

Enhancement of Th1 Lung Immunity Induced by Recombinant *Mycobacterium bovis* Bacillus Calmette-Guérin Attenuates Airway Allergic Disease

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Mycobacterium bovis Bacillus Calmette-Guérin (BCG) has been shown to down-regulate experimental allergic asthma, a finding that reinforced the hygiene hypothesis. We have previously found that recombinant BCG (rBCG) strain that express the genetically detoxified S1 subunit of pertussis toxin (rBCG-S1PT) exerts an adjuvant effect that enhances Th1 responses against BCG proteins. Here we investigated the effect of this rBCG-S1PT on the classical ovalbumin-induced mouse model of allergic lung disease. We found that rBCG-S1PT was more effective than wild-type BCG in preventing Th2-mediated allergic immune responses. The inhibition of allergic lung disease was not associated with increased concentration of suppressive cytokines or with an increased number of pulmonary regulatory T cells but was positively correlated with the increase in IFN- γ -producing T cells and T-bet expression in the lung. In addition, an IL-12-dependent mechanism appeared to be important to the inhibition of lung allergic disease. The inhibition of allergic inflammation was found to be restricted to the lung because when allergen challenge was given by the intraperitoneal route, rBCG-S1PT administration failed to inhibit peritoneal allergic inflammation and type 2 cytokine production. Our work offers a nonclassical interpretation for the hygiene hypothesis indicating that attenuation of lung allergy by rBCG could be due to the enhancement of local lung Th1 immunity induced by rBCG-S1PT. Moreover, it highlights the possible use of rBCG strains as multipurpose immunomodulators by inducing specific immunity against microbial products while protecting against allergic asthma.

Keywords: airways allergic disease; eosinophils; rodent; Th1/Th2 cytokines; knockout mice

Allergic airway inflammation and airway hyperreactivity (AHR) driven by T helper (Th) 2 cells are hallmarks of allergic asthma. There has been a rapid increase in allergic asthma and other atopic disorders in industrialized countries, the cause of which is still a matter of debate (1, 2). Epidemiologic studies suggest a direct relationship between high living standards and hygiene conditions and an increased risk to develop allergic diseases (3–6). The Hygiene Hypothesis proposes that a reduced contact with infectious diseases (e.g., tuberculosis and measles) may favor atopy (2, 7–9). More specifically, epidemiologic data obtained by Shirakawa and colleagues (10) indicated an asso-

CLINICAL RELEVANCE

The recombinant Bacillus Calmette-Guerin strain that expresses the genetically detoxified S1 subunit of pertussis toxin induces an enhanced lung Th1 immunity that in turn attenuates allergic lung disease. The major immunologic mechanism involved in the protection appears to be mediated by local lung immunity against Bacillus Calmette-Guerin. Our work highlights the possible use of recombinant Bacillus Calmette-Guerin strains as multipurpose immunomodulators by inducing specific immunity against microbial products while protecting against allergic asthma.

ciation between the exposures to *Mycobacterium tuberculosis* and a reduced risk of developing asthma. Experimentally, it has been shown that *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) and other mycobacterial infections can down-regulate the establishment of allergen-induced Th2-type cytokine responses (11–15). However, it appears that the degree of suppression of allergic features is dependent on the mycobacterial strain and its preparation (live or dead microorganisms), dose, timing, and route of administration (16, 17).

Two major immunologic mechanisms have been identified for the control of Th2 responses by mycobacterial infections: (1) induction of regulatory T cells (Treg) that secrete suppressive cytokines, which prevent the development or block the activities of Th2 effector cells (18), or (2) development of allergen-specific Th1 cells or Th1-immune deviation, which antagonizes Th2 responses via IFN- γ (1, 19).

An experimental model with recombinant BCG (rBCG) expressing β -galactosidase was tested and found to be effective in preventing Th2 sensitization and consequent β -galactosidase-driven Th2 responses (20). However, in this study, lung-specific immune responses were not investigated.

BCG has been explored for over a decade as a promising live vector for the presentation of heterologous antigens from a variety of pathogens. Specifically, we have constructed strains that express tetanus toxin fragment C (rBCG-FC) (21), a non-toxic fragment of the diphtheria toxin; CRM₁₉₇ (rBCG-DD) (22); and the genetically detoxified S1 subunit of pertussis toxin, S1PT (rBCG-S1PT) (23), aiming at the development of a tetra-valent vaccine against tuberculosis, tetanus, diphtheria, and pertussis (21). Bacterial toxins may have adjuvant properties. When we combined rBCG-FC with rBCG-DD, we observed a reciprocal adjuvant effect on antibody production against both bacterial components, whereas the rBCG-S1PT strain did not induce antibody production against PT but induced strong PT-specific IFN- γ production (21–23). We also found that the expression of the toxin derivatives modified the immune response induced against BCG mycobacterial antigens when

(Received in original form January 30, 2009 and in final form August 7, 2009)

This work was supported by grants from FAPESP to M.R. (04/14297-6) and to L.C.C.L. (99/05202-1).

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This article contains an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 43. pp 243–252, 2010

Originally Published in Press as DOI: 10.1165/rmb.2009-0040OC on October 5, 2009

Internet address: www.atsjournals.org

compared with wild-type (WT) BCG (unpublished results). Together these results indicate that, depending on the derivative expressed by the rBCG strains, an enhanced Th1 skewing condition can be achieved.

In the present work we investigated whether these rBCG strains would protect against allergic asthma-like lung disease. We found that rBCG-S1PT was the most effective strain in preventing Th2-mediated lung immune responses. The inhibition of allergic lung disease was not associated with an increased number of pulmonary regulatory T cells or with increased secretion of suppressive cytokines but was associated with the increase in IFN- γ -producing T cells and T-bet expression in the lung. An IL-12-dependent mechanism appeared to be crucial to the inhibition of lung allergic disease. Moreover, the inhibition of allergic inflammation was observed when allergen challenge was given by the intranasal route but not by the intraperitoneal route.

MATERIALS AND METHODS

Mice

Female BALB/c mice (6–8 wk old) were weight matched and used throughout this study. In some experiments we used IL-12 knockout (KO) and IFN- γ KO mice on C57BL/6J background because these KOs animals in BALB/c background were not available in our breeding unit. All mouse strains were kept under standard pathogen-free conditions and were bred for many generations in the breeding unit (São Paulo, Brazil). Mice were treated according to Animal Welfare guidelines of Institute of Biomedical Sciences.

