

Protective T cell immunity against respiratory syncytial virus is efficiently induced by recombinant BCG

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Respiratory syncytial virus (RSV) is one of the leading causes of childhood hospitalization and a major health burden worldwide. Unfortunately, because of an inefficient immunological memory, RSV infection provides limited immune protection against reinfection. Furthermore, RSV can induce an inadequate Th2-type immune response that causes severe respiratory tract inflammation and obstruction. It is thought that effective RSV clearance requires the induction of balanced Th1-type immunity, involving the activation of IFN- γ -secreting cytotoxic T cells. A recognized inducer of Th1 immunity is *Mycobacterium bovis* bacillus Calmette–Guérin (BCG), which has been used in newborns for decades in several countries as a tuberculosis vaccine. Here, we show that immunization with recombinant BCG strains expressing RSV antigens promotes protective Th1-type immunity against RSV in mice. Activation of RSV-specific T cells producing IFN- γ and IL-2 was efficiently obtained after immunization with recombinant BCG. This type of T cell immunity was protective against RSV challenge and caused a significant reduction of inflammatory cell infiltration in the airways. Furthermore, mice immunized with recombinant BCG showed no weight loss and reduced lung viral loads. These data strongly support recombinant BCG as an efficient vaccine against RSV because of its capacity to promote protective Th1 immunity.

Th1 cell response | RSV | immunopathology | T cell immunity

Respiratory syncytial virus (RSV) is an enveloped, negative, single-stranded RNA virus belonging to the Paramyxoviridae family with a genome that encodes for 11 proteins (1). This virus is the leading cause of viral bronchiolitis and pneumonia worldwide, infecting more than 70% of children in the first year of life and nearly 100% of children by age 2 years (2). Despite being highly infectious, RSV does not induce an effective immunological memory, and repeated infections are therefore very frequent (3, 4). Although symptoms associated with RSV infection usually manifest as rhinitis in adults, in premature infants, the elderly, and immunosuppressed individuals RSV infection frequently leads to severe symptoms and airway obstruction (5, 6). Furthermore, it has been proposed that exposure to RSV infection early in life can lead to increased susceptibility to recurrent allergic wheezing and asthma during the following years (7). Considering epidemiological data, RSV is responsible for a health problem that is extremely expensive for individuals, governments, and health care systems. Unfortunately, to date there are no commercially available vaccines against this pathogen. Vaccine trials for RSV were first carried out with a formalin-inactivated RSV formulation (FI-RSV) in the mid-1960s (8). However, vaccinated children experienced exacerbated pulmonary disease and required hospitalization upon subsequent RSV infection, whereas nonvaccinated control children experienced significantly milder symptoms (8, 9). The failure of FI-RSV remained unexplained for at least 2 decades, primarily because of the poor understanding of the immune responses triggered by RSV

infection. However, recent studies have suggested that the FI-RSV vaccine failed because of the fact that it promoted an allergic-like Th2 immune response against the virus (10–12). This particular Th2-type response is characterized by the activation and proliferation of CD4⁺ T cells that secrete a pattern of cytokines that promote increased and accelerated infiltration of eosinophils and neutrophils into the lung tissues. Furthermore, this allergic-like cellular environment dampens CD8⁺ cytotoxic T cell activation and effector functions, such as the secretion of IFN- γ (13). As a result, clearance of RSV is delayed, lung damage is induced, and virus dissemination is promoted.

Over the last few years, several experimental approaches aimed at developing an effective vaccine against RSV have been designed and assessed, such as attenuated RSV particles (14), recombinant viruses (different from RSV) that express RSV antigens (15–17), purified RSV proteins administered with bacterial adjuvants (17, 18), RSV proteins packed as immune stimulating complexes (19), and RSV sequence peptides applied together with adjuvants (20). Although several RSV vaccine candidates may currently be at the end of their corresponding clinical trials around the world, most of these approaches unfortunately promise to be expensive to the point of being unaffordable for middle/low socioeconomic groups. Alternatively, the use of recombinant bacteria for RSV antigens as candidate vaccines against this virus has not been evaluated.

