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Efficient Lung Recruitment of Respiratory Syncytial Virus-Specific Th1 Cells Induced by Recombinant Bacillus Calmette-Guérin Promotes Virus Clearance and Protects from Infection

Kelly M. Cautivo,* Susan M. Bueno,* Claudia M. Cortes,*† and Alexis M. Kalergis*‡

Infection by the respiratory syncytial virus (RSV) can cause extensive inflammation and lung damage in susceptible hosts due to a Th2-biased immune response. Such a deleterious inflammatory response can be enhanced by immunization with formalin- or UV-inactivated RSV, as well as with vaccinia virus expressing the RSV-G protein. Recently, we have shown that vaccination with rBCG-expressing RSV Ags can prevent the disease in the mouse. To further understand the immunological mechanisms responsible for protection against RSV, we have characterized the T cell populations contributing to virus clearance in mice immunized with this BCG-based vaccine. We found that both CD4+ and CD8+ T cells were recruited significantly earlier to the lungs of infected mice that were previously vaccinated. Furthermore, we observed that simultaneous adoptive transfer of CD8+ and CD4+ RSV-specific T cells from vaccinated mice was required to confer protection against virus infection in naive recipients. In addition, CD4+ T cells induced by vaccination released IFN-γ after RSV challenge, indicating that protection is mediated by a Th1 immune response. These data suggest that vaccination with rBCG-expressing RSV Ags can induce a specific effector/memory Th1 immune response consisting on CD4+ and CD8+ T cells, both necessary for a fully protective response against RSV. These results support the notion that an effective induction of Th1 T cell immunity against RSV during childhood could counteract the unbalanced Th2-like immune response triggered by the natural RSV infection. The Journal of Immunology, 2010, 185: 000–000.

Respiratory syncytial virus (RSV) is the leading cause of respiratory tract infections in infants and young children worldwide (1). Globally, the World Health Organization estimates that RSV causes up to 64 million infections and 160,000 deaths annually (2). Repeated reifications are very common, suggesting that adaptive immunity after RSV infection is inefficient and nonlasting (3). Therefore, the development of a vaccine or antiviral drugs against this virus is a public health priority. Regrettably, no effective vaccines are currently available, in part due to the unknown immunopathological mechanisms of RSV infection. In 1960, the formalin-inactivated vaccine against RSV (FI-RSV) led to a predisposition for exacerbated pulmonary disease in infants, which was attributed in part to an imbalanced Th2-biased immune-inflammatory response (4). Accordingly, it has been reported that in those cases of RSV-induced bronchiolitis, there is a correlation between Th2-biased immune response and exacerbated pulmonary disease (4–8). However, this notion has been challenged by the finding of high levels of Th1 cytokines in children suffering from RSV infection (9, 10). Nevertheless, studies in mice have suggested that FI-RSV induced an exacerbated pulmonary disease response characterized by the production of non-protective Abs (11). This was probably due to a deficient TLR activation in B cells (12). The pathological manifestations caused by RSV in mice that were previously vaccinated with FI-RSV were characterized by pulmonary eosinophilia and mixed Th1/Th2 CD4+ T cell responses (13–15). Similar observations were made in mice immunized with recombinant vaccinia virus expressing the RSV-G protein (16). Thus, it is thought that a Th1-polarized T cell immune response would be required to overcome the RSV-induced pathogenesis and efficiently clear the virus (3, 17–20).

Recently, we have shown that immunization with live attenuated bacillus Calmette-Guérin (BCG) strains expressing RSV Ags can confer long-term protection against RSV (3). The use of attenuated recombinant bacteria-expressing RSV Ags constitutes an interesting vaccination approach, because live attenuated bacteria can be significantly more immunogenic than isolated purified proteins. We...
observed that immunization with our rBCG vaccine induced a T cell-mediated immune response in BALB/c mice, which protected them against a subsequent infection with virulent RSV (3). Although the response was shown to involve RSV-specific CD4+ and CD8+ T cells with a Th1 effector profile, the mechanism of immune protection induced by the rBCG vaccine needs to be defined. To elucidate the mechanism of protection conferred by the rBCG vaccine is crucial to understand the nature of the immune response that is required to overcome the immunopathology caused by RSV (2, 3, 14, 21, 22). To approach this question, we have evaluated the contribution of CD4+ and CD8+ T cells to the protection against RSV infection in mice that have been immunized with rBCG. We observed that recombination activating gene (RAG)-1–deficient mice immunized with rBCG were seriously affected by RSV infection, confirming the role of T cells in the protection mechanism. Moreover, postinfection, RAG–1–deficient (RAG-1 KO) mice showed an even more severe disease and persistent viral loads than infected wild-type (WT) mice, suggesting that adaptive immunity is essential for RSV clearance. We observed that in WT mice immunized with rBCG, IFN-γ–secreting T cells were readily expanded after RSV infection. Vaccinated mice showed faster kinetics of T cell recruitment to the lung mucosa after an airway challenge with the virus. Furthermore, adoptive transfer of CD4+ T cells from vaccinated mice dampened the immunopathogenesis caused by RSV infection. Protection was enhanced when both CD4+ and CD8+ T cell populations from mice vaccinated with rBCG were simultaneously transferred to naive recipients, suggesting a synergistic effect between these two T cell subsets. Our data suggest that immunization with rBCG promotes the efficient recruitment of RSV-specific T cells with a Th1 effector profile to the lungs of infected mice, which mediate virus clearance and prevent the immunopathology caused by RSV.

**Materials and Methods**

**RSV preparation**

HEP-2 cells (American Type Culture Collection, Manassas, VA) were used to propagate RSV serogroup A, strain 13018-8 (clinical isolate obtained from Instituto de Salud Pública de Chile, Santiago, Chile), as described previously (21). Briefly, HEP-2 monolayers were grown in T75 flasks with OPTIMEM medium (Life Technologies Invitrogen, Carlsbad, CA). Flasks containing 10 ml culture medium were inoculated with 2 × 106 PFU RSV and incubated at 37˚C. For the following 48 h, supernatants were replaced with fresh medium until visible cytopathic effect was observed. The contents of the flasks were harvested and cell debris removed by two rounds of centrifugation at 5000 × g for 10 min. Viral titers in supernatants were quantified in 96-well plates with HEP-2 cells, as described previously (23). Screening for detection and quantification of synctia was done after cell fixation with 1% paraformaldehyde-PBS and 0.4% crystal violet staining solution. RSV preparations were routinely evaluated for LPS and contamination.