Bacterial Strains, Growth Conditions, and Vaccine Preparation

M. bovis WT BCG Moreau and recombinant BCG strains expressing the FC, CRM197, or S1PT toxin fragments were grown in Middlebrook 7H9 medium supplemented with albumin-dextrose-catalase (Difco, Detroit, MI), with or without kanamycin (20 mg/ml), at 37°C, using stationary tissue culture flasks. BCG and rBCG vaccines were prepared from midlog-phase liquid cultures. The liquid cultures were centrifuged at $4,000 \times g$, resuspended in 50% glycerol PBS, and maintained frozen at -80°C until used. Immediately before BCG or rBCG instillation, cells were thawed and diluted in saline to reach the appropriate concentrations. The BCG and rBCG preparations were tested for endotoxin content, and we found that the endotoxin levels for all preparations were below the limit of detection as measured by amoebocyte lysate assay (LAL QCL-1000 kit; Biowhittaker, Walkersville, MD).

Alum Gel Preparation

Alum ($\text{Al}(\text{OH})_3$) gel was prepared by precipitating 0.184 M ammonium aluminum sulfate dodecahydrate ($\text{AlH}_4\text{NO}_8\text{S}_2\text{O}_{12}\text{H}_2\text{O}$) with an excess of 1 N NaOH, roughly 2.5V/1V. After precipitation, $\text{Al}(\text{OH})_3$ was suspended in water (Milli Q; Millipore, Etobicoke, ON, Canada) and washed five times at 3,000 rpm for 15 minutes. The final precipitate was dissolved in water, and the concentration of $\text{Al}(\text{OH})_3$ was calculated by determining the dry weight of 1 ml solution. For sensitization, alum gel stock solution (50 mg/ml) was dissolved in 10 ml PBS containing ovalbumin (OVA) (10 $\mu\text{g}/\text{ml}$) in a multitube rotator for 30 minutes.

BCG and rBCG Immunization, OVA/Alum Sensitization, and OVA Challenge

To test the effect of BCG and rBCG on pulmonary allergic reaction induced by OVA, groups of six to eight mice were immunized intranasally with 10^6 CFU of BCG or rBCG-S1PT or with a mixture of 10^7 of rBCG-FC and rBCG-DD diluted in 50 μl of saline 30 days before starting LPS-free OVA (Sigma-Aldrich, St. Louis, MO) sensitizations and challenges (Day -30). Mice were sensitized on Days 0 and 7 by subcutaneous injection (0.4 ml total volume) in the nape of the neck with 4 μg OVA adsorbed to 2.0 mg of alum. On Days 14 and 21, mice were challenged intranasally with 10 μg of OVA in 50 μl of

PBS as previously described (24). In some experiments, the OVA challenge was given intraperitoneally, and the peritoneal allergic inflammation was determined by washing the peritoneal cavity with 3 ml of cold PBS. Mice were anesthetized intramuscularly with 100 μl of a solution containing ketamine (Ketamina Agener; União Química Farmacêutica Nacional S/A, São Paulo, Brazil) and xylazine (Bayer, Pittsburgh, PA) before sensitization or challenge to ensure a complete instillation to the lungs as previously described (25).

To check the growth and immune response against BCG or rBCG strains, we killed five mice per group 1 or 4 weeks after nasal instillation and plated the lungs in Middlebrook 7H10 medium supplemented with albumin-dextrose-catalase (Difco), with or without kanamycin (20 mg/ml), for 30 days at 37°C. After 7 days, the number of CFU/lung was similar to the initial inoculum (i.e., 10^6 CFU for BCG and rBCG-S1PT groups and 10^7 CFU for rBCG-FC/DD group), confirming that the mycobacteria was properly delivered (data not shown). At 30 days postinfection, the CFU/lung was still very high in animals that received BCG or rBCG, and the infection was cleared at Day 120 (see Figure E1 in the online supplement).

Determination of Airway Responsiveness

Airway responsiveness to increasing doses of inhaled methacholine (3, 6, 12, and 25 mg/ml) in conscious unrestrained mice was determined using a single-chamber, whole-body plethysmograph (Buxco Electronics Inc., Wilmington, NC) as previously described (26, 27). After each nebulization with methacholine, recordings were taken for 5 minutes. The Penh values measured during each 5-minute sequence were averaged and expressed for each methacholine concentration. Although the noninvasive method is considered controversial, it was established that when the C57BL/6 animals are challenged once or twice with OVA via the airway route, the Penh measurements correlate with lung resistance and compliance (28). Preliminary results in our laboratory confirmed these findings in BALB/c animals (data not shown).

Bronchoalveolar Lavage and Peritoneal Exudate Cell Lavage

Immediately after assessment of AHR, mice were deeply anesthetized by an intraperitoneal injection of urethane (Sigma-Aldrich) at 15 mg per 10 g body weight. The abdominal cavity was opened, and blood samples from the inferior cava vein were collected for serum antibody determinations. The trachea was cannulated, and lungs were lavaged twice with 0.5 and 1.0 ml of cold PBS. After total cell counting, cytospin preparations of bronchoalveolar lavage (BAL) cells were stained with Diff-Quik (Baxter-Dade AG, Duding, Germany) and differential cell counts were performed on 200 cells on the basis of morphology and staining characteristics. Peritoneal lavage was performed with 5 ml of ice-cold PBS. Total and differential cell counts of BAL fluid and peritoneal exudate cell (PEC) lavage were determined by hemocytometer and cytospin preparation stained with Instant-Prov (Newprov, Brazil). Supernatants from BAL and PEC were collected and frozen at -20°C for cytokines measurements.

Measurement of OVA-Specific IgG1, IgG2a, and IgE Antibodies

OVA-specific antibodies were assayed by sandwich ELISA as previously described (24). Briefly, serum samples were titrated for optimal dilutions for testing different isotypes. For OVA-specific IgE, IgG1, and IgG2a (Southern Biotech, Birmingham, AL), we used, respectively, 1/10, 1/10,000, and 1/100 dilutions. For OVA-specific IgE determinations, plates were coated with anti-IgE, followed by washings and the addition of serum samples (1/10). Subsequently, biotin-labeled OVA was added to the wells, and bound OVA-biotin was revealed. OVA-specific IgE levels of samples were deduced from an internal standard arbitrarily assigned as 1,000 units. For OVA-specific IgG1 and IgG2a antibodies, serum samples were plated on 96 wells previously coated with OVA (2 $\mu\text{g}/\text{well}$). The bound antibodies were revealed with goat antimouse IgG1 or IgG2a, followed by peroxidase-labeled rabbit ant goat antibodies (all from Southern Biotech). The concentrations of each OVA-specific isotype were estimated by comparison with IgG1 and IgG2a standards run in parallel as previously described (24).