Mycobacterium bovis bacillus Calmette–Guérin (BCG) is currently used worldwide as a vaccine against tuberculosis and has been used by more than 1 billion humans since its introduction in 1921. In both adults and newborns, BCG induces cell-mediated immune responses and Th1 cytokines that persist for at least 1 year after vaccination (21–23). Because of the fact that BCG vaccination has been shown to be safe in newborns, infants, and adults, this bacterium arises as an attractive vaccine vector candidate for recombinant antigens. Indeed, several studies have shown that recombinant BCG strains efficiently induce Th1 responses against several pathogens and diseases (24–32).

Here, we have evaluated whether BCG strains expressing RSV antigens promote a protective immune response against RSV infection in mice. Our data show that vaccination with recombinant

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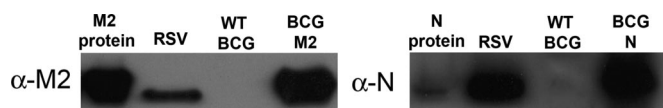
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BCG strains expressing RSV proteins N or M2 induces protective immunity against viral infection. The BCG-based vaccine was able to reduce weight loss, lung viral protein loads, and polymorphonuclear cell infiltration in the airways of RSV-challenged mice. This protective response is consistent with increased secretion of IFN- γ and IL-2 by T cells stimulated in vitro with RSV proteins. In addition, adoptive transfer of T cells obtained from mice vaccinated with recombinant BCG can protect naive mice from RSV challenge. In summary, our results suggest that recombinant BCG expressing RSV antigens can be used as a safe and protective vaccine against virulent RSV by inducing an efficient Th1-polarized, T cell-mediated specific immunity.

Results

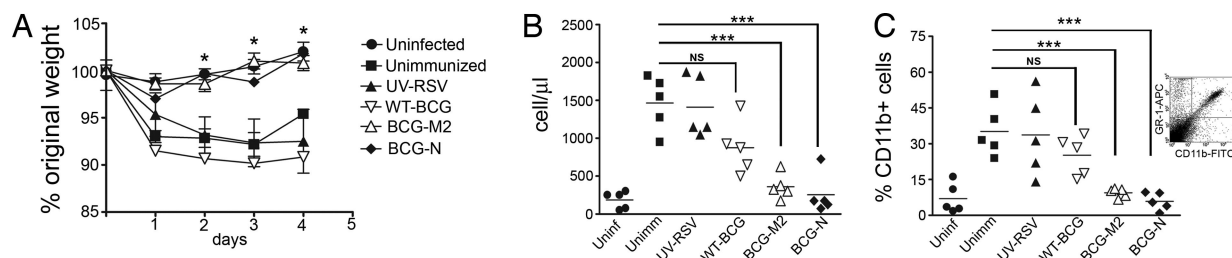
Immunization with Recombinant BCG Reduces the Severity of RSV-Induced Disease. Genes coding for RSV-N and RSV-M2 proteins were cloned into the integrative plasmid pMV361 (24) to produce the recombinant BCG-N and BCG-M2 strains. These antigens were chosen based on their previously reported capacity to induce RSV-specific cytotoxic CD8⁺ T cell responses (33, 34). Here, we tested whether expression of these antigens in BCG could promote protective T cell immunity against RSV.

As shown in Fig. 1, N and M2 proteins were efficiently expressed by both recombinant BCG strains. Quantitative Western blot assay revealed that these recombinant BCG strains expressed ≈ 2.3 ng/10⁶ cfu N protein and 5.7 ng/10⁶ cfu M2 protein (data not shown).

To evaluate whether recombinant BCG strains can protect animals from virulent RSV challenge, 4- to 6-week-old BALB/c mice were immunized with 10^8 cfu of either BCG-N or BCG-M2 using a single subdermal injection in the dorsal flank. Control groups received PBS-Tween 0.02% (unimmunized), 1×10^7 pfu of