**Mouse immunization and RSV challenge**

BCG strains expressing the N or M2 protein from RSV and purified N and M2 protein were obtained as described previously (3). The BCG strain expressing OVA Ag (BCG-OVA) was kindly provided by Dr. Steven Porcelli from The Albert Einstein College of Medicine (Bronx, NY). RAG–1–deficient, BALB/c, and C57BL/6 mice were 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. Five- to 6-wk-old BALB/c and RAG-deficient mice (six to eight animals per group) received a s.c. injection in the right dorsal flank with 1 × 107 CFU BCG-WT or rBCG strains expressing RSV N-protein (BCG-N) or M2-protein (BCG-M2). In addition, groups of mice immunized with 10 µg purified N or M2 proteins from RSV combined with Alum (Thermo Fisher Scientific, Rockford, IL) were included. After 10 d, mice were boosted with the respective BCG strain, as indicated above. Twenty-one days postimmunization, mice were anesthetized with a mixture of ketamine and xylazine (20 mg/kg and 1 mg/kg, respectively) and challenged by intranasal infection with 1 × 107 PFU RSV. Body weight was recorded daily during BCG immunizations and RSV challenges.

**Expansion of RSV-specific T cells**

Spleens from naive mice or immunized mice were removed and mechanically homogenized in 1× PBS (when indicated, in some experiments, mice were previously infected with RSV). After erythrocyte lysis with ACK buffer (150 mM NH4Cl, 10 mM KHCO3, 0.15 mM EDTA), cells were resuspended at a final concentration equal to 5 × 106 cells/ml in RPMI 1640 medium, supplemented with 10% FBS, 1 mM nonessential amino acids, 2 mM glutamine, 1 mM pyruvate, 100 µg/ml penicillin G, 100 µg/ml streptomycin, 50 µg/ml gentamycin, and 50 µM 2-ME. Splenocytes from BALB/c mice were left untreated or stimulated with 10 µg/ml of either N-RSV protein or M2-RSV protein or 10 µM M2_82-90 (SYGGSINN) peptide. In addition, splenocytes from C57BL/6 mice were stimulated with 10 µg/ml of either N-RSV protein or 10 µM antigenic peptides derived from N-RSV (N67-68: ANHKTFTG; N68-74: NGVINSYVL) (Genscript, Piscataway, NJ). As a positive control, splenocytes were stimulated with 2 µM Con A. After 5 d of incubation (37˚C, 5% CO2), culture supernatants were analyzed for cytokine production by sandwich ELISA, and the expression of activation markers was measured on the surface of cells by flow cytometry, as described below.

**Detection of cytokine secretion**

IFN-γ was determined in supernatants from lung homogenates after 5 d of in vitro stimulation or directly in bronchoalveolar lavages (BALS) of infected mice, as described previously (3, 24). Briefly, 96-well plates were coated overnight at 4˚C with 1.25 µg/well purified capture anti–IFN-γ Ab (clone R4-6A2, BD Pharmingen, San Diego, CA) and blocked with 200 µl 3% BSA in PBS for 2 h at room temperature (RT). Wells were washed, and 200 µl culture supernatant was added and incubated for 12 h at 4˚C. Later, plates were washed and incubated with 0.85 µg/well biotinylated anti–IFN-γ Ab (clone XMG1.2, BD Pharmingen) for 1 h at RT. Detection, the samples were incubated for 1 h at RT with streptavidin-HRP (dilution 1/1500, BD catalog number 554066, BD Pharmingen) and revealed with 50 µl HRP substrate (3-3’-5-5’-tetramethylbenzidine, 1 mg/ml). To stop the reaction, 50 µl H2SO4 2 M were added, and then absorbance was measured at 450 nm in an ELISA plate reader.

**Intracellular cytokine staining**

Intracellular IFN-γ was measured as described previously (25). Briefly, lung cells were collected and cultured in RPMI 1640 medium. Then, cells were incubated overnight with 10 µg/ml N-protein and treated with 10 µg/ml brefeldin A (Invitrogen) for 5 h at 37˚C and 5% CO2 and then stained with CD4+APC (clone GK1.5, BD Pharmingen) and CD8-PerCP (clone 53-6.7, BD Pharmingen) Abs for 1 h at 4˚C. Cells were then washed and fixed (2% formaldehyde in PBS), permeabilized (0.5% saponine, 0.5% BSA, 1× PBS), and stained with PE-conjugated anti-mouse IFN-γ (clone XMG1.2, BD Pharmingen). Finally, cells were washed, resuspended in PBS, and analyzed in a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

**T cell adoptive transfers**

Spleen and lymph node cells from mice immunized either with BCG-WT or BCG-N were cultured in the presence of N-protein, as described above. Controls, unimmunized mice and mice immunized with BCG-OVA were included in these assays. After 5 d of in vitro stimulation with 10 µg/ml of their respective cognate proteins (N-RSV or OVA), CD4+ and CD8+ T cells were purified using MACS T cell isolation kits (Miltenyi Biotec, Auburn, CA). A total of 1 × 106 purified CD4+, CD8+, or a 1:1 mixture of CD4+ and CD8+ T cells were injected i.v. into syngeneic recipient mice. After 24 h, mice were challenged with 1 × 106 PFU RSV, as described above. To neutralize the activity of IFN-γ, 150 µg anti–IFN-γ (clone R4-6A2, BD Pharmingen) was administrated i.v. to recipient mice simultaneously with the adoptive transfer of purified T cells. After 24 h, mice were challenged with 1 × 106 PFU RSV and 1 d later inoculated with 100 µg neutralizing anti–IFN-γ Ab. Body weight was scored on a daily basis during 6 d postinfection. In addition, infiltration of inflammatory cells in BALS and lungs, as well as eosinophilia and viral loads, were determined after RSV infection as described below.