Cytokine Measurements

Cytokine protein concentration in the BAL fluid or PEC lavage was quantified by using ELISA kits specific for IL-4, IL-5, IFN- γ , IL-10, and IL-12 (BD Biosciences PharMingen, San Diego, CA) for IL-13 (R&D Systems, Minneapolis, MN) and for TGF β 1 by ImmunoAssay System (Promega, Madison, WI). IL-17 was measured with purified and biotinylated antibodies and standard purified cytokines from Biosciences PharMingen according to the manufacture's recommendations and as previously described (24). Values are expressed as pg/ml deduced from standards run in parallel with recombinant cytokines.

Histologic Analyses

After BAL collection, lungs were perfused via the right ventricle with 10 ml of PBS to remove residual blood, immersed in 10% phosphate-buffered Formalin for 24 hours, and immersed in 70% ethanol until embedded in paraffin. Lung sections (5 μ m) were stained with hematoxylin/eosin for evaluation of peribronchial and perivascular lung inflammation or with periodic acid-Schiff (PAS)/hematoxylin for evaluation of mucus production as previously described (29). Briefly, a quantitative digital morphometric analysis was performed using the application program Metamorph 6.0 (Universal Images Corp., CA). The circumference area of bronchi and the PAS-stained area were electronically measured, and the mucus index was determined by the formula (PAS stained area/bronchial circumference area) $\times$ 100.

ELISPOT

Lung cells, purified from lung tissue after collagenase (2 mg/ml) and DNase (1 mg/ml) (Sigma-Aldrich) digestion or BAL cells isolated from mice submitted to the experimental protocols were incubated for 20 hours with α -CD3 (1 μ g/ml) or only media as control (nonstimulated) in ELISPOT plates (BD Biosciences) coated with antibodies against IL-4 or IFN- γ . The number of spot-forming cells (SFC) secreting IL-4 or IFN- γ was detected through ELISPOT technique according to the manufacture's recommendations (BD Biosciences). The average number of spots in triplicate wells was calculated by subtracting non-stimulated wells and considered as the number of SFC/ 10^5 cultured BAL/lung cells.

Foxp3 Staining

Foxp3 was accessed by intracellular staining. Briefly, cells isolated from lung tissue after collagenase and DNase treatments were surface stained with PE-labeled anti-CD4 (clone L3T4) and Cy5-labeled anti-CD25 (clone PC61) antibodies (BD Biosciences), fixed, permeabilized (BD Cytofix/Cytoperm kit; BD-PharMingen), stained with FITC-labeled anti-Foxp3 antibody (clone FJK-16S; e-Biosciences, San Diego, CA), and analyzed in a FACSCalibur (BD Biosciences).

Real-Time PCR

After perfusion, the upper right lobule was placed in Trizol reagent (Invitrogen, Carlsbad, CA) and homogenized with a Fisher Powergen 125 homogenizer. Samples were incubated at room temperature for 5 minutes, and then 0.2 ml of chloroform per 1 ml of Trizol was added to perform the RNA extraction. Samples were centrifuged at $10,000 \times g$ at 4°C for 20 minutes, and the aqueous phase was transferred to clean tube. After 0.5 ml of isopropanol was added, the samples were incubated for 10 minutes at room temperature and centrifuged at $4,000 \times g$ at 4°C for 30 minutes. The pellet containing the total RNA was washed twice with 75% ethanol and air dried. RNA samples were resuspended in 0.1 ml of diethylpyrocarbonate-treated water and stored at -80°C . To obtain the RNA concentration and purity, samples were spectrophotometrically measured at 260/280 nm. Complementary DNA was synthesized using 3 mg of RNA through a reverse transcription reaction (Superscript II, Gibco). Real-time PCR quantitative analyses were performed in an ABI Prism 5700 Sequence Detection System using the SYBR-green fluorescence quantification system (Applied Biosystems, Warrington, UK) for quantitation of amplicons. The standard PCR conditions were 95°C (10 min), 56°C (1 min), and 72°C (2 min), followed by the standard denaturation curve. The sequences of the primers, the predicted amplicon sizes, and the melting temperatures (Mt) used were as follows: β -actin sense ATGTTTGA GACCTTCAACAC, antisense CACGTCACACTTCATGATGG,

which results in a 495-base pair (bp) amplification product (Mt, 56°C); T-bet sense AACCAGTATCTGGTCCCA, antisense TGT CGCCACTGGAAGGATAG (436 bp; Mt, 57°C); GATA-3 sense AAGGCATCCAGACCCGAAAC, antisense ACCCATGGCGGTG ACCATGC (255 bp; Mt, 55°C). PCR conditions for each target were conscientiously optimized with regard to primer concentration, absence of primer dimmer formation, and efficiency of amplification of target genes and housekeeping gene control. SYBR Green Master Mix (Applied Biosystems, Foster City, CA), 400 nM specific primers, and 2.5 ng complementary DNA were used in each reaction. The threshold for positivity of real-time PCR was determined based on negative controls. The relative levels of gene expression were calculated according to the instructions in the User's Bulletin (P/N 4303859) from Applied Biosystems by reference to the β -actin in the sample, using the cycle threshold (Ct) method as previously described (30). Briefly, Ct is the point at which the exponential increase in signal (fluorescence) crosses a somewhat arbitrary signal level (usually 10 times background). The mean Ct values from duplicate measurements were used to calculate the expression of the target gene, with normalization to β -actin, and then compared with the target-internal control subjects to calculate the fold increase expression using the 2-DCt formula. Negative controls without RNA and without reverse transcriptase were also performed. The results show one of two representative experiments.

Statistical Analysis

ANOVA was used to determine the levels of difference between all groups. Comparisons of all pairs were performed by Tukey-Kramer honest significant difference test. Values for all measurements are expressed as means $\pm$ SEM, and *P* values for significance were set to 0.05.

