISA kit (BD Biosciences Pharmingen). Following the manufacturer's instructions,

96-well plates were coated overnight at 4°C with 1:5 of the respective capture rat anti-mouse isotype Ig in PBS buffer. The plates were blocked for 1 h at RT with PBS-3% BSA. Serial dilutions of sera were added to the wells and incubated for 1 h at RT. For detection of Ig, HRP-linked goat anti-mouse IgGs were added to each well and incubate for 1 h at RT. Color was developed adding substrate solution provided in the kit, and the OD was read at 450 nm. Then, the titers in ELISA were expressed as the $\log_{10}$ of the reciprocal of the final dilution. The seroconversion was determined relative to $\log_{10}$ titers of preimmune sera.

Adoptive transfer to unimmunized mice of stimulated CD4⁺ and CD8⁺ T cells from rBCG-P-hMPV immunized mice

After 7 d of stimulation with phosphoprotein of hMPV, spleen cells from rBCG-P-hMPV immunized or WT-BCG-immunized mice were purified using MACS T cell isolation kits (Miltenyi Biotec) according to the manufacturer's instructions. A total of 2×10^6 of CD4⁺, CD8⁺, or a 1:1 mixture of CD4⁺/CD8⁺ T cells was injected via caudal vein in recipient BALB/c mice. Twenty-four hours after T cell transfer recipient mice were intranasally challenged with 1×10^6 PFU hMPV. Body weight of infected mice was recorded daily until the day 6, when the mice were euthanized and BAL and lung were analyzed for signs of infection, as described above.

Results

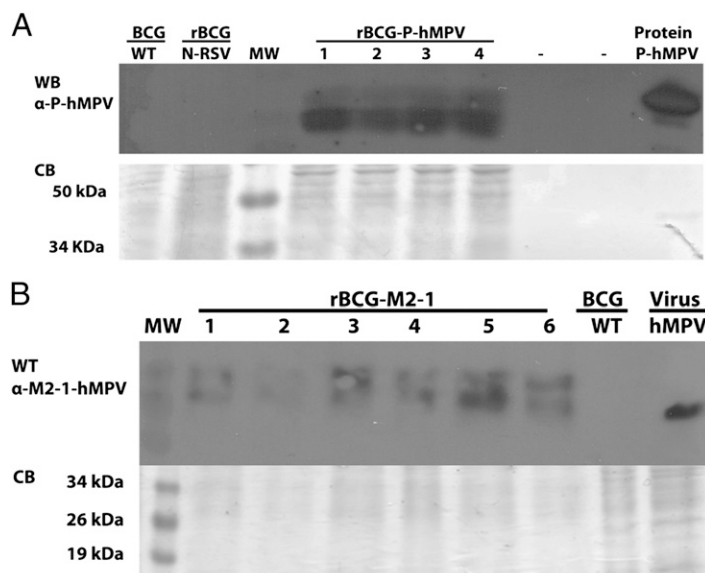
hMPV phosphoprotein and M2-1 protein are efficiently expressed by rBCG

To produce the rBCG strains expressing hMPV P and M2-1 protein, the P and M2-1 encoding genes were cloned into the integrative plasmid pMV361 (26) and transformed to a Danish BGC strain, as described in *Materials and Methods*. At least four rBCG clones were able to produce P (rBCG-P-hMPV) and five were able to express the M2-1 protein (rBCG-M2-1-hMPV), as demonstrated by Western blot (Fig. 1). Four clones of each recombinant strain were used for preliminary screening and finally one of each rBCG strain was further used to perform immunization assays in mice.

Immunization with rBCG prevents viral-induced disease in mice

To evaluate whether immunization with rBCG strains expressing hMPV proteins prevents hMPV infection, BALB/cJ mice were immunized with 1×10^8 CFU rBCG-P-hMPV or rBCG-M2-1-hMPV and control groups received 100 μ l PBS-Tween 20 at 0.05% (unimmunized) or 1×10^8 CFU WT-BCG. As additional controls, mice immunized with either viable or UV-inactivated hMPV (Live-hMPV and UV-hMPV, respectively) were included. Also, an uninfected

FIGURE 1. Expression of hMPV-P and M2-1 proteins by rBCG strains. BCG was electroporated with plasmid pMV361-P or pMV361-M2-1 and selected on solid 7H10 medium supplemented with 20 μ g/ml kanamycin. Two SDS-PAGE gels were prepared and loaded with 25 μ g whole protein preparations in each lane. Coomassie blue (CB) stained gel shows the molecular mass markers and protein loads for each strain. Expression of rhMPV proteins was assessed by Western blot analysis using mouse monoclonal antisera specific for the phosphoprotein (A) or M2-1 (B) protein. (A) Total proteins of four clones of rBCG-P were loaded in each lane. As positive controls, a recombinant phosphoprotein (P-hMPV) was included in blot. As negative control, whole proteins prepared from WT-BCG (nonrecombinant) and rBCG-N-RSV (recombinant) strain expressing the nucleoprotein of RSV were included. (B) Total protein of five clones of rBCG-M2-1-hMPV was loaded in each lane. As positive controls, viral protein extracts (virus hMPV) were included in blot. As negative control, whole protein prepared from WT-BCG (nonrecombinant) was included.



group of mice was included. Fourteen days after immunization, one single boost was performed as described in *Materials and Methods*, and 7 d later all animals (except the uninfected group) were challenged with 1×10^6 PFU hMPV. We observed that unimmunized mice started to lose weight from the first day after infection and recovered at day 6 postinfection (Fig. 2A). Similar to unimmunized mice, WT-BCG-, Live-hMPV-, and UV-hMPV-immunized mice showed weight loss close to 10% at day 2 postinfection (Fig. 2A). On the contrary, mice immunized with rBCG-P-hMPV showed no significantly loss of body weight after hMPV infection as compared with unimmunized mice ($p < 0.0001$ by two-way ANOVA; Fig. 2A). Although mice immunized with rBCG-M2-1-hMPV showed less weight loss than did unimmunized mice, they had a significant loss when compared with uninfected mice ($p = 0.029$ by two-way ANOVA).