**Lung histopathology and immunofluorescence assays**

Lungs were obtained from mice 6 d post RSV infection and frozen in Tissue Freezing Medium (Jung, Wetzlar, Germany) at −80˚C. Slices of 7 µm were obtained by using a Leica CM1510S cryostat (Leica Microsystems, San Jose, CA).
Deerfield, IL) and stained either with H&E or phenol red for eosinophil detection, as previously described (26). For detection of apoptotic cells, a DeadEnd Fluorometric TUNEL System (Promega, Madison, WI) was used according to the manufacturer’s recommendation. Briefly, tissue sections were fixed in 4% formaldehyde-PBS solution, washed in PBS, and treated with proteinase K (20 μg/ml). Cell suspensions were stained with anti-CD4 (allophycocyanin), anti-CD8 (PerCP), anti-CD11b (PE), or anti-Gr-1 (allophycocyanin) Abs for 30 min. Then, cells were fixed and permeabilized in 70% ice-cold ethanol at −20˚C. Next, tissue sections and cell suspensions were washed by immersion in PBS and treated with 100 μl Equilibration Buffer (Promega). Finally, slides were washed and treated with 50 μl incubation buffer (45 μl equilibration buffer, 5 μl nucleotide mix, 1 μl TrTdT enzyme) for 1 h at 37°C. The reaction was stopped with saline-sodium citrate buffer. For immunofluorescence assays, 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 RT. Pre-staining, lung sections were hydrated, starting with 95% ethanol for 30 min, transferred to 75% ethanol for 5 min, then to 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× PBS) for at least 30 min at RT. Then, slides were incubated with different Abs against RSV: biotin-conjugated goat polyclonal anti-RSV Ab (United States Biologicals, Swampscott, MA), purified mouse monoclonal anti-F RSV Ab (United States Biologicals), and rabbit polyclonal anti-N RSV. Abs were diluted 1:100 in blocking solution and incubated overnight at 4°C. Sections were then incubated with FITC-conjugated streptavidin (1:200, BD Pharmingen), Alexa Fluor 488 goat anti-rabbit IgG (1:200, Molecular Probes, Invitrogen), or FITC- or Alexa Fluor 488 goat anti-mouse IgG/IgM (1:200, BD Pharmingen) for 1 h at RT and washed with PBS. CD11b and cytokeratin (P-actin) were stained with Hoescht 33342 (5 μg/ml) and rhodamine phalloidin (1:30, Molecular Probes, Invitrogen) for 30 min, respectively. Coverslips were mounted and examined in a Fluoview FV1000 Laser Scanning Confocal Microscope (Olympus, Melville, NY). The images were processed using FV10-ASW 1.7 software (Olympus).

### FACS analyses of BALs and lung T cells

Six days postinfection, mice were terminally anesthetized by i.p. injection of a mixture of ketamine and xylazine (20 mg/kg and 1 mg/kg, respectively). BALs were collected as previously described (26) and stained with anti-CD11b-FITC (clone CBRM1.5, BD Pharmingen) and Gr-1 (Ly-6G)-APC (clone RB6-8C5, BD Pharmingen) Abs. For pulmonary T cell studies, the CD11b-FITC (clone CBRM1.5, BD Pharmingen) and Gr-1 (Ly-6G)-APC (clone RB6-8C5, BD Pharmingen) Abs were diluted 1:100 and incubated in EDTA-PBS (5 mM) to inactivate the collagenase activity. Then, tissues were homogenized using two sterile slides and filtered and incubated in a shaker (300 rpm) at 37˚C for 15 min. Then, tissues were incu bated in blocking solution (4% BSA in 1× PBS) for at least 30 min at RT. Then, slides were incubated with different Abs against RSV: biotin-conjugated goat polyclonal anti-RSV Ab (United States Biologicals, Swampscott, MA), purified mouse monoclonal anti-F RSV Ab (United States Biologicals), and rabbit polyclonal anti-N RSV. Abs were diluted 1:100 in blocking solution and incubated overnight at 4°C. Sections were then incubated with FITC-conjugated streptavidin (1:200, BD Pharmingen), Alexa Fluor 488 goat anti-rabbit IgG (1:200, Molecular Probes, Invitrogen), or FITC- or Alexa Fluor 488 goat anti-mouse IgG/IgM (1:200, BD Pharmingen) for 1 h at RT and washed with PBS. CD11b and cytokeratin (P-actin) were stained with Hoescht 33342 (5 μg/ml) and rhodamine phalloidin (1:30, Molecular Probes, Invitrogen) for 30 min, respectively. Coverslips were mounted and examined in a Fluoview FV1000 Laser Scanning Confocal Microscope (Olympus, Melville, NY). The images were processed using FV10-ASW 1.7 software (Olympus).

### Determination of viral load by quantitative real-time PCR

Total RNA was isolated from 100 mg lung tissue using TRIZol Reagent (Invitrogen). Five micrograms RNA were reverse transcribed to cDNA using the Improm-II Reverse transcription system kit (Promega), according to the manufacturer’s instructions. Primers used for RSV-N gene detection in quantitative real-time PCR (QPCR) reactions were Fwd: 5′-GAG ACA GCA TTG ACA CTC CT-3′ and Rev: 5′-CGA TGT GTT ACA TCC ACT-3′, with an amplicon length of 214 bp. Detection of mouse β-actin was used as a housekeeping reference gene, using primers Fwd: 5′-GAG CAT CAC CCT CAC TGA GTA C-3′ and Rev: 5′-TCT TCA GGT GTG CTG TCA G-3′ with an amplicon length of 384 bp. The products were detected using Brilliant QPCR Master Mix (Stratagene, La Jolla, CA) in an Mx3000P thermocycler (Stratagene). Relative gene expression data analyses were performed using the comparative cycle threshold method. Standard curves for QPCR were generated by using RT-PCR-RA-N/RSV or RT-PCR-β-actin as templates. The cycle threshold results were entered in the standard curve with the quantity log.