UV-inactivated RSV (UV-RSV), 10^8 cfu of either nonrecombinant BCG (WT-BCG) or ovalbumin-recombinant BCG (BCG-OVA). Twenty-one days after immunization, all animals were challenged with 1×10^7 pfu of virulent RSV, and body weight was determined daily for 4 days after infection. As shown in Fig. 2*A*, significant weight loss was observed for unimmunized animals or mice immunized either with UV-RSV or WT-BCG, as well as BCG-OVA (data not shown). These data are consistent with previous studies indicating that naive mice challenged with RSV can show significant weight loss as early as 24 h after infection (35–37). In sharp contrast, mice immunized with either BCG-N or BCG-M2 showed no significant weight loss after RSV challenge, similarly to uninfected mice (Fig. 2*A*). These observations were confirmed when disease severity was evaluated using computerized axial tomography (CAT) scan. Signs of pulmonary inflammation could be observed in unimmunized animals 3 days after RSV challenge as pneumonia foci on CAT scan images [supporting information (SI) Fig. S1 and Movie S1]. In contrast, mice immunized with recombinant BCG expressing RSV antigens showed neither pneumonia foci nor lung inflammation after RSV challenge, and their pulmonary parenchyma was similar to that of uninfected mice (Fig. S1 and Movies S1–S3). These data suggest that vaccination with BCG-N or BCG-M2 can significantly reduce RSV-related disease symptoms.

Immunization with Recombinant BCG Prevents Recruitment of Inflammatory Cells into the Airways. Consistent with the data shown above, RSV challenge caused massive infiltration of inflammatory cells into the airways of unimmunized mice, as well as in mice immunized either with WT-BCG, UV-RSV (Fig. 2*B*), or BCG-OVA (data not shown). Flow cytometry revealed that most infiltrating cells were positive for the surface marker CD11b (Fig. 2*C*). Because CD11b-positive cells in bronchoalveolar lavages (BALs) were also positive for Ly-6G/Ly-6C marker (GR1) (Fig. 2*C Inset*), it is likely that most of the infiltrating cells were neutrophils. Cytospin preparation of BAL fluid after RSV challenge confirmed substantial infiltration of neutrophils in the airways of unimmunized mice and mice immunized with WT-BCG or UV-RSV (Fig. 3), as well as mice immunized with BCG-OVA (data not shown). Contrarily, after RSV challenge no significant recruitment of CD11b⁺ cells to the airways was observed for mice immunized either with BCG-N or BCG-M2 recombinant strains (Fig. 2*C*). In addition, immunization with recombinant BCG strains also prevented RSV-induced neutrophil invasion of BALs (Fig. 3). These results suggest that vaccination with BCG-N or BCG-M2 can significantly reduce the recruitment of inflammatory cells to the airways after RSV infection.



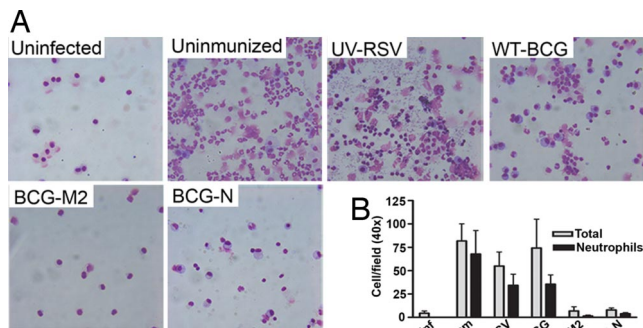


Fig. 3. Immunization with BCG-M2 or BCG-N prevents airway infiltration by neutrophils after RSV infection. (A) Four days after RSV challenge, BALs obtained from unimmunized or immunized mice with BCG-M2, BCG-N, WT-BCG, or UV-RSV were spun on glass slides, stained with May-Grunwald and Giemsa, and observed under a light microscope at 40 $\times$ magnification. As a control, BALs from uninfected mice were included. (B) Graph shows the amount of total cells (gray) and neutrophils (black) per field, as quantified by light microscopy at 40 $\times$ magnification in at least 6 random fields per sample. Bars represent means $\pm$ SE.