RESULTS

Immunomodulatory Effects of Recombinant BCG Strains on Airway Allergic Responses

Because we found that rBCG strains expressing different toxin derivatives appear to induce toxins-specific or mycobacterial-specific Th1 skewing responses (21–23 and unpublished results), we first determined the effects of intranasal administration of different rBCG strains on the airway allergic responses induced by OVA sensitizations and challenges in comparison with WT BCG. Briefly, BALB/c mice received intranasal WT BCG (BCG/OVA group) or rBCG-S1PT (rBCG-S1PT/OVA group as Th1-inducing strain) or a mixture of rBCG-FC and rBCG-DD strains (rBCG-FC/DD/OVA group as Th2-inducing strains) 30 days before OVA sensitizations and challenges. Two additional groups were used: One group consisted of mice that received PBS before the OVA sensitization (PBS/OVA group), and the other group consisted of nonmanipulated control mice. We used the later group as control because preliminary results obtained in animals that received only alum (sham-sensitized) and were challenged twice with OVA were similar to the nonmanipulated control group. As expected, mice that received PBS and were sensitized and challenged with OVA developed allergic airway inflammation, as demonstrated by the increased number of total cells and eosinophils in BAL when compared with control mice (Figures 1A and 1B). The total number of cells was decreased in mice receiving rBCG-S1PT but not in mice receiving the BCG or rBCG-FC/DD combination (Figure 1A).

When comparing the number of eosinophils of the PBS/OVA group with those obtained in BCG-treated animals, we found that only animals receiving the rBCG-S1PT strain exhibited a significant decrease (roughly 77% inhibition) in the number of eosinophils in BAL (Figure 1B). The number of lymphocytes was lower, although not statistically significant, in rBCG-S1PT- or BCG-treated mice as compared with PBS/

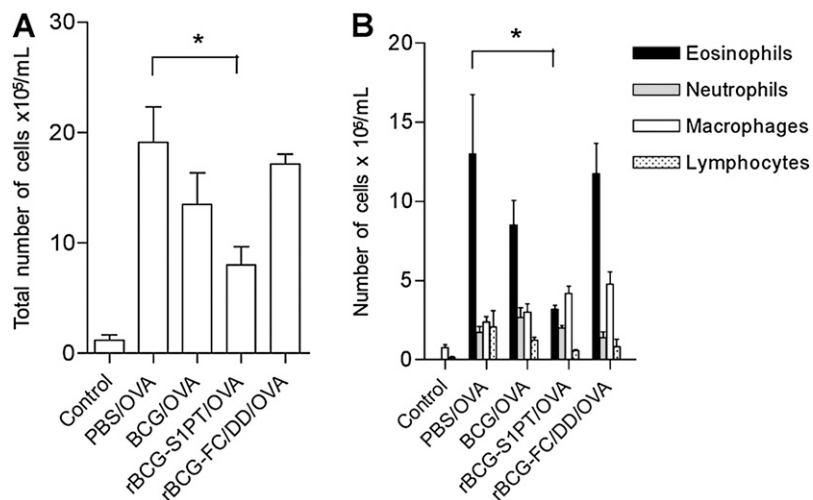


Figure 1. Effect of *Bacillus Calmette-Guérin* (BCG) and recombinant *Bacillus Calmette-Guérin* (rBCG) administration on ovalbumin (OVA)-induced airway inflammation. BALB/c mice received intranasal BCG (BCG/OVA group), rBCG (rBCG/OVA groups), or PBS (PBS/OVA group) 30 days before subcutaneous OVA/Alum sensitizations and intranasal OVA challenges. The control group consisted of nonmanipulated animals. The experiments were performed 24 hours after the last OVA challenge. (A) Brochoalveolar lavage (BAL) total cell counts. (B) BAL differential cell counts. Results are expressed as means $\pm$ SEM for groups of five mice and are representative of three experiments. *Significant differences ($P < 0.05$) when compared with the PBS/OVA group.

OVA (Figure 1B), whereas the numbers of other cell types were comparable among the studied groups (Figure 1B). These results demonstrate that the intranasal administration of rBCG strains can prevent OVA-induced airway inflammation, with rBCG-S1PT being the most effective in preventing the establishment of the allergic symptoms.

Effect of Intranasal Administration of rBCG Strains on OVA-Specific Antibody Production

We evaluated whether the rBCG strains could also modulate the levels of OVA-specific IgG1, IgG2a, and IgE isotypes. As expected, the production of OVA-specific IgG1, IgG2a, and IgE antibodies was increased in allergic mice when compared with the control group (Figures 2A–2C). No significant differences were observed in the production of OVA-specific IgG1 among the groups sensitized and challenged with OVA (Figure 2A), and the changes in OVA-specific IgG2a levels, a Th1-affiliated isotype, or OVA-specific IgE, a Th2-associated isotype, were modest (Figures 2B and 2C). IgG2a levels were slightly higher in mice that received BCG or rBCG-S1PT than in the PBS/OVA group (Figure 2B), whereas the levels of OVA-specific IgE antibodies induced by OVA-sensitized and OVA-challenged animals were similar in all BCG groups, except in the rBCG-S1PT/OVA group, where they were slightly lower (Figure 2C). Thus, the overall OVA-specific isotype profile of BCG or rBCG groups was similar to PBS/OVA group with a more Th2 profile than Th1, indicating that BCG or rBCG administration was not effective in modifying OVA-specific systemic humoral immunity.

Intranasal Administration of rBCG-S1PT Prevents the Development of Lung Pathology

It is well established experimentally that allergic pathology is associated with AHR and mucus hyperproduction. Because rBCG-S1PT administration was efficient in downmodulating airway allergic inflammation, in the next experiments we only used rBCG-S1PT and WT BCG for comparison. Determination of AHR in response to increasing doses of β -methacholine by whole-body plethysmography revealed that OVA-sensitized and OVA-challenged mice showed a substantial increase in methacholine response when compared with control mice (Figure 3A). BCG or rBCG-S1PT administration prevented methacholine responsiveness, with the administration of rBCG-S1PT being more effective than BCG, as revealed by the area under the curve obtained from Penh values (Figure 3B). BCG or rBCG-S1PT treatments on their own did not alter the Penh values or total and differential cell counts in BAL when compared with the control group (Figure E2). Next we determined goblet cell metaplasia, and a similar inhibitory effect of BCG or rBCG-S1PT on allergic responses was observed for mucus production, quantified by PAS-positive goblet cell staining. Figure 3C shows that PAS-positive goblet cells staining and peribronchovascular inflammation was more attenuated in the rBCG-S1PT/OVA group than in the BCG/OVA group. The reduction of mucus production was confirmed by morphometry with measurement of mucus index (Figure 3D). We then determined type-2 cytokines in BAL fluid by measuring the levels of IL-4, IL-5, and IL-13. The IL-4 production was negligible in animals that received BCG or rBCG-S1PT when