To evaluate the severity of airway inflammation in hMPV-infected mice, the amount of infiltrating cells in BALs was evaluated. As shown in Fig. 2B, a massive infiltration of cells was detected in BALs after hMPV infection in unimmunized mice.

WT-BCG-, Live-hMPV-, and UV-hMPV-immunized mice showed similar cell infiltration in BALs than in unimmunized mice. On the contrary, mice immunized with rBCG-P-hMPV ($p < 0.0001$) and rBCG-M2-1-hMPV ($p < 0.05$) showed significantly less cell infiltration in airway as compared with unimmunized mice.

To identify the phenotype of inflammatory cells infiltrating airways after a challenge with the virus, cells from BALs were analyzed by FACS. We observed that an important proportion of infiltrating cells were GR-1⁺/CD11b⁺ (probably neutrophils) in BALs of unimmunized, WT-BCG-, Live-hMPV-, and UV-hMPV-immunized mice as compared with uninfected mice (Fig. 2C). On the contrary, rBCG-P-hMPV-immunized mice showed an amount of GR-1⁺/CD11b⁺ cells in their BALs similar to that of uninfected mice (Fig. 2C), which was significantly less than the amount of granulocytes present in BALs of unimmunized mice ($p < 0.0001$). Although the percentage of GR-1⁺/CD11b⁺ cells in BALs of rBCG-M2-1-hMPV immunized mice is apparently low, it was not significantly lower compared with unimmunized mice ($p = 0.0863$), and also it was higher than the infiltration shown by uninfected mice ($p < 0.05$).

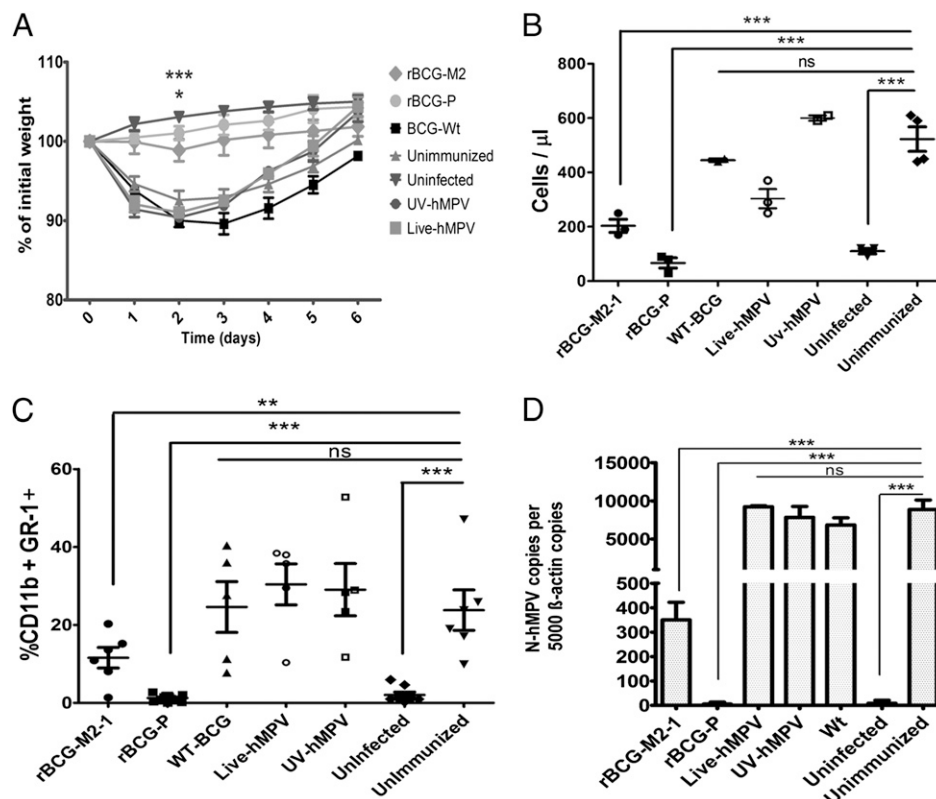


FIGURE 2. Immunization with rBCG-hMPV strains protect mice from hMPV-induced lung pathology. Groups of BALB/cJ mice (5–6 wk of age) received subdermal immunization with 1×10^8 CFU rBCG-M2-1-hMPV (rBCG-M2), rBCG-P-hMPV (rBCG-P), WT-BCG, Live-hMPV, or UV-hMPV and infected with 1×10^6 PFU hMPV. Unimmunized and uninfected mice were included as control groups. (A) Body weight loss after hMPV infection. Weight loss due to hMPV infection for rBCG-P-hMPV- or rBCG-M2-1-hMPV-immunized mice was significantly lower than for unimmunized mice ($***p < 0.0001$, $*p = 0.05$ by two-way ANOVA). Total cell count (B) and percentage of double-positive CD11b⁺/Gr1⁺ cells (C) in BALs of mice 6 d after hMPV infection. Cells recovered from BALs were counted and stained with an FITC-labeled anti-CD11b Ab and an allophycocyanin-labeled anti-Gr-1 Ab and analyzed by flow cytometry. The amount of both total cells (B) and granulocytes (C) were significantly lower in mice immunized with rBCG-P-hMPV as compared with unimmunized mice ($***p < 0.0001$ by Student *t* test between rBCG-P-hMPV-immunized mice and unimmunized mice). In contrast, the amount of total cells and granulocytes in BALs of WT-BCG-, Live-hMPV-, and UV-hMPV-immunized mice were not significantly different from unimmunized mice (not significant [ns] by Student *t* test between WT-BCG-, Live-hMPV-, and UV-hMPV-immunized mice compared with uninfected mice). Although the total cell counts of rBCG-M2-1-hMPV-vaccinated mice were significantly lower than unimmunized mice ($*p < 0.05$ by Student *t* test), granulocyte cell counts were not different to unimmunized mice (not significant [ns] by Student *t* test). (D) Total RNA from lungs of control and infected animals were obtained and reverse transcribed to quantify the number of N-hMPV copies by real-time PCR. Data are expressed as the number of N-hMPV gene copies per 5000 copies of β-actin gene (not significant [ns] by Student *t* test between WT-BCG-, Live-hMPV-, and UV-hMPV-immunized mice compared with unimmunized mice; $***p < 0.0001$ by Student *t* test for rBCG-P-immunized, rBCG-M2-1-immunized, and uninfected mice compared with unimmunized mice).