Immunization with Recombinant BCG Prevents Histopathology and Viral Load in Lungs of RSV-Infected Animals. Five days after RSV challenge, lung tissue from unimmunized or WT-BCG-immunized mice showed an elevated pulmonary histopathology score (HPS, Fig. 4B), as evidenced by significant inflammatory cell infiltration in alveoli and peribronchial tissues (Fig. 4A). Interestingly, mice immunized with UV-RSV and challenged with RSV showed increased lung inflammation, as evidenced by higher infiltration of inflammatory cells in alveoli, bronchioles and peribronchiolar tissues (Fig. 4). Consistent with the massive infiltration of inflammatory cells, increased eosinophil infiltration (Fig. S2) and mieloperoxidase activity (data not shown) was observed in the lung tissue of unimmunized and UV-RSV-immunized mice. These findings suggest that the pronounced weight loss observed in control mice after RSV infection is related to neutrophil infiltration in the airways and lung inflammation. In contrast, mice immunized with BCG-N or BCG-M2 showed reduced HPS (Fig. 4B), as evidenced by significantly less cellular infiltration in the lungs in response to RSV challenge and lung histology equivalent to uninfected mice (Fig. 4A). Consistently, no significant eosinophil infiltration in lung tissue was observed for mice immunized either with BCG-M2 or BCG-N (Fig. S2).

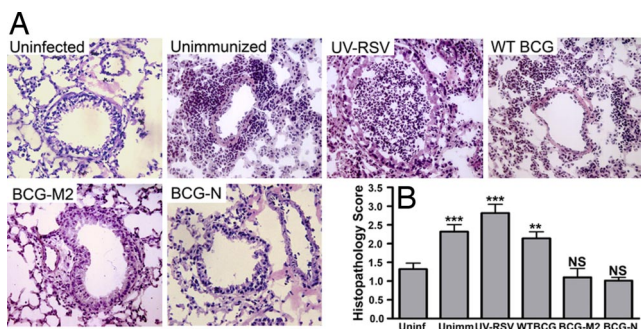


Fig. 4. Immunization with BCG-M2 and BCG-N reduces inflammatory cell infiltration into the lungs after RSV infection. (A) Four days after RSV infection, mouse lungs were removed and fixed with paraformaldehyde, and 5- μ m cuts were stained with hematoxylin/eosin. Significant polymorphonuclear cell infiltration can be observed in unimmunized, UV-RSV, and WT-BCG-immunized mice. Photos are representative of 3 to 6 independent experiments. (B) HPS for each group: 0, no cellular infiltration; 1, minimal cellular infiltration; 2, slight cellular infiltration; 3, moderate cellular infiltration; 4, severe cellular infiltration, as described in *Methods* (NS indicates nonsignificant; **, $P < 0.0036$, ***, $P < 0.0001$, Student's t tests between uninfected and each individual group).

Presence of viral proteins in lung tissues after RSV challenge was determined by immunohistochemistry and immunofluorescence using an HRP-labeled IgG RSV-specific antiserum and a biotin-labeled anti-RSV-F antibody, respectively. As shown in Fig. 5A, positive staining for RSV proteins could be detected in respiratory epithelia of unimmunized, UV-RSV- and WT-BCG-immunized mice. These data suggest that RSV replication takes place in lungs of control mice after viral challenge. On the contrary, RSV proteins could not be detected in the respiratory epithelia of mice immunized with recombinant strains of BCG-N or BCG-M2 (Fig. 5A). These results are consistent with quantitative real-time PCR data showing that viral RNA loads in lungs were reduced significantly only in BCG-N-immunized and BCG-M2-immunized mice, compared with unimmunized, WT-BCG or UV-RSV-immunized mice (Fig. 5B). These observations support the notion that vaccination with BCG-N or BCG-M2 can induce protective immunity in mice, reducing viral loads in lungs and preventing excessive inflammatory cell recruitment after RSV infection.