### Cytotoxicity assay

The cytotoxic capacity of CD8+ T cells was determined by colorimetric quantification of lactate dehydrogenase (LDH) released from target cells using the CytoTox 96 Non-Radioactive Cytotoxicity assay (Promega) (27). RSV-infected J774.A1 macrophages (ATCC TIB-67, American Type Culture Collection) were used as target cells. Briefly, effectors and target cells were mixed at different ratios (E:T) and plated on a 96-well U-bottomed plate. The incubation was done for 6 h at 37°C at 5% CO2. Post-incubation, samples were centrifuged and supernatants collected and incubated for 30 min at RT with the Substrate Mix provided in the kit to detect LDH activity. A stop solution was added, and the absorbance of the sample was measured at 490 nm. The amount of cell-mediated cytotoxicity was calculated by subtracting the spontaneous LDH released from the target and effector cells from the LDH released by lysed target cells, as indicated by the manufacturer (28, 29), using the following equation: % cytotoxicity: ((O.D₄₉₀ target cells − spontaneous O.D₄₉₀ effector cells − spontaneous O.D₄₉₀ target cells)/(maximum O.D₄₉₀ target cells − spontaneous O.D₄₉₀ target cells)) × 100.

### Statistical analysis

GraphPad Prism 5.00 (GraphPad, La Jolla, CA) was used for graphing and statistical analyses. Unpaired, two-sided t tests were used for two-group analysis, whereas one-way ANOVA was used to compare three or more groups in the same experiment. The p values <0.05, 0.01, and 0.001 were considered significant.

### Results

**Immunization with rBCG induces IFN-γ secretion and protects against RSV infection**

To evaluate the cellular immune response induced by vaccination with an RBCG strain, BALB/c mice were immunized with BCG-WT, BCG-N, or BCG-M2. Single-cell suspensions were obtained from spleens and lymph nodes of immunized mice and treated as described in Materials and Methods. As shown in Fig. 1A, cell suspensions from mice immunized with BCG-N secreted significant amounts of IFN-γ in response to specific antigenic stimulation (Fig. 1A). In agreement with these data, we observed a significant increase in CD69 expression in both CD4+ and CD8+ T cells in cell suspensions obtained from mice immunized with BCG-N (Fig. 1B). Moreover, similar results were obtained with mice immunized with another RSV rBCG strain, BCG-M2 (Supplemental Fig. 1A, 1B). No significant IFN-γ secretion could be measured in supernatants of cell suspensions derived from mice immunized with BCG-WT (Fig. 1A) or unimmunized mice (data not shown), which is consistent with the notion that IFN-γ was produced specifically by cells primed by RSV Ags. Spleen and lymph node cells from mice immunized either with purified N or M2 proteins in Alum secreted amounts of IFN-γ significantly lower than those induced by rBCG (data not shown).

In addition, we measured activation of CD8+ T cells specific for RSV-derived peptides in spleens of mice immunized with BCG-N or BCG-M2 and infected with the virus (Fig. 1C, Supplemental Fig. 1C). As shown in Fig. 1C, CD8+ T cells from BCG-N–immunized mice produced significant amounts of IFN-γ upon stimulation with N57–64 and N360–368 peptides (Fig. 1C). Similar results were obtained with mice immunized with BCG-M2 (Supplemental Fig. 1C). CD8+ T cells from these animals secreted significant amounts of IFN-γ upon M protein or M2 protein antigenic peptide stimulation (Supplemental Fig. 1C).

Furthermore, to corroborate the protective capacity of the immune response triggered by BCG-N and BCG-M2, immunized mice were intranasally challenged with RSV, and the severity of the infection was determined (Fig. 1D, 1E, Supplemental Fig. 1D, 1E). Consistent with our previous work (3), mice immunized with BCG-N or BCG-M2 and challenged with RSV showed a significant decrease of both polymorphonuclear cells (PMNs; Gr1+/CD11b+ cells) in the BALs (Fig. 1D, Supplemental Fig. 1D) and viral RNA loads in the lungs (Fig. 1E, Supplemental Fig. 1E), as compared with unimmunized or BCG-WT–immunized mice. Although some protection against RSV was obtained by immunizing with purified N or M2 protein, this vaccination approach was less effective than rBCG (data not shown).
FIGURE 1. Immunization with BCG-N induces secretion of IFN-γ and activation of Ag-specific T cells. Twenty-one days postimmunization with either BCG-WT or BCG-N, spleen cells were collected to evaluate the specific T cell response. The collected cells were stimulated with 10 μg/ml purified RSV-N. As a positive control, cells were stimulated with Con A. A, IFN-γ secretion detected by ELISA in the supernatant of splenocytes 5 d poststimulation with RSV-N. Data shown are means ± SEM of four independent experiments. B, Flow cytometry detection of CD69 expression by T cells derived from BCG-N–immunized mice, in response to RSV-N stimulation, respectively. After 72 h of incubation with RSV-N, spleen cells were stained with anti-CD8, anti-CD4, and anti-CD69 Abs and analyzed by flow cytometry. As a positive control of activation, cells were stimulated with Con A. Histograms show the mean fluorescence intensity (MFI) of CD69+ cells gated in CD4+ (left panel) or CD8+ (right panel), respectively. A total of 40,000 events was acquired in all the experiments using a FACScalibur cytometer and analyzed with the FACS Express v3 software (BD Biosciences). Histograms are representative of four independent experiments. C, Twenty-one days postimmunization with BCG-N, groups of C57BL/6 mice were intranasally challenged with 1 × 10^7 PFU RSV. Spleens and lymph nodes were collected for expansion of RSV-specific T cells with 10 μg/ml of N-protein, 10 μM of N37-64 peptide, or 10 μM N360-368 peptide or left untreated as a negative control. After 72 h of incubation, CD8+ T cells were analyzed for IFN-γ production by intracellular cytokine
These data support the notion that rBCG strains can efficiently protect against disease caused by RSV and induce an effective viral clearance. Further, these findings also suggest that immunization with BCG-N and BCG-M2 promotes the expansion of RSV-specific T cells that produce IFN-γ upon antigenic stimulation, which could contribute to protective viral immunity.