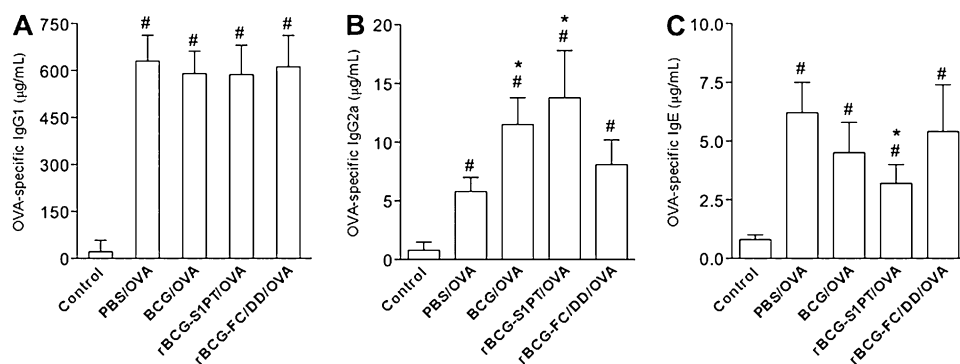


Figure 2. Modulation of OVA-specific antibody production by rBCG strains. BALB/c mice received intranasal BCG (BCG/OVA group), rBCG (rBCG/OVA groups), or PBS (PBS/OVA group) 30 days before OVA/Alum sensitizations and OVA challenges. The control group consisted of nonmanipulated animals. The experiments were performed 24 hours after the last OVA challenge. (A) OVA-specific IgG1, (B) OVA-specific IgG2a, and (C) OVA-specific IgE were determined in the sera by sandwich ELISA. Results are expressed as means $\pm$ SEM for groups of five mice and are representative of three experiments. *Significant differences ($P < 0.05$) when compared with PBS/OVA group. #Significant differences ($P < 0.05$) when compared with control group.

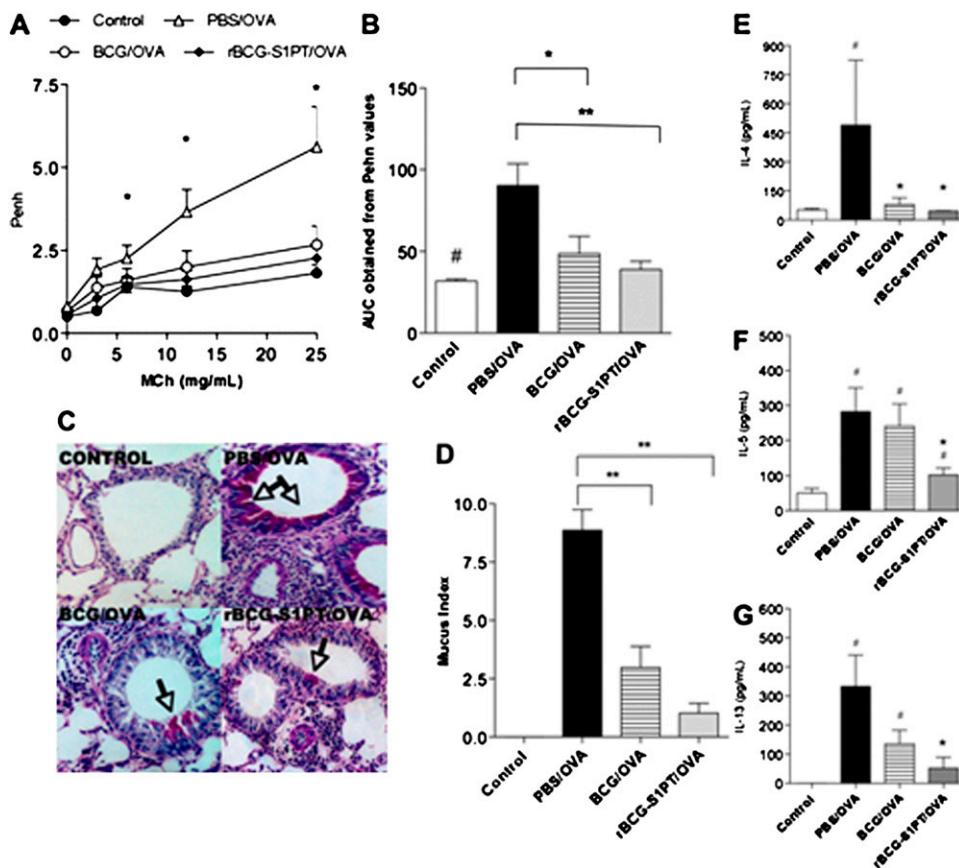


Figure 3. BCG or rBCG-S1PT prevents the development of allergic lung pathology. BALB/c mice received intranasal BCG (BCG/OVA group), rBCG-S1PT (rBCG-S1PT/OVA group), or PBS (PBS/OVA group) 30 days before OVA/Alum sensitizations and OVA challenges. The control group consisted of nonmanipulated animals. The experiments were performed 24 hours after the last OVA challenge. (A) Airway hyperreactivity to increasing doses of methacholin. (B) Area under the curve (AUC). (C) Lung histology. (D) Mucus index (note mucus secretion by Goblet cells indicated by PAS staining [red arrows]). Cytokine secretion in BAL: (E) IL-4, (F) IL-5, and (G) IL-13. Results are expressed as means $\pm$ SEM for groups of five mice and are representative of three experiments. *Significant differences ($P < 0.05$) when compared with PBS/OVA group. #Significant differences ($P < 0.05$) when compared with control group.

compared with the PBS/OVA group, reaching control values (Figure 3E). The levels of IL-5 or IL-13 were also decreased in the BCG/OVA or rBCG-S1PT/OVA groups, but statistical significance was obtained only when comparing the rBCG-S1PT/OVA group with the PBS/OVA group (Figures 3F and 3G). These data confirm and extend the notion that rBCG-S1PT infection is the most effective strain among the strains tested for the prevention of lung allergic pathology.