Mice Immunized with Recombinant BCG Exhibit RSV-Specific T Cells That Secrete Th1 Cytokines

To evaluate whether humoral or cellular adaptive immunity is induced by BCG-N and BCG-M2, IgG titers specific for N or M2 were determined at 7, 14, and 21 days after immunization. No significant increase in anti-N or anti-M2 IgG serum titers could be observed for any of the groups of immunized animals, compared with the unimmunized controls (data not shown). These data suggest that a T cell immune response rather than a humoral response might preferentially be induced by immunization with recombinant BCG. To evaluate this notion, cytokine secretion was determined for T cells obtained from spleens 21 days after immunization in response to antigenic stimulation. Cells were restimulated in vitro for 5 days with recombinant N or M2 proteins, and IFN- γ , IL-2, and IL-4 were measured in culture supernatants. As shown in Fig. 6A, spleen cell suspensions obtained from mice immunized with BCG-N secreted considerable amounts of IFN- γ and IL-2 only in response to N protein. Similarly, cell suspensions obtained from mice vaccinated with BCG-M2 secreted significant amounts of IFN- γ and IL-2 only when stimulated with M2 protein. In contrast, IFN- γ and IL-2 were not detected in the supernatant of cells derived from unimmunized mice or mice immunized with UV-RSV or BCG-WT (Fig. 6A). Although only a modest increase in IL-4 secretion could be detected for T cells obtained from mice immunized with UV-RSV (Fig. 6A), no significant secretion of any Th2-type cytokines, such as IL-4, IL-10, and IL-5, could be measured for T cells obtained from mice immunized with BCG-M2 or BCG-N (data not shown).

Furthermore, a significant increase in CD4 $^{+}$ and CD8 $^{+}$ T cells expressing the early activation marker CD69 was observed only in cell suspensions derived from mice immunized with BCG-N or BCG-M2 after stimulation with recombinant N or M2 proteins, respectively (Fig. 6B). Expression of IFN- γ could be detected in these CD4 $^{+}$ T cells expressing CD69 (Fig. 6C). In contrast, no increase in CD4 $^{+}$ T cells expressing CD69 or IFN- γ was observed in unimmunized mice or mice immunized with WT-BCG or UV-RSV (Fig. 6B and data not shown).

To confirm that protection against RSV was mediated by virus-specific T cells induced by immunization with recombinant BCG, adoptive transfer experiments were performed. Purified CD8 $^{+}$ and CD4 $^{+}$ T cells obtained from mice immunized with BCG-N were adoptively transferred into naive BALB/c mice. One day after the transfer, mice were challenged with RSV as described in *Methods*. As shown in Fig. 6D, mice that received either CD4 $^{+}$ or CD8 $^{+}$ T cells from BCG-N-immunized mice were only partially protected against RSV infection, as shown by a less severe weight loss (Fig. 6D) and neutrophil airway infiltration (Fig. 6D) after virus challenge. In contrast, complete protection against RSV challenge was obtained after the simultaneous transfer of CD4 $^{+}$ and CD8 $^{+}$ T cells from BCG-N-immunized mice. Mice receiving both T cell popu-

specific to viral antigens. As a result, primary infection does not confer immune protection against subsequent reinfections with antigenically similar RSV strains (40–42). Another example of immune modulation is observed after RSV infection and/or vaccination with inactivated virus, which can induce a detrimental Th2 immune memory. This type of immune response can promote airway inflammation, leading to lung injury after a second exposure to RSV (11, 43). Because of the complexity of the detrimental immune response induced by RSV, more than 2 decades of research have been required to identify a potentially successful approach to generate a safe and protective vaccine against this pathogen. It has been suggested that protection against RSV could be achieved by means of immunization methods capable of promoting a balanced, RSV-specific, Th1-type immune response, which could clear viral infection without excessive or damaging inflammation of the infected tissues. Because several genetically modified RSV strains have failed to promote protective immunity even in the presence of Th1-type cytokines (7), it would seem likely that the choice of vector expressing RSV antigens is a critical parameter for conferring immune protection against the virus.

In this study we demonstrate that vaccination with recombinant BCG strains expressing RSV antigens can dampen RSV-induced disease. Moreover, recombinant BCG strains expressing RSV antigens efficiently promote protective Th1-like immunity against this virus, which fosters clearance of this pathogen with no evident inflammation and lung injury. Interestingly, it is likely that this feature is a consequence related directly to BCG and its capacity to promote a balanced Th1 immune response. It is thought that the capacity of BCG to induce Th1-skewed immune responses would be in part mediated by the cell wall lypomannan and lipoarabinomannan, which promote IL-12 secretion by dendritic cells (44). It seems that this feature of BCG is fundamental for inducing a protective immune response against RSV, because vaccination with other bacterial vectors expressing the same RSV antigens, such as attenuated strains of *Salmonella*, failed to confer protection against the virus (Fig. S4).