**Recruitment of CD4+ and CD8+ T cells to the lungs of RSV-infected mice is accelerated by immunization with rBCG-N**

According to our results, we have shown that immunization with rBCG strains expressing RSV-N or RSV-M2 proteins can efficiently trigger protective T cell responses (3). These viral proteins are the main antigenic targets of RSV-specific CTL responses, among which the nucleoprotein N is a major carrier of CTL epitopes in human and cattle (30–32). Furthermore, N protein is the most conserved viral protein between RSV human isolates (33, 34), and other strategies that had used RSV-N protein for vaccine design were also effective (32, 35–38). Therefore, our next aim was to study the cellular mechanism by which BCG-N induces protection against RSV infection. We evaluated whether immunization with BCG-N could increase the efficiency of RSV-specific T cell immunity. We measured whether immunization with BCG-N could increase the efficiency of RSV-specific T cell immunity. Immunized BALB/c mice were included as controls. Significantly more CD4+ T cells could be detected 4 d postinfection in the lungs of mice immunized with BCG-N compared to naive mice or mice immunized with BCG-WT.

**FIGURE 2.** Immunization with BCG-N changes the proportion of CD4+ and CD8+ T cells in the lung upon RSV infection. Populations of T cells that migrate to the lungs in naive and BALB/c mice immunized either with BCG-N or BCG-WT were analyzed after 4, 6, and 10 d postinfection with 1 × 10⁷ PFU RSV. The infection with RSV was performed 21 d postvaccination. The lung tissue was disrupted and the cells were analyzed by FACS (see Materials and Methods). Data in the graph are the percentages of CD4+ T cells (A) and CD8+ T cells (B) in lungs from uninfected mice or infected mice that were unimmunized, immunized with BCG-N, or immunized with BCG-WT. Data show means ± SEM of three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001, Student t test and one-way ANOVA.
of mice immunized with rBCG-N, which declined by day 10 post-
infection (Fig. 2A). In contrast, naive and BCG-WT–immunized
mice challenged with RSV showed an increase of CD4+ T cells
only after 6 d postinfection, which remained at high levels up to
day 10 postinfection (Fig. 2A). Consistent with the observations
made for CD4+ T cells, the efficiency of CD8+ T cell recruitment
to the lungs after RSV infection was also significantly enhanced
by immunization with BCG-N by day 6 postinfection (Fig. 2B). In
contrast, naive or BCG-WT–immunized mice challenged with
RSV showed no increase for this population of CD8+ T cells in the
lungs during the entire evaluation period. Furthermore, 21 d after
RSV infection, the percentage of CD4+ and CD8+ T cells in the
lungs of both unimmunized and immunized mice exhibited normal
levels, equivalent to naive controls (data not shown). These data
suggest that in BCG-N–immunized mice, T cells are recruited
significantly more efficiently to lungs after RSV infection than in
naive mice. It is likely that an earlier recruitment of CD4+ and
CD8+ T cells induced by BCG-N immunization could contribute
to the clearance of RSV in the lungs.

BCG-N–induced immune protection against RSV requires
normal T cell function

To test whether cells of the innate immune response, such as NK
cells, could be responsible for the protective immune response
observed in our experiments, RAG-1–deficient mice lacking T and
B cells were immunized with either BCG-N or BCG-WT and then
infected with RSV. In contrast to WT mice, RAG-1 KO mice
immunized with BCG-N showed significant weight loss after RSV
infection, equivalent to unimmunized mice infected with RSV (Fig.
3A). Additionally, a significantly higher number of infiltrating cells
were detected in BALs from RAG-1 KO mice immunized with
BCG-N and infected with RSV as compared with uninfected mice
(Fig. 3B, 3C). May Grünwald Giemsa staining suggested that BAL-
infiltrating cells were mostly neutrophils and eosinophils (Fig. 3C).
However, the percentage of Gr1+/CD11b+ cells was slightly re-
duced in BALs of RAG-1 KO mice immunized with either BCG-N
or BCG-WT, as compared with unimmunized RAG-1 KO mice
(Fig. 3B). Furthermore, we observed that numbers of NK cells in-
filtrating the lungs increased in an equivalent proportion for both
control and immunized mice upon RSV infection (Fig. 3B). In ad-
inistration, after RSV infection, secretion of IFN-γ was slightly in-
creased in supernatants of BALs derived from BCG-immunized
RAG-1 KO mice when compared with unimmunized controls
(Fig. 3D), suggesting that BCG might improve airway innate im-
mune response independently of viral Ag recognition by T cells.

When viral RNA was measured in the lungs of infected mice by
means of QPCR, equivalent viral loads were observed for control
and immunized RAG-1 KO mice as well as for unimmunized WT
mice at day 6 postinfection (Fig. 3E). It is noteworthy to observe
that 12 d postinfection viral loads showed a significant decrease
only in WT mice. In contrast, persistently high RSV loads were
detected in the lungs of both control and BCG-N–immunized
mice.
RAG-1 KO mice (Fig. 3F). These data suggest that T cells are required for RSV clearance, which is consistent with virus persistence in lungs of RAG-1-deficient mice lacking T cells. It is also evident from these results that innate immunity by itself is not able to protect against RSV infection.