Additionally, we measured viral RNA in the lungs of mice immunized and challenged with hMPV. We observed high amounts of N-hMPV RNA in the lungs of unimmunized or WT-BCG-, Live-hMPV-, and UV-hMPV-immunized mice (Fig. 2D). On the contrary, mice immunized with rBCG-P-hMPV or rBCG-M2-1-hMPV showed reduced viral RNA loads in their lungs as compared with unimmunized animals ($p < 0.0001$). However, the rBCG-M2-1-hMPV-immunized animals showed higher amounts of viral RNA as compared with mice immunized with rBCG-P-hMPV ($p < 0.001$).

Furthermore, the presence of the N-hMPV protein in lungs of infected mice was evaluated. We did not detect N-hMPV proteins in the lungs of either uninfected mice or mice immunized with rBCG-P-hMPV (Fig. 3). On the contrary, high levels of N-hMPV protein was detected in both unimmunized mice and mice immunized with WT-BCG. However, N-hMPV protein was detected in mice immunized with rBCG-M2-1-hMPV, but the amount of fluorescence was much lower than the level observed for unimmunized or WT-BCG immunized mice (Fig. 3). These results are consistent with qPCR experiments showing that viral RNA loads in lungs were significantly reduced for rBCG-P-hMPV- and rBCG-M2-1-hMPV-immunized mice as compared with unimmunized or WT-BCG-immunized mice (Fig. 2D). These data suggest that

immunization with rBCG-P-hMPV or rBCG-M2-1-hMPV can significantly reduce lung infection by hMPV and viral-induced disease.

Immunization with rBCG-P-hMPV prevents the lung histopathology caused by hMPV

Six days after hMPV challenge, lung tissue was obtained and analyzed for histopathology. Fig. 4A shows that unimmunized, WT-BCG-immunized, and rBCG-M2-1-hMPV-immunized mice had significant inflammatory cell infiltration in alveoli and peribronchial tissues. In contrast, mice immunized with rBCG-P-hMPV showed reduced histopathology scores as evidenced by both a significantly reduced cellular infiltration in lungs after hMPV challenge and lung histopathology equivalent to uninfected mice. Histopathology scores performed in at least three independent experiments confirm these observations (Fig. 4B). These findings suggest that weight loss observed in control mice after hMPV infection might be related to inflammatory cell infiltration in the airways and lung inflammation. These observations support the notion that vaccination with rBCG-P-hMPV can prevent viral replication in lungs and prevent excessive inflammatory cell recruitment after hMPV infection. However, vaccination with

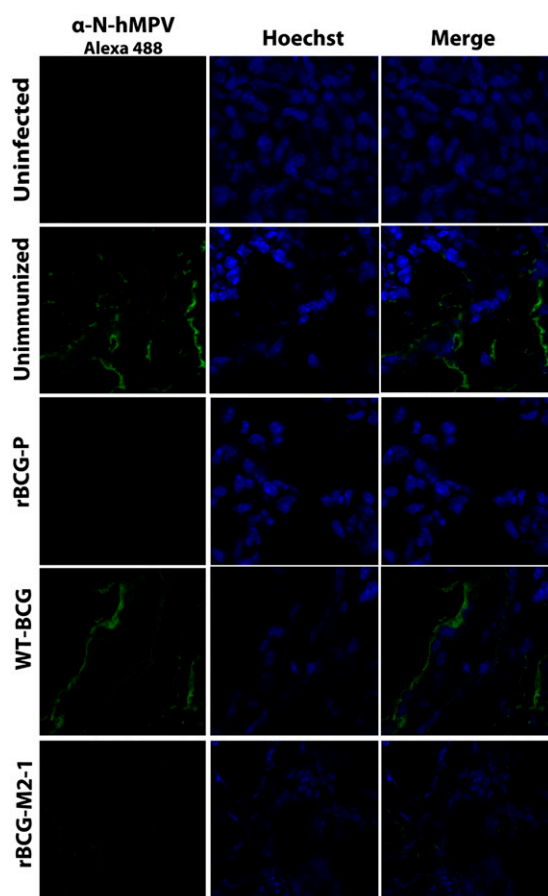


FIGURE 3. Immunization with rBCG-P-hMPV reduces the amount of viral proteins in lung tissues after hMPV infection. Six days after infection, lungs were removed, fixed on OCT at -80°C , and stained with a monoclonal anti-N-hMPV Ab and revealed with an Alexa Fluor 488 anti-mouse Ab. Fluorescence counterstaining derives from a nuclear staining with Hoechst. N-hMPV⁺ (green) staining is observed in lungs of unimmunized, rBCG-M2-1-hMPV-immunized, and WT-BCG-immunized mice. Data are shown at original magnification $\times 40$ and are representative of three to six independent experiments.