BCG has been applied for several decades as a vaccine against tuberculosis (45) and, more recently, was used successfully as a carrier to promote a Th1 immune response against antigens from other bacterial and viral pathogens, such as measles (24), *Borrelia* (46, 47), *Bordetella pertussis* (48, 49), and *Pneumococcus* (50). In this study we show that recombinant BCG strains expressing either protein N or protein M2 from RSV can promote a cell-mediated, Th1-like, RSV-specific immune response. Our data show that vaccination with these recombinant BCG strains promotes secretion of IFN- γ and IL-2 by RSV-specific T cells residing in spleens of vaccinated mice. As a result, weight loss and recruitment of inflammatory cells to the airways, a process induced by RSV infection, were prevented with the RSV-specific immune response induced by recombinant BCG strains. Furthermore, immunization with BCG expressing RSV proteins significantly reduced virus presence in infected lung tissue after challenge and prevented excessive lung inflammation. On the contrary, unimmunized, WT-BCG-immunized, or UV-RSV-immunized animals showed significant weight loss and inflammatory infiltration into the airways. According to previous studies, RSV-immunized mice showed enhanced inflammatory infiltration within airways and lungs compared with unimmunized animals because of the adverse Th2 immune response promoted by RSV antigens (10). It is noteworthy that after RSV infection, mice immunized with WT-BCG showed a moderate reduction in the amount of inflammatory cells infiltrating the airways. However, differences observed were not statistically significant when compared to unimmunized animals (Fig. 2). This finding is consistent with previous studies showing that BCG immunization can promote IFN- γ secretion by T cells after unspecific stimulation, and reduce allergic responses to inhaled antigens (51, 52). In addition, it has been demonstrated that immunization of newborns with BCG induces a strong Th1 immune

response, despite the fact that newborns are prone to Th2 responses upon antigenic challenge (23, 53). Further, several epidemiological studies have provided evidence supporting the notion that *Mycobacterium* exposure can prevent atopic disorders in humans (54, 55). Nevertheless, the observation that significant weight loss followed RSV infection in mice immunized with WT-BCG would indicate that the unspecific immunity induced by BCG is not sufficient to prevent RSV-induced disease (Fig. 2A). On the contrary, our data suggest that protection against RSV requires specific T cell-mediated immunity involving CD4⁺ and CD8⁺ T cells secreting Th1-type cytokines (Fig. 6D). This notion is further supported by the observation that BCG strains expressing RSV antigens failed to confer protection to RSV in RAG-deficient mice (Fig. S3).

It is likely that pathogen-associated molecular patterns derived from recombinant BCG can modify the nature of adaptive immunity to RSV antigens by promoting a Th1-like T cell response, which can counteract the allergic-like immune response triggered by natural RSV infection. Our results support the notion that vaccination with BCG expressing RSV antigens induces a Th1 immune response, which protects against RSV infection. Considering that administration of BCG to newborns has been demonstrated to be safe, strains of BCG expressing RSV antigens are candidates for a potential new vaccine that could be administered to young children to prevent disease induced by RSV infection during infancy.

Methods

Mice and RSV Production. BALB/c mice were obtained from The Jackson Laboratory and maintained at the Pontificia Universidad Católica de Chile animal facility (Santiago, Chile). All animal work was performed according to institutional guidelines. RSV serogroup A, strain 13018-8, is a clinical isolate provided by the Public Health Institute of Chile. Sequence comparison analyses reveal that this clinical strain is 96% identical to the standard RSV long strain (Fig. S5). RSV was propagated over HEp-2 cells, as described in *SI Methods*. RSV preparations were routinely evaluated for lipopolysaccharide and mycoplasma contamination. Recombinant production of RSV proteins was performed as described in *SI Methods*.

Generation of BCG Expressing RSV Proteins and Preparation of Vaccine Doses. BCG strains expressing the N or M2 proteins (BCG-N and BCG-M2, respectively) were generated as described in *SI Methods*. Expression of N or M2 proteins by BCG strains was assessed by Western blot analysis using rabbit antisera against N or M2 protein, respectively. Vaccine doses of BCG-N, BCG-M2, or nonrecombinant BCG were prepared as described in *SI Methods*. BCG-OVA was generated as described previously (56).