**Adoptive T cell transfer from BCG-N–immunized mice prevents RSV pathology**

To confirm that virus-specific T cells protect against RSV infection, adoptive transfer experiments were performed. WT BALB/c mice were used as recipients to evaluate the response of transferred immune T cells and their effect on the endogenous T cell repertoire of naive mice after RSV infection. Spleen cells obtained from mice immunized with BCG-N and BCG-WT were cultured in vitro for 5 d in the presence of N-RSV protein to induce activation and proliferation of Ag specific CD8+ and CD4+ T cells. Next, T cells were purified as described in the Materials and Methods and adoptively transferred to naive BALB/c mice. One day post transfer, mice were challenged with RSV (Fig. 4A).

Although mice that received individual populations of either CD4+ or CD8+ T cells from BCG-N-immunized mice were partially protected against RSV, complete protection was only observed when both T cell subsets were simultaneously transferred (Fig. 4B–D). Mice that received both T cell subsets showed neither significant weight loss after RSV infection (Fig. 4B) nor inflammatory cellular infiltration in BALs (Fig. 4C). Furthermore, lung histopathology and the amount of eosinophils infiltrating lungs were significantly reduced in mice receiving both CD4+ and CD8+ T cells from BCG-N-immunized mice (Fig. 4D). In contrast, no protection was observed when transferred T cells derived from BCG-WT–immunized mice (Fig. 4B). As expected, RSV infection caused significant weight loss in these control animals (Fig. 4B) and massive infiltration of CD11b+/Gr1+ cells to the lungs (Fig. 4C, lower panel). When tissue damage caused by RSV on the airways was measured by TUNEL, we observed that control and CD8+ T cell-transferred mice showed a significant increase on apoptosis of lung epithelium (Fig. 4E, white arrows) and infiltrating inflammatory cells (Fig. 4E, white arrowheads) at day 6 postinfection. In contrast, no significant TUNEL staining was observed in tissues from mice adoptively transfected with both N-specific CD4+ and CD8+ T cells (Fig. 4E). Flow cytometry analyses suggested that TUNEL–positive cellular infiltrate in naive and CD8+ transferred mice was mainly PMNs (Gr1+/CD11b+) but not lung T cells (Fig. 4F).

Adoptive T cell transfer experiments were also performed using RAG-1 KO mice, and protection against RSV infection was evaluated (Supplemental Fig. 2). After T cell transfer, RAG-1 KO mice were challenged with RSV, and 6 d later, cell suspensions from spleen, lymph nodes, and lungs were obtained for T cells measurement by flow cytometry. CD4+ and CD8+ T cells were only detected in transferred RAG-1 KO mice (Supplemental Fig. 2A). The percentage of PMNs in BALs was reduced in RAG mice that received CD4+, CD8+, or both T cell subsets (Supplemental Fig. 2B) as compared with nontransferred RAG-1 KO mice. Nontransferred WT mice were included as control of RSV infection, which showed high levels of inflammatory cells in BALs after RSV infection.

Finally, to rule out the possibility that RSV protection could be due to the unspecific activation of innate immunity, we performed adoptive transfer experiments using T cells purified from mice immunized with BCG-OVA (Supplemental Fig. 3) (39). Splenocytes and lymph node cells from these immunized mice were stimulated for 5 d with 10 μg/ml OVA protein, and purified T cells were transferred to naive BALB/c mice. During in vitro activation, high levels of IFN-γ were secreted only in response to specific stimulation with OVA protein (Supplemental Fig. 3A). However, no protection against RSV was observed in mice transferred with these T cells (Supplemental Fig. 3B–D). These data support the notion that protective immunity against RSV infection is achieved only after transferring RSV-specific T cells that were primed by immunization with BCG recombinant for RSV Ags (Fig. 4).

**T cells expanded by BCG-N immunization reduce RSV loads in lungs**

In agreement with the data shown above, the simultaneous transfer of CD4+ and CD8+ T cells from BCG-N–immunized mice led to a significant reduction of RSV loads in the lungs of naive recipients, as demonstrated by the quantification of the RSV RNA in lungs by QPCR (Fig. 5A, Supplemental Fig. 2C). Moreover, transfer of solely CD4+ T cells was enough to achieve a significant reduction of RSV RNA in the lungs of recipient mice. In contrast, although the transfer of CD8+ T cells caused a significant viral load reduction in the lungs, RNA levels were higher when compared with mice receiving either CD4+ T cells alone or both T cell subsets simultaneously (Fig. 5A). Consistent with the QPCR data, RSV proteins were detected in the bronchial and alveolar airway epithelium of nontransferred controls and mice receiving CD8+ T cells only (Fig. 5B). On the contrary, RSV proteins could not be detected in the lungs of mice adoptively transferred simultaneously with CD4+ and CD8+ T cells from BCG-N–immunized mice (Fig. 5B). These results support the notion that N-RSV–specific CD4+ and CD8+ T cells are required and sufficient to prevent the immunopathology caused by RSV infection in the lungs.

**CD4+ T cells infiltrating the lungs of recipient mice secrete IFN-γ**

Next, we evaluated the phenotype of T cells residing at the lungs of mice adoptively transferred with T cells from BCG-N–immunized mice 4 d after RSV challenge. Specifically, we measured intracellular IFN-γ production and CD69 expression on T cells in response to stimulation with RSV-N protein, as described in the Materials and Methods. We observed that the percentage of IFN-γ–producing CD4+ T cells in lungs of mice transfected with CD4+, CD8+, or CD4+/CD8+ T cells was significantly higher than the amount observed in nontransferred mice (Fig. 6A). In contrast, whereas a high percentage of CD8+ T cells from mice adoptively transfected with CD8+ T cells produced IFN-γ, endogenous CD4+ T cells from these mice showed only a marginal production of this cytokine (Fig. 6B). Remarkably, the adoptive transfer of CD4+/CD8+ T cells resulted in a high amount of IFN-γ–producing pulmonary CD8+ T cells, suggesting that a synergic response occurs when both T cell populations are simultaneously transferred to mice that are subsequently exposed to RSV (Fig. 6B). As expected, CD4+ and CD8+ T cells from control (nontransferred) mice showed no significant production of IFN-γ (Fig. 6). Moreover, lung CD4+ T cells from CD4+ and CD4+/CD8+ transferred mice also expressed CD69 poststimulation with RSV-N protein (Fig. 6A). On the contrary, CD8+ transferred mice showed no significant expression of this activation marker in CD4+ or CD8+ T cells (Fig. 6B). Likewise, CD8+ T cells from mice transferred with CD4+ or CD8+ T cells did not significantly express the CD69 activation marker. However, CD8+ T cells from mice transferred with CD4+/CD8+ T cells expressed this activation marker (Fig. 6B).