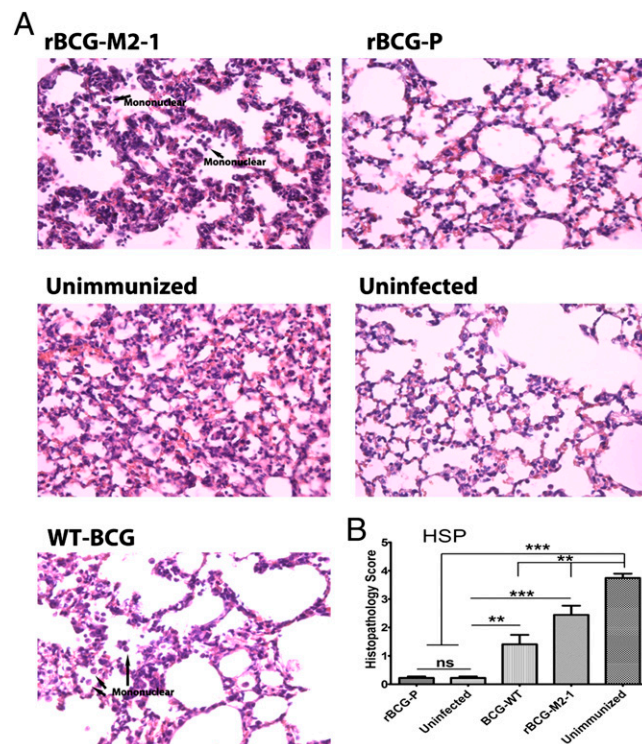


FIGURE 4. Immunization with rBCG-P-hMPV reduces histopathology damage in the lungs after hMPV infection. (A) Six days after hMPV infection, lungs were removed, fixed in paraformaldehyde, and included in paraffin, and then 5- μm cuts were stained with H&E and photographed at original magnification $\times 40$. Significant polymorphonuclear cell infiltration, perivascular lymphocytic infiltrate, and pneumonia can be observed in unimmunized and WT-BCG-immunized mice. Also, similar histopathology is observed in rBCG-M2-1-hMPV-immunized mice. No evident inflammation is observed in lungs from animals immunized with rBCG-P-hMPV. Images are representative of three to six independent experiments. (B) Histopathology score for each group was recorded as described in *Materials and Methods*. Reduced histopathology score is observed for rBCG-P-hMPV-immunized mice, similar to uninfected mice. In contrast, WT-BCG- and rBCG-M2-1-hMPV-immunized mice present a significantly higher histopathology score as compared with uninfected mice (** $p = 0.0085$, *** $p < 0.0001$ by Student t test).

rBCG-M2-1-hMPV did not reduce significantly the immunopathology of infection in the airways and therefore is a vaccine prototype less efficient than rBCG-P-hMPV. Therefore, the next set of experiments was performed only with mice immunized with the rBCG-P-hMPV strain.

rBCG-P-hMPV-immunized mice have reduced T cell infiltration in airways after hMPV infection

Next, T cell recruitment to the BALs and lungs after hMPV infection was quantified. As shown in Fig. 5, 6 d postinfection with hMPV the numbers of CD4⁺ and CD8⁺ T cells in the BALs and lungs of mice immunized with rBCG-P-hMPV were equivalent to the number of T cells found in uninfected mice. In contrast, higher amounts of both CD4⁺ and CD8⁺ T cells were found in BALs and lungs of unimmunized mice or mice immunized with WT-BCG, Live-hMPV, or UV-hMPV (Fig. 5). These results suggest that immunization with rBCG-P-hMPV could either prevent the recruitment of T cells to BALs and lungs after hMPV infection or promote an early recruitment of these cells upon hMPV infection, which left the tissue after 6 d of infection. This last hypothesis correlates with the reduced amounts of viral proteins and RNA found after 6 d postinfection in rBCG-P-hMPV-immunized mice.

Cytokine profile in airways after hMPV infection is compatible with a cellular-based immune response in rBCG-P-hMPV-immunized mice

Cytokine production on airways (BAL) and lung parenchyma of hMPV-infected mice was detected after 6 d postinfection using ELISA for BALs and qPCR for lungs. We observed low levels of IFN- γ in BALs of rBCG-P-hMPV-immunized mice after the challenge with hMPV as compared with unimmunized mice (Fig. 6A). In contrast, high levels of IFN- γ were found in BALs of WT-BCG-, Live-hMPV-, and UV-hMPV-immunized mice, which were similar to the amounts detected in unimmunized mice. Similar results were observed in lungs (Fig. 6D), where the relative amount of mRNA for this cytokine was reduced in mice immunized with rBCG-P-hMPV and in uninfected mice.

For IL-4, the amount in BALs (Fig. 6B) was reduced in all groups, as was the relative amount of mRNA for this cytokine in lungs (Fig. 6E). However, the amount of IL-10 in BALs (Fig. 6C) of mice immunized with rBCG-P-hMPV was significantly reduced as compared with unimmunized mice ($p < 0.0001$). In contrast, higher amounts of this cytokine were detected in lungs (Fig. 6F) of BCG-WT-, Live-MPV-, and UV-hMPV-immunized mice, which were not significantly different from the levels of IL-10 in BALs