rBCG-S1PT Infection Does Not Increase the Production of Suppressive Cytokines or the Number of CD4⁺Foxp3⁺ Regulatory T Cells in BAL

It was previously shown that subcutaneous injection of *Mycobacterium vaccae* induces allergen-specific IL-10- and TGF- β -secreting regulatory T (Treg) cells, which dampen the allergic responses (18). To determine whether Treg cells or suppressive cytokines could be involved in the inhibition induced by our BCG strains, we compared the production of suppressive cytokines and the number of Treg cells in mycobacteria-treated allergic mice. The levels of IL-10 or TGF- β in the BAL fluid of allergic mice were increased in relation to the control group, but the groups that received mycobacteria showed comparable levels of these cytokines (Figures 4A and 4B). Although suppressive cytokines were not augmented in mycobacteria-treated mice, Treg cells could still be involved in down-regulation of allergic responses. To better visualize Tregs in our model, we stained BAL cells with a Treg-specific transcription factor Foxp3 fluorescent antibody and determined the frequency and the number CD4⁺ T cells expressing Foxp3. The percentage of Treg cells was comparable in the PBS/OVA (9.1%), BCG/OVA (9.8%), or the rBCG-S1PT/OVA groups (10.3%) (Figure 4C). Nevertheless, we determined that the absolute number of Treg cells present in BAL was decreased in

mycobacteria-treated groups when compared with the PBS/OVA group (Figure 4E). In fact, the decreased number of Treg cells in infected mice reflected the decreased number of CD4⁺ T lymphocytes present in the BAL of these animals (Figure 4D). These data indicate that BCG or rBCG-S1PT administration does not augment the influx of Treg cells to the BAL after OVA challenge in sensitized mice.

Intranasal Administration of rBCG-S1PT Decreases IL-4 and Increases IFN- γ -Producing Cells in the Airways Correlating with GATA-3 and T-bet Expression, Respectively

Because OVA-specific systemic antibody response was more skewed toward a Th2 pattern, we asked whether BCG or rBCG administration was affecting lung immunity. To establish the hallmarks of T-cell immunity that predominate in the airways of our experimental groups, we first determined, by ELISPOT, the number of IFN- γ - or IL-4-secreting T cells by BAL or parenchymal lung cells, which were cultured with or without anti-CD3 antibody, and the number of SFC was quantified. Additionally, we investigated the master transcription factors of Th2 (GATA-3) and Th1 (T-bet) immunity (31) in whole lung by real-time PCR. We found a significant increase in IFN- γ SFCs in BAL cells of animals treated with mycobacteria in relation to the allergic group, the latter of which showed results similar to that of the control group (Figure 5A). The production of IFN- γ was directly correlated with T-bet expression (Figure 5C). Conversely, the PBS/OVA group showed the highest number of IL-4 SFC in the BAL and lungs (Figures 5D and 5E), and this was correlated with the highest GATA-3 expression (Figure 5F). The number of IL-4 SFC and the expression of GATA-3 decreased in BCG- or rBCG-S1PT-treated animals when compared with the PBS/OVA group (Figures 5D5F). These results indicate that

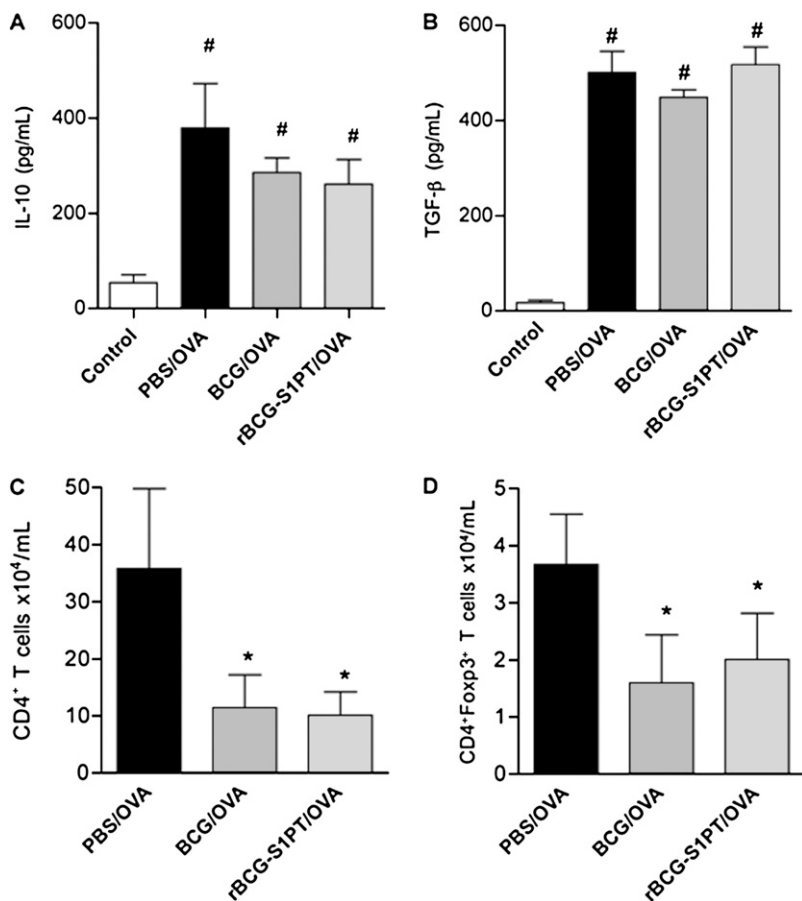


Figure 4. Effect of BCG or rBCG-S1PT on T-cell regulatory activity in the BAL. BALB/c mice received intranasal BCG (BCG/OVA group), rBCG-S1PT (rBCG-S1PT/OVA group), or PBS (PBS/OVA group) 30 days before OVA/Alum sensitizations and OVA challenges. The control group consisted of nonmanipulated animals. The experiments were performed 24 hours after the last OVA challenge. (A) IL-10 and (B) TGF- β secretion in the BAL. (C) Representative FACS dot plot of double immunofluorescence-stained CD4⁺ T cells gated on lymphocytes (peritoneal exudate-conjugated anti-CD4 and intracellular staining with FITC-conjugated anti-Foxp3 antibodies). (D) Quantitative analysis of (D) CD4⁺ and (E) CD4⁺Foxp3⁺ T cells in the BAL of treated animals. Results are expressed as means $\pm$ SEM for groups of five mice and are representative of three experiments. *Significant difference ($P < 0.05$) when compared with PBS/OVA group. #Significant difference ($P < 0.05$) when compared with control group.

rBCG-S1PT administration is superior to BCG in inducing lung Th1 immunity.