**RSV-infected cells are killed by T cells primed by BCG-N immunization**

Next, we evaluated whether T cells purified from lungs of recipient mice could directly induce cytotoxicity on RSV-infected cells. Adoptive transferred and control mice were infected with
RSV, and 8 d postinfection, CD8⁺ T cells were purified and used as effector cells on cytotoxicity assays. We observed that CD8⁺ T cells from mice transferred either with CD8⁺ or CD4⁺/CD8⁺ T cells caused the specific lysis of RSV-infected J774.1 cells (Fig. 6C). In contrast, CD8⁺ T cells from control mice or CD4⁺ recipient mice showed only minor cytotoxic activity.

IFN-γ blockade suppresses protective immunity induced by BCG-N immunization

To evaluate the contribution of IFN-γ secreted by transferred T cells upon RSV infection, we performed CD4⁺/CD8⁺ T cell transfer experiments in the presence of a neutralizing anti–IFN-γ mAb (40–42) (Fig. 7A). We observed that blockade of IFN-γ...
FIGURE 5. Adoptive transfer of BCG-N–primed CD4+ and CD8+ reduces viral loads in lungs. BALB/c mice were transferred with CD4+, CD8+, or both CD4+/CD8+ T cells from BCG-N–immunized mice and 24 h later were intranasally challenged with 1 × 10^7 PFU RSV. A. RSV RNA detection after 6 d of infection in lung from mice transferred with N-specific T cells and infected with RSV using QPCR. Data in graph are the average of three independent experiments, and bars are SE. B. Viral proteins were observed by immunofluorescence (green fluorescence) in a confocal microscope using an anti–F-RSV Ab, an anti–pan-RSV Ab, or an anti-N RSV Ab. Nuclei and cytoplasm (F-actin) were stained with Hoescht (blue fluorescence) and rhodamine phalloidin (red fluorescence), respectively. Confocal images are representative of three independent experiments (original magnification ×60). **p < 0.01; ***p < 0.001, Student t test and one-way ANOVA.

Discussion

RSV infection is the cause of a major public health burden, especially among infants, and has an extremely high socioeconomic impact worldwide. Several studies have shown that RSV can modulate host immunity by blocking the activation, expansion, and function of cytotoxic and memory T cells (14, 15, 22, 43, 44). As a result, primary infection does not confer immune protection against subsequent reinfections with antigenically similar RSV strains (45, 46). Another example of immune modulation is observed after RSV infection and/or vaccination with inactivated virus, which can induce a detrimental Th2 immune memory (4). This type of immune response can promote airway inflammation, leading to lung injury after a second exposure to RSV (13, 16, 47).

Our findings support the notion that both CD4+ and CD8+ T cell populations from BALB/c mice immunized with BCG expressing either N- or M2-proteins from RSV upregulate CD69 and secrete IFN-γ in response to stimulation with RSV proteins or antigenic peptides. These data suggest that RSV-specific CD4+ T cells have acquired a Th1-polarized phenotype, which is effective at controlling RSV infection with no significant inflammation or injury to the lungs. Furthermore, these T cells migrate more efficiently to the lungs upon RSV challenge, an event that seems to be crucial to prevent viral replication and airway inflammation. The pulmonary T cell recruitment induced by RSV in naive mice peaked at day 6 postinfection and remained high for several weeks, which is likely to contribute to the immunopathogenesis caused by the virus. In contrast, BCG-N–immunized mice showed an earlier T cell recruitment to the lungs, which remained there only for a limited number of days. In addition, adoptive transfer of both CD4+ and CD8+ T cells from BCG-N–immunized mice contributed in a synergic manner to the antiviral response that finally cleared RSV. In contrast, only partial protection or no protection at all was achieved by individual transfer of CD4+ or CD8+ T cells.

Our results also suggest that production of IFN-γ by N-RSV–specific T cells was fundamental to promote protective immunity against the virus, because neutralization of this cytokine abolished the capacity of transferred T cells to protect against infection. It has been shown that IFN-γ can upregulate the expression of MHC class I and II molecules (48, 49), thereby enhancing the cellular immune response to viral infection and suppressing the proliferation of Th2-type T cells. Such a response promotes the development of Th1-type T cells, reducing the development of allergic airway immune responses (50, 51). Our data also suggest that protective immunity required that IFN-γ was produced by RSV-specific T cells. Consistent with this notion, no protection against RSV infection was observed in RAG-deficient mice or BALB/c mice immunized with BCG-N or BCG-WT, respectively.

These findings suggest that N-specific CD4+ T cells induced by BCG-N vaccination are important mediators of the protective immune response against RSV because these cells were able to activate and promote the differentiation of CD8+ naive T cells into cytotoxic and IFN-γ-secreting cells, which contributed to clear RSV infection. In agreement with this notion, we observed an early recruitment of CD4+ T cells to lungs of BCG-N–immunized mice upon RSV infection. The accelerated recruitment of these RSV-specific T cells to the lungs correlated with reduced infection and inflammation in the airways shown by immunized mice.

Furthermore, we have observed that the adoptive transfer of CD4+ and CD8+ T cells from vaccinated mice led to the expansion of IFN-γ-producing CD8+ T cells after RSV challenge. These CD8+ T cells showed higher cytotoxic activity on target cells infected with RSV. The observation that CD8+ T cells in BCG-N–immunized mice were recruited to the lungs 6 d after RSV infection caused significant weight loss in mice transferred with CD4+/CD8+ T cells from BCG-N–vaccinated animals when IFN-γ was neutralized (Fig. 7B). In addition, treatment with anti–IFN-γ Ab led to higher levels of viral RNA in lungs of mice transferred with CD4+/CD8+ T cells from BCG-N–vaccinated animals as compared with mice not treated with the Ab (Fig. 7C). In agreement with these data, IFN-γ blockade caused a significantly higher neutrophil airway infiltration postinfection with RSV (Fig. 7D). These results suggest that production of IFN-γ by transferred T cells is fundamental for the protection against RSV infection.