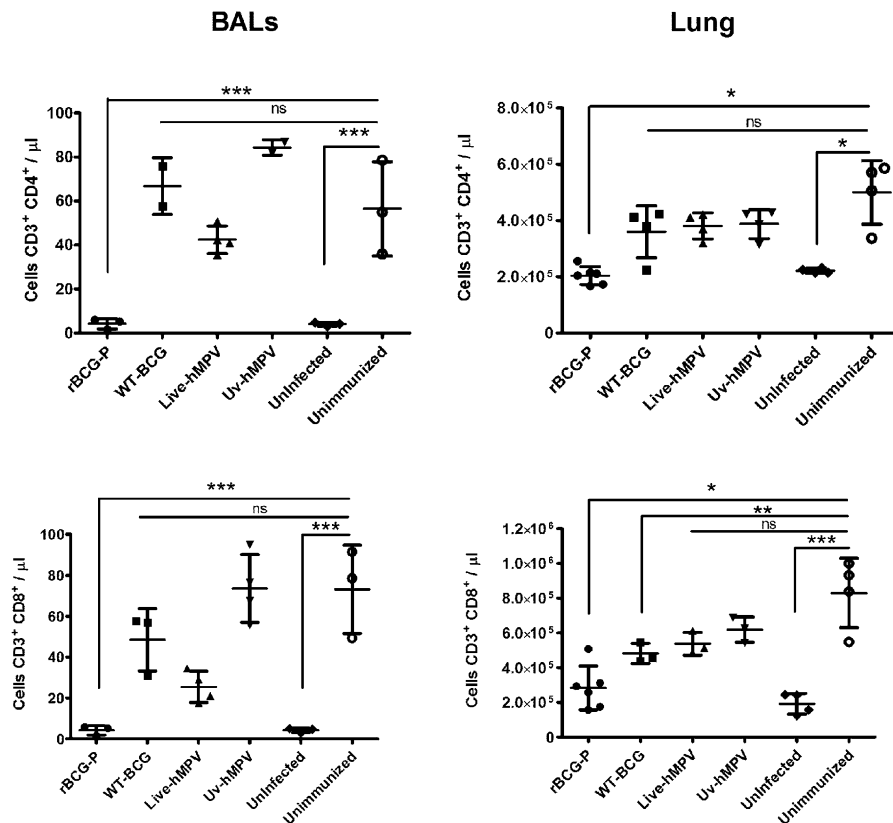


FIGURE 5. Immunization with rBCG-P-hMPV decreases the recruitment of CD4⁺ and CD8⁺ T cells in the lung upon hMPV infection. The recruitment of T cells was assessed 6 d postinfection with 1×10^6 PFU hMPV in mice previously immunized with rBCG-P-hMPV, WT-BCG, Live-hMPV, or UV-hMPV or in unimmunized BALB/cJ mice. *Upper panels*, Number of CD4⁺ T cells in BALs and lungs of immunized and challenged mice. *Lower panels*, Number of CD8⁺ T cells in BALs and lungs of immunized and challenged mice. CD4⁺ T cells were significantly lower in mice immunized with rBCG-P-hMPV and in uninfected mice as compared with unimmunized mice (*** $p < 0.0001$ by Student t test between rBCG-P-hMPV immunized or uninfected mice compared with unimmunized mice; not significant [ns] by Student t test between WT-BCG-, Live-hMPV-, or UV-hMPV-immunized mice compared with unimmunized mice). In lungs, for CD4⁺ T cells only the rBCG-P-hMPV-immunized and uninfected mice were significantly reduced compared with unimmunized mice (* $p < 0.05$ by Student t test between uninfected or rBCG-P-hMPV-immunized compared with unimmunized mice; not significant [ns] by Student t test between WT-BCG-, Live-hMPV-, or UV-hMPV-immunized mice compared with unimmunized mice). The CD8⁺ T cells were significantly reduced in lungs of mice immunized with rBCG-P-hMPV or WT-BCG and in uninfected mice as compared with unimmunized mice (*** $p < 0.0001$, ** $p < 0.001$, * $p < 0.05$ by Student t test between uninfected or animals immunized with WT-BCG or rBCG-P-hMPV, respectively, compared with unimmunized mice; not significant [ns] by Student t test between Live-hMPV- or UV-hMPV-immunized mice compared with unimmunized mice).

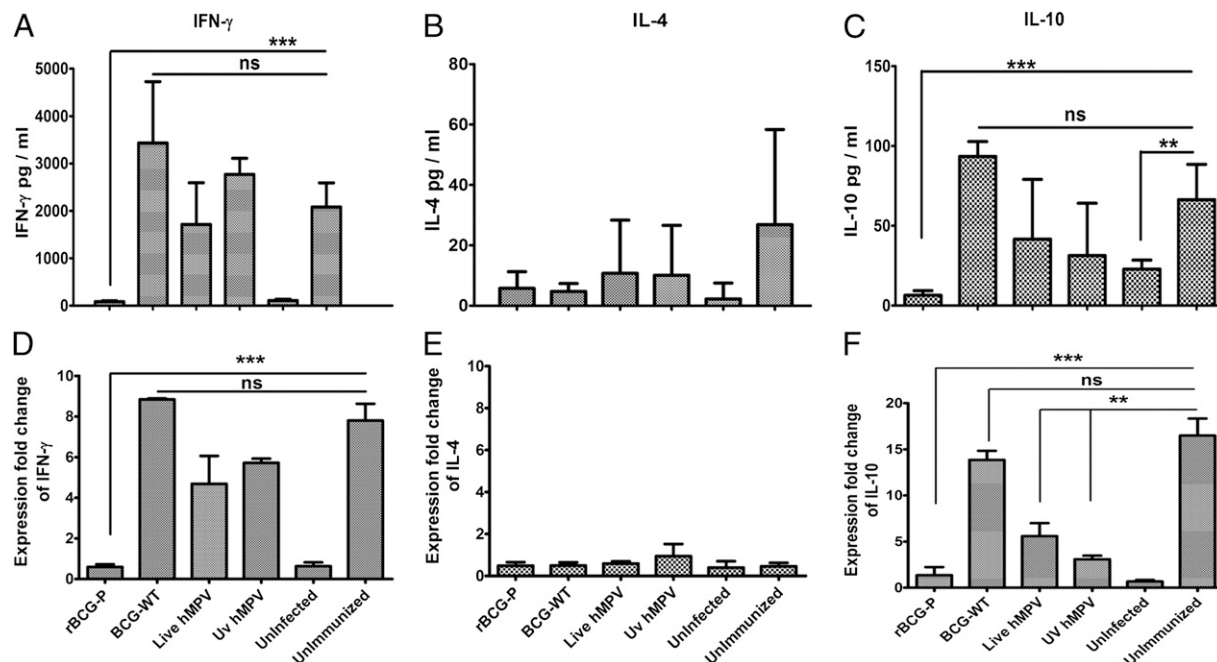


FIGURE 6. Cytokine production in airway and lung of rBCG-P-MPV-immunized mice. The production of IFN- γ , IL-4, and IL-10 was quantified by ELISA in supernatant of bronchoalveolar lavage (*upper panels*) or by qPCR (*lower panels*) in homogenate lungs of mice after 6 d of hMPV infection of mice immunized with rBCG-P-hMPV, WT-BCG, Live-hMPV, UV-hMPV, or in unimmunized mice. As a control, a group of uninfected mice was included. The levels of IFN- γ (**A, D**) and IL-10 (**C, F**) were low in airways and lungs of rBCG-P-hMPV-immunized mice. On the contrary, high levels of IFN- γ were observed in airways and lung of WT-BCG-, Live-hMPV-, and UV-hMPV-immunized mice. No differences were observed for production of IL-4 (**B, E**) either in BAL or lungs of any experimental group (not significant [ns], *** p < 0.0001, ** p < 0.001, * p < 0.05 by Student t test compared with unimmunized mice).

of unimmunized mice. Quantitative PCR assays in lung tissues to detect this cytokine supported the observations made in BALs.