IL-12 Production Is Involved in the Downmodulation of Allergic Airway Inflammation by rBCG-S1PT

Because rBCG-S1PT administration strongly induced T-bet expression in the lung with the appearance of IFN- γ secreting cells, we sought to define the role of IFN- γ in our model by using IFN- γ KO mice. The major drawback of this approach is that mice lacking IFN- γ develop a severe airway neutrophilia accompanied by overt cachexia 30 days after BCG or rBCG-S1PT administration (data not shown), precluding the use of these animals for this purpose. Because IL-12 is required for optimal development of IFN- γ -producing T cells (32), we used IL-12 KO animals in our model. Administration of mycobacteria to IL-12-deficient mice did not result in a wasting disease as was observed in IFN- γ KO animals (data not shown). In IL-12-deficient mice, we found no significant difference in AHR between the nonmanipulated control group and the allergic group (data not shown). Therefore, we only studied the effect of rBCG-S1PT administration on allergic airway inflammation in IL-12-deficient mice, and for comparison we used WT C57BL/6 (B6) mice. Figure 6A shows that OVA-sensitized and OVA-challenged WT mice displayed a significant influx of inflammatory cells in BAL when compared with control mice. As expected, the migration of inflammatory cells was minimal in the animals treated with rBCG-S1PT. In contrast, in IL-12-deficient mice, the rBCG-S1PT/OVA group showed a robust cellular influx, similar to that of the PBS/OVA group (Figure 6A). Regarding eosinophil cell counts (Figure 6B), we found that in the WT mice, administration of rBCG-S1PT almost completely inhibited

airway eosinophilia (95% of inhibition), whereas in IL-12-deficient mice, the inhibition was only partial (57% of inhibition). Next, we measured the levels of type-2 cytokines in the BAL and found that in WT animals, rBCG-S1PT reduced the levels of IL-5 and IL-13, almost reaching control levels (Figures 6C and 6D). Conversely, in IL-12-deficient mice, the levels of IL-5 in the rBCG-S1PT/OVA group were similar to those of the PBS/OVA group, whereas IL-13 levels were slightly decreased (Figures 6C and 6D). Collectively, these data indicate that IL-12 is involved in the down-regulation of Th2-mediated allergic inflammation induced by rBCG-S1PT treatment.

Intranasal Administration of rBCG-S1PT Does Not Affect OVA-Induced Peritoneal Allergic Inflammation

To further characterize the mechanisms by which rBCG-S1PT affects airway allergic disease, we designed a protocol to test whether the inhibition is due to a local effect on lung environment or due to a systemic effect on OVA-induced Th2-mediated allergy. For this, we administered rBCG-S1PT to sensitized animals as done previously, but the OVA challenge was given intraperitoneally instead of intranasally. We found that in OVA-sensitized animals, the intraperitoneal OVA challenge increased the number of total peritoneal cells and eosinophils when compared with the control group (Figures 7A and 7B). BCG or rBCG administration failed to inhibit allergic peritoneal inflammation, as determined by total and eosinophil cell counts (Figures 7A and 7B). Accordingly, the levels of IL-5 in peritoneal washes were also not inhibited in these groups (Figure 7C). These results clearly indicate that inhibition of allergic inflammation by rBCG appears to be restricted to the lung.