Adoptive transfer of BCG-N–primed CD4+ and CD8+ T cells from lungs of our experimental animals 24 h later were intranasally challenged with 1 × 10^7 PFU RSV. A. RSV RNA detection after 6 d of infection in lung from mice transferred with N-specific T cells and infected with RSV using QPCR. Data in graph are the average of three independent experiments, and bars are SE. B. Viral proteins were observed by immunofluorescence (green fluorescence) in a confocal microscope using an anti–F-RSV Ab, an anti–pan-RSV Ab, or an anti-N RSV Ab. Nuclei and cytoplasm (F-actin) were stained with Hoescht (blue fluorescence) and rhodamine phalloidin (red fluorescence), respectively. Confocal images are representative of three independent experiments (original magnification ×60). **p < 0.01; ***p < 0.001, Student t test and one-way ANOVA.
FIGURE 6. IFN-γ is secreted by CD4+ T cells in lungs of recipient mice. To evaluate the phenotype of T cells in lungs of adoptively transferred mice after RSV infection, BALB/c mice were transferred with T cells as described in Materials and Methods. Twenty-four hours later, mice were intranasally infected with RSV (1 × 10^7 PFU). Four days postinfection, lungs were collected and mechanically disaggregated. The cells were cultured in RPMI 1640 in the presence of RSV-N and tested for IFN-γ secretion, CD69 expression, and cytotoxicity capacity. A, Upper panel shows representative dot plots with the percentage of CD4+ T cells producing IFN-γ in lungs, and the graph shows the quantitative analysis of dot plot data from three independent experiments. Lower panel shows representative histograms with the MFI of CD69+ in gated CD4+ population (black line, cells stimulated with 10 μg N-protein; gray line, unstimulated cells). Graph shows the quantitative analysis of histograms data from three independent experiments. B, Analyses similar to those described for CD4+ T cells in A were performed for CD8+ T cells, in which the IFN-γ secretion by lung CD8+ T cells is shown in the upper panels, and the MFI of CD69+ cells gated in CD8+ population is shown in the lower panels. C, LDH release from J774.1 cells infected with RSV, which were incubated with CD8+ T cells purified from lungs of transferred mice 8 d after RSV infection. Infected J774.1 cells and T cells were incubated at different E:T cell ratios, and specific lysis was determined as described in Materials and Methods. Bar graph shows the specific lysis of RSV-infected cells at E:T ratio equal to 10:1 in three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001, Student t test and one way ANOVA.
infection would suggest that IFN-γ secretion and cytotoxic activity of Ag-specific CD8+ T cells might be contributing to the recovery in later stages of infection rather than being an initial host defense mechanism. However, although the viral load was high in lungs of mice transferred with CD8+ T cells only (as compared with mice transferred with CD4+ T cells only), some protection was observed when compared with nontransferred control mice.

As we have previously shown (3), immunization of BALB/c mice with BCG-WT showed a slight decrease in several parameters of RSV disease (weight loss, cellular infiltrate, and lung inflammation), which is not evident in the unvaccinated control. In agreement with these observations, previous studies and our results in RAG-deficient mice support the notion that BCG might modulate the pulmonary innate immune microenvironment (52). However, this response by itself is not capable of reverting RSV infection and disease. Probably, IFN-γ secreted in response to BCG WT alone led to the attenuation of the strong and damaging Th2 reactions that are generally associated with the subsequent intranasal RSV challenge, as is the case with our UV-RSV control (3) or FI-RSV and recombinant vaccinia virus expressing the RSV-G protein in other reports (15, 53). A prominent hypothesis to explain these results is that the prior immune history of a host can change the cytokine milieu of a particular tissue or organ (in this case the lung). Thus, subpopulations of immune cells, such as T cells, can undergo a biased differentiation in response to prior Ag exposures and cytokine production. In support of this notion is the observation that addition of IFN-γ during neonatal RSV infection can contribute to reduce the disease during adult rechallenge (54). This concept is also supported by our experiments in RAG-deficient mice. These results indicate that immunization with either BCG-WT or BCG-N increased the levels of IFN-γ in lungs and BALs during RSV infection. Thus, Th1-type milieu might explain the reduced percentage of PMN in BALs of vaccinated RAG-1 KO mice. However, in this study we have shown that despite the presence of IFN-γ during infection in lungs of RAG-1 KO mice, the absence of T and/or B cells was responsible for the lack of protective antiviral response.

Because several genetically modified RSV strains have failed to promote protective immunity, even in the presence of Th1-type cytokines (42), both vector and the Ag selection are critical parameters for conferring immune protection against this virus. Along these lines, in this study, two RSV proteins were evaluated as Ags for the elicitation of protective immune response: N and M2. As shown in this and other studies using different delivery methodologies, the N protein appears to be an appropriate Ag for promoting RSV immunity. An additional advantage for the use of N as Ag is its high sequence homology between the two antigenic subgroups known for RSV (32, 36, 37). Our data support the notion that delivery of N-RSV by BCG is an appropriate way of immunization against RSV, which confers protective T cell immunity that clears viral infection and prevents immunopathology in the airways.

In summary, our results show that CD4+ and CD8+ T cells from mice immunized with BCG-N are recruited early to lungs of RSV-infected mice, promote an efficient clearance of the virus without significant inflammation signs, and prevent RSV-induced disease. This promising immunization approach successfully modifies the cellular and cytokine milieu in such a way that RSV-N Ag elicits an effective response against the virus, avoiding the damaging effects of the inflammatory response against natural infection.

Disclosures
A patent application on this vaccine has been filed.
References


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