To further explore the cytokine profiles of all experimental groups, the relative expression of TNF- α (Supplemental Fig. 1A), TGF- β 1 (Supplemental Fig. 1B), and IL-5 (Supplemental Fig. 1C) was evaluated by qPCR in lungs of immunized and infected animals. The expression levels of these cytokines were similar between mice immunized with rBCG-P-hMPV and in uninfected mice. One interesting observation is that only the group of mice immunized with UV-hMPV showed a significantly higher expression of IL-5 (Supplemental Fig. 1C) after hMPV infection, supporting the notion that immunization with inactivated hMPV induces a strong Th2 type immune response (6, 7). Collectively, the cytokine profile observed in BALs and lungs of rBCG-P-hMPV-immunized mice upon infection with hMPV is compatible with a Th1 cellular immune response. These results also correlate with the amount of T cells infiltrating lungs (Fig. 5). Again, it is possible that the protective, Th1-based cellular response against hMPV promoted by the immunization with rBCG-P-hMPV takes place early after infection and, after 6 d, most T cells producing Th1 cytokines left the lungs due to an efficient and early clearance of the infecting virus.

Immunization with rBCG-P-hMPV promotes anti-P-hMPV IgG Abs upon hMPV challenge

To further gain insights into the nature of the immune response elicited by rBCG-P-hMPV, seroconversion of specific Abs for hMPV phosphoprotein was determined in immunized and unimmunized mice. Reduced levels of anti-phosphoprotein Abs were detected in rBCG-P-hMPV-immunized mice before infection (1:16), which significantly increased to 1:128 after hMPV infection (compare Supplemental Fig. 2A with 2B). Similar to unimmunized mice, a reduced seroconversion was observed in WT-BCG im-

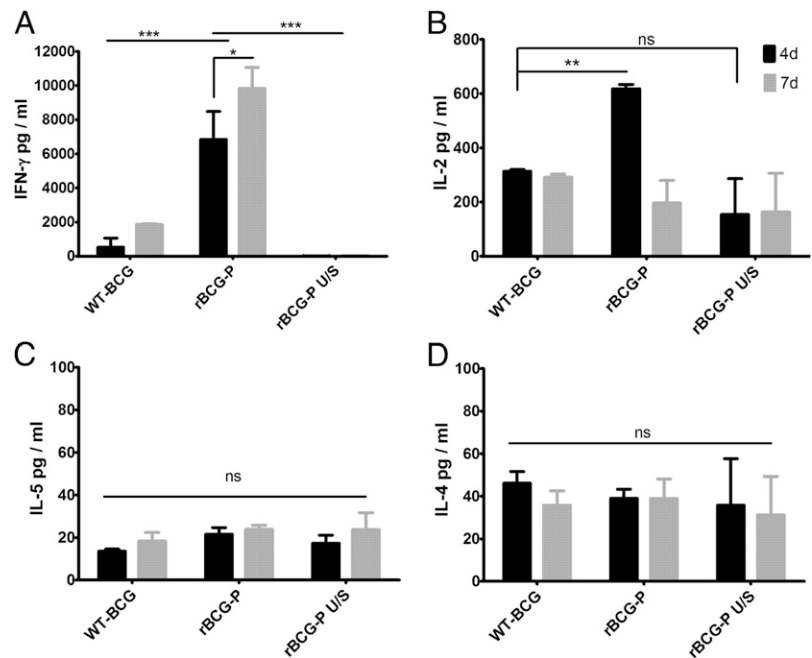
nized mice after hMPV infection. Higher titers of anti P-hMPV were observed in Live-hMPV and UV-hMPV before and after infection with hMPV (compare Supplemental Fig. 2A with 2B).

We also determined the Th1/Th2 Ig profile of immunized and unimmunized mice. The seroconversion of IgG isotype was expressed as reciprocal OD₄₅₀ relative to preimmune sera of each mouse. Two-fold seroconversion was observed in all groups compared with uninfected mice (p < 0.0001 and p < 0.005). As shown in Supplemental Fig. 2, no significant difference was observed in IgG1 (Supplemental Fig. 2C), IgG2a (Supplemental Fig. 2D), or total IgG (Supplemental Fig. 2E) titers when comparing pre-infected and postinfected mice sera in all groups. Furthermore, no differences in the magnitude of seroconversion were observed in all groups.

T cell transfer from rBCG-P-hMPV-immunized mice prevents hMPV-induced disease

To evaluate whether T cells in rBCG-P-hMPV-immunized mice mediated protection against hMPV, splenocytes were obtained from immunized mice and treated *in vitro* with 10 μ g/ml rhMPV-P protein to induce activation of Ag-specific T cells. At days 4 and 7 of culture, secretion of IL-2 and IFN- γ was measured as a parameter of T cell activation. As shown in Fig. 7, significant increased secretion of IFN- γ (Fig. 7A) and IL-2 (Fig. 7B) in response to stimulation with hMPV-P protein could only be detected in cells derived from rBCG-P-hMPV-immunized mice as compared with unstimulated splenocytes (rBCG-P-U/S). Low secretion of these cytokines was observed also in cells obtained from WT-BCG-immunized mice. Additionally, no significant increment or secretion of IL-5 (Fig. 7C) and IL-4 (Fig. 7D) could be detected for splenocytes in response to phosphoprotein as compared unstimulated splenocytes, which is in agreement with the cytokine profile previously described in the lungs of rBCG-P-hMPV-immunized