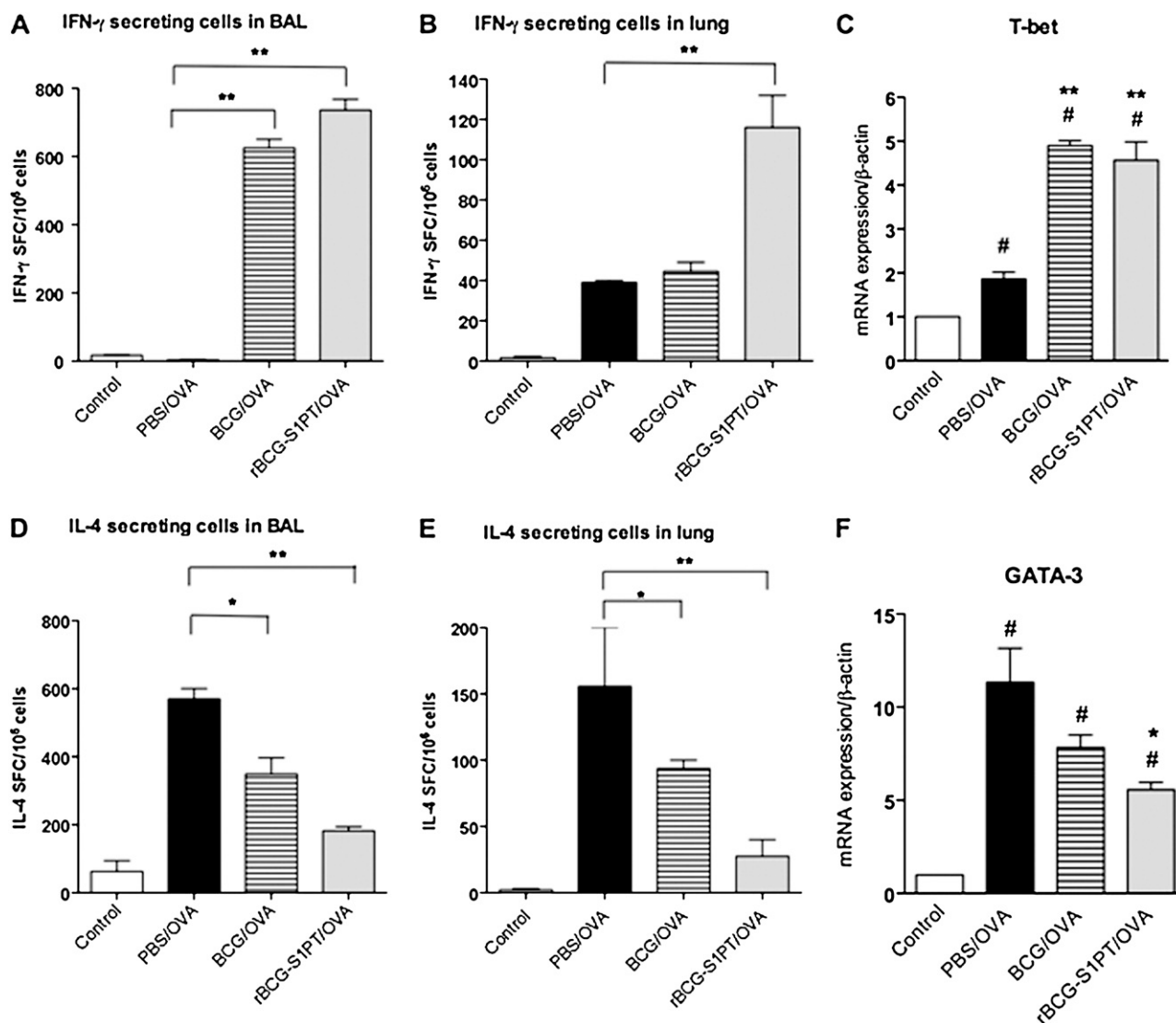


Figure 5. BCG or rBCG-S1PT increases IFN- γ -producing cells and decreases IL-4-producing cells in BAL and lung tissue and alters the expression of the master regulators of Th1/Th2 differentiation BALB/c mice received intranasal BCG (BCG/OVA group), rBCG-S1PT (rBCG-S1PT/OVA group), or PBS (PBS/OVA group) 30 days before OVA/Alum sensitizations and OVA challenges. The control group consisted of nonmanipulated animals. BAL and lung cells were harvested 24 hours after the last OVA challenge and were incubated for 24 hours with α -CD3 (1 μ g/ml) or only media as control (nonstimulated) in ELISPOT plates coated with capture antibodies against IFN- γ or IL-4. The numbers of spot-forming cells (SFC) secreting IFN- γ and IL-4 (A and B) in the BAL and (B and E) in the lung tissue were detected through ELISPOT. The average number of spots in triplicate wells was calculated by subtracting nonstimulated wells and considered as the number of SFC/10⁶ cultured cells per ml. The expression of Th1/Th2 master regulators was analyzed by real-time PCR. The upper right lung lobule was removed, and total RNA from lung tissue of treated mice was extracted as specified in MATERIALS AND METHODS, and the mRNA expression of (C) T-bet and (F) GATA-3 was quantified as its ratio to β -actin. Results are representative of two independent experiments.

DISCUSSION

BCG, a live attenuated *M. bovis*, is used in many countries as a vaccine against human tuberculosis and is an inducer of a Th1-like response. Cellular and humoral immune responses are observed in animal models immunized with BCG. Therefore, this microorganism constitutes an ideal live vector for the presentation of foreign antigens or allergens to the immune system.

The present study investigated the potential use of previously constructed rBCG strains (21–23) in modulating allergic features in the mouse model of allergic airway disease induced by OVA. We took advantage of the fact that rBCG strains expressing different bacterial toxin derivatives display diverse

immunomodulatory effects against mycobacterial antigens. More recently it was demonstrated that rBCG-S1PT exhibits an enhanced therapeutic effect against bladder cancer when compared with WT BCG (33).

In line with the above findings, we found that the rBCG-S1PT prevented the development of asthma-like responses. Indeed, the inhibition of airway eosinophilic inflammation, AHR, type 2 cytokine production, and mucus hypersecretion was more pronounced in animals treated with rBCG-S1PT than with BCG or rBCG-FC with rBCG-DD (see Figure 1). In addition, we found that the protection induced against lung allergy was long lasting because after 4 months of rBCG-S1PT administration, the allergic lung responses were still attenuated (data not shown). In conclusion, we provide data indicating that

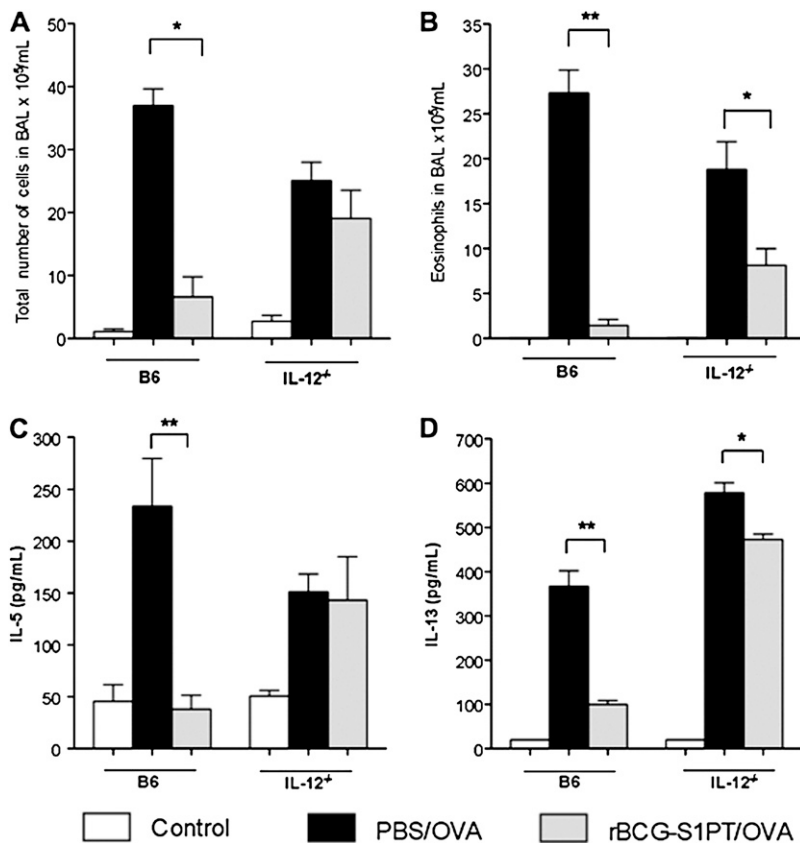


Figure 6. Prevention by rBCG-S1PT of allergic airway inflammation induced by OVA is partially dependent on IL-12. Wild-type (WT) or IL-12 KO B6 mice received intranasal 10⁶ CFU of rBCG-S1PT (rBCG-S1PT/OVA group) or PBS (PBS/OVA group) 30 days before subcutaneous OVA/Alum sensitizations and intranasal OVA challenges (intranasal