Mouse Immunization and RSV Challenge. Four- to 6-week-old BALB/c mice (5–8 animals per group) received a subdermal injection with 1×10^8 cfu of WT-BCG, BCG-N, or BCG-M2 in the right dorsal flank. As controls, unimmunized mice or mice vaccinated with 1×10^7 pfu of UV-inactivated RSV were included in each experiment. At 7, 14, and 21 days after vaccination, serum samples were obtained from the tails of mice, and anti-N or anti-M2 antibodies (IgG) were detected by ELISA using 500 ng/well recombinant N or M2 proteins and serial dilutions of mouse sera. Twenty-one days after vaccination, mice were anesthetized with 150 μ L of a 0.8% ketamine–0.1% xylazine solution in PBS and challenged intranasally with 1×10^7 pfu of RSV in 75- μ L inoculums. Body weight was determined daily after vaccination and for 4 days after infection.

FACS Analyses of BAL Cells. After 4 days of infection, mice were terminally anesthetized, and lungs were inflated through the trachea 3 times with PBS. Recovered cells were stained with trypan blue and counted with a Neubauer chamber. A total of 200 μ L was spun onto glass slides, air dried, and stained with May-Grünwald and Giemsa (Merck). Visual quantification of neutrophils was performed in 6 random fields per sample on an Olympus BX51 light microscope at 40 $\times$ magnification. BALs were centrifuged at $300 \times g$ for 5 min, resuspended in 100 μ L of PBS, and stained with 0.1 μ L of anti-CD11b-FITC and GR-1 (Ly-6G)-APC (BD PharMingen) for 30 min on ice. Data acquisition was performed on a FACS-Calibur cytometer (BD Biosciences) and analyzed by using WinMDI 2.8 software (The Scripps Research Institute, La Jolla, CA; <http://facs.scripps.edu/help/html/read1ptl.htm>).

Lung Histopathology. Lungs of control and infected mice were removed, and the right upper lobes were frozen in Tissue Freezing Medium (Jung) at -80°C . Five-micrometer slices were prepared on a Leica CM 1510 S cryostat and stained

with hematoxylin/eosin and evaluated for HPS as described in *SI Methods*. RSV detection in lung tissues was performed by immunohistochemistry and immunofluorescence, as described in *SI Methods*. Eosinophil infiltration was detected in 5- μ m lung sections using the EoProbe staining kit according to the manufacturer's instructions (BioFX Laboratories) and was analyzed on a fluorescence microscope (Olympus).

Cytokine Secretion by RSV-Specific T Cells. Spleen cells from unimmunized or mice immunized with WT-BCG, BCG-M2, BCG-N, or UV-RSV were obtained as described in *SI Methods* and incubated with 10 μ g/mL of either recombinant protein N or M2. Recombinant proteins were produced as described in *SI Methods*. After 5 days of incubation, cytokine secretion was determined on supernatants by sandwich ELISA as described previously (57). Cytokine secretion was expressed as fold increase relative to unstimulated cells (treated only with PBS). CD69 expression and IFN- γ secretion by T cells were evaluated by flow cytometry, as described in *SI Methods*.

Adoptive Transfers of RSV-Specific T Cells to Naive Mice. Spleen and lymph node cells from unimmunized mice or mice immunized with WT-BCG or BCG-N were

cultured in the presence of N protein as described above. After 5 days, CD4⁺ and CD8⁺ cells were purified with CD4⁺ or CD8⁺ MACS T cell isolation kits according to the manufacturer's instructions (Miltenyi Biotec). A total of 10⁶ purified CD4⁺, CD8⁺, or a 1:1 mixture of CD4⁺ and CD8⁺ T cells were injected intravenously into syngeneic recipient mice. Recipient mice were challenged with 1 $\times$ 10⁷ pfu of RSV 24 h later. Body weight was scored daily during the 5 days after infection, and BALs were analyzed for the presence of GR1⁺ and CD11b⁺ cells at day 5.

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