FIGURE 7. Spleen cells of rBCG-P-hMPV-immunized BALB/cJ mice secrete Th1 cytokines. Spleens of WT-BCG- or rBCG-P-hMPV-immunized BALB/cJ mice were removed and cells were incubated with 10 μ g purified rhMPV-P protein or PBS during 7 d. The supernatant of stimulated and unstimulated rBCG-P-hMPV and WT-BCG spleen cells were assayed for the presence of (A) IFN- γ , (B) IL-2, (C) IL-5, and (D) IL-4 cytokines at 4 and 7 d of culture. Stimulated spleen cells derived from rBCG-P-hMPV secrete significantly higher amounts of IFN- γ as compared with WT-BCG or unstimulated rBCG-P-hMPV spleen cells ($***p < 0.001$ by Student *t* test). Also, the amounts of IFN- γ increased between days 4 and 7 ($*p < 0.05$ by Student *t* test). The stimulated spleen cells from rBCG-P-hMPV-immunized mice secreted a significantly higher amount of IL-2 at day 4, which decreased to basal level at day 7, as compared with unstimulated cells ($**p < 0.001$, not significant [ns] by Student *t* test). There were no significant increments of IL-4 and IL-5 secretion in spleen cells from rBCG-P-hMPV-immunized compared with unstimulated rBCG-P-hMPV spleen cells and also not in WT-BCG (not significant [ns] by Student *t* test).



mice. These data suggest that P-hMPV-specific T cells from rBCG-P-hMPV-immunized mice show a Th1-type pattern of cytokine secretion.

Furthermore, to evaluate whether P-hMPV-specific Th1 cells have the capacity to confer protection against hMPV infection, purified CD4⁺ and CD8⁺ T cells were obtained from either rBCG-P-hMPV- or WT-BCG-immunized mice and adoptively transferred to unimmunized BALB/c mice after 7 d of stimulation with recombinant phosphoprotein. Unimmunized mice received individual (either CD4⁺ or CD8⁺) or simultaneous (CD4⁺ plus CD8⁺) T cell subsets though i.v. injection. Twenty-four hours after T cell transfer, recipient mice were challenged with hMPV and weight loss was recorded during 6 d. As shown in Fig. 8A, mice that received T cells from rBCG-P-hMPV-immunized mice showed no weight loss after infection as compared with unimmunized mice ($p < 0.0001$). In contrast, mice that received T cells from WT-BCG-immunized mice showed an equivalent body weight loss as compared with unimmunized mice (Fig. 8B). Consistently, compared with uninfected mice, a reduced infiltration of inflammatory cells was observed at 6 d postinfection in mice transferred with T cells from rBCG-P-hMPV-immunized mice ($p < 0.0001$; Fig. 8C). In contrast, significant infiltration was observed in the airways of mice receiving T cells from WT-BCG-immunized mice, similar to what was observed in unimmunized mice. These data are consistent with the high viral loads shown by mice receiving T cells from WT-BCG-immunized mice (Fig. 8D). On the contrary, when compared with unimmunized mice, viral loads were significantly lower in mice transferred with T cells from rBCG-P-hMPV-immunized mice ($p < 0.0001$). These results suggest that either CD4⁺ or CD8⁺ T cells from mice immunized with rBCG-P-hMPV are sufficient to protect against hMPV infection.

Discussion

From its discovery in 2001 (1), hMPV has been associated with an increasing proportion of respiratory illnesses of upper and lower airways of children and the elderly (28). We have previously shown that rBCG strains expressing hRSV Ags can elicit a protective

immune response against this paramyxovirus. Such a recombinant vaccine was capable of significantly reducing the clinical manifestations and inflammatory damage caused by hRSV infection in BALB/cJ mice (21, 22). In this study, we show that mice immunized with a novel rBCG strain expressing the hMPV phosphoprotein (rBCG-P-hMPV) have a protective T cell immunity against hMPV to a similar extent to what was previously described for hRSV. The specificity and efficacy of the rBCG-P-hMPV vaccine was supported by its capacity to restrain viral dissemination in the infected tissue and control lung inflammation following hMPV challenge. Furthermore, transfer of hMPV-P-specific T cells led to virus clearance and disease prevention on recipient animals. In contrast, immunization with WT-BCG failed to prevent disease development or to expand hMPV-specific T cells able to prevent disease development on recipient animals, demonstrating that the expression of hMPV Ags by BCG is required to generate virus immunity. The mechanism of protection seems to rely on the induction of specific Th1 cells able to secrete IFN- γ in response to the phosphoprotein. Moreover, owing to reduced seroconversion before hMPV challenge, which did not differ significantly from controls, our results suggest that rBCG-elicited immune responses must be mainly T cell-dependent, which can promote B cell activation after hMPV challenge. Importantly, given the detection of IL-10 and TGF- β 1 in the lung parenchyma of rBCG-P-hMPV-immunized mice, this response seems to be accompanied by a still unidentified regulatory subset of immune cells, most likely regulatory T cells or tolerogenic dendritic cells. Such a balanced hMPV-specific Th1-type immune response was capable of promoting the clearance of viral infection without excessive or damaging inflammation of the infected airways. In contrast, vaccination of mice with either infectious or UV-inactivated hMPV resulted in significant hMPV-induced disease.

Previous studies have shown that hMPV-immunized mice can undergo enhanced inflammatory infiltration in airways as compared with unimmunized animals, due to a potentially adverse Th2 immune response promoted by hMPV. Therefore, a strain of BCG expressing the hMPV-P protein could be considered an efficient candidate vaccine to prevent hMPV infection in young children. A

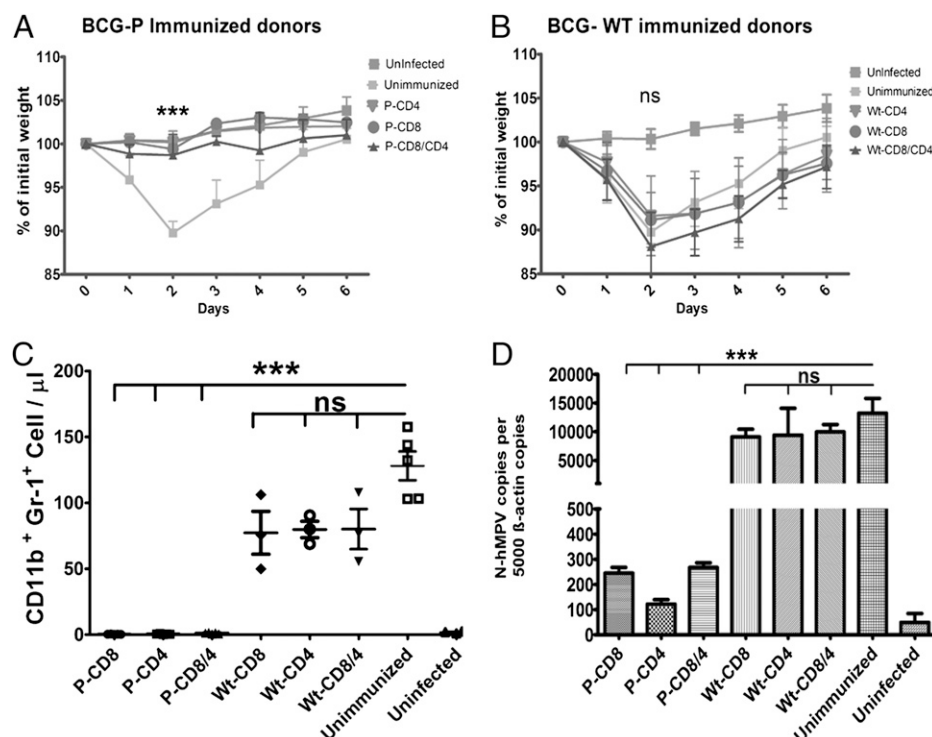


FIGURE 8. T cells from rBCG-P-MPV-immunized mice are sufficient to promote immunity to hMPV infection. CD4⁺ and CD8⁺ T cells were purified from stimulated spleen cells of WT-BCG- or rBCG-P-hMPV-immunized mice. Then, 2 million purified T cells from WT-BCG- or rBCG-P-hMPV-immunized mice were injected via caudal vein to recipient BALB/cJ mice. Twenty-four hours after transfer, mice were intranasally challenged with 1×10^6 PFU hMPV. **(A)** Body weight loss of mice transferred with rBCG-P-hMPV T cells. Mice transferred with either CD4⁺ or CD8⁺ or both T cells from rBCG-P-hMPV-immunized mice showed no body weight loss as compared with unimmunized mice (***p < 0.0001 by two-way ANOVA). **(B)** Body weight loss of WT-BCG transferred mice after hMPV infection. Unimmunized mice transferred with WT-BCG T cells lost body weight comparable to unimmunized mice (not significant [ns] by two-way ANOVA). **(C)** BAL from unimmunized mice transferred with rBCG-P-MPV T cells. Graphs show significantly reduced infiltration of granulocytes in mice that received T cells from rBCG-P-hMPV-immunized mice as compared with unimmunized mice or mice transferred with T cells from WT-BCG immunized mice (***p < 0.0001 by Student *t* test). The cellular number of infiltrated granulocytes in BALs from WT-BCG transferred mice show no significant differences with infiltration of unimmunized mice (not significant [ns] by Student *t* test). **(D)** Viral loads were quantified as copies of the N-hMPV gene by RT-qPCR as described above. The viral loads in lungs of mice transferred with T cells from rBCG-P-hMPV-immunized mice were significantly lower compared with unimmunized mice (***p < 0.0001). In contrast, the WT-BCG transferred mice showed no significant difference in viral load compared with unimmunized mice (not significant [ns], ***p < 0.0001 by Student *t* test).

Th1-balanced immune response seems to be a direct consequence of using BCG as vector to express the hMPV Ag. Indeed, it is thought that the BCG lipoarabinomannan and lipomannan molecules can promote the differentiation of naive CD4⁺ T cell into Th1 cells (29). Additionally, a recent study has demonstrated Th1 polarization capacity when T cell epitopes are expressed by rBCG (30).

The observed reduction of disease manifestations, such as weight loss or recruitment of inflammatory cells to the airways, in rBCG-P-hMPV-immunized animals is consistent with the reduction of CD4⁺ and CD8⁺ lymphocyte infiltration in pulmonary parenchyma. Because inflammation largely influences the outcome of cellular immune responses, either by promoting the infiltration of migrating T cells into sites of infection or by the direct secretion of antiviral cytokines, the reduced infiltration of T cells at the time of analysis may be due to a swift kinetic of T cell response and disease resolution in lungs. Accordingly, we observed strong IFN- γ and IL-2 secretion in response to hMPV phosphoprotein after in vitro restimulation of splenocytes of rBCG-P-vaccinated mice, which is consistent with a T cell memory response and Th1 polarization. Such a pattern of cytokine secretion was hMPV Ag-specific, as splenocytes with no stimulation failed to secrete IFN- γ and IL-2. More importantly, as evidenced by adoptive transfer experiments, rBCG-P-hMPV was able to confer strong effector phenotypes to both CD4⁺ and CD8⁺ T cells, which were able to

prevent disease in unimmunized BALB/cJ mice, which showed protective hMPV immunity equivalent to actively immunized animals.

BCG has been applied for several decades as a vaccine against tuberculosis (31, 32) and, more recently, it has been used successfully as a carrier to promote a Th1 immune response against Ags from other bacterial, parasitic, and viral pathogens, such as *Bordetella pertussis* (33), HIV (34), and *Plasmodium falciparum* (35). Also, the effect of the BCG itself at improving protection in respiratory virus infection has been observed (36). Therefore, given the potential uses of BCG as vaccine vector for a number of pathogens, the vaccine described in this study could be a promising prophylactic tool to prevent and control hMPV disease burden.

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Disclosures

A patent for the BCG-hMPV vaccine has been filed by S.M.B., A.M.K., and C.E.P. The other authors have no financial conflicts of interest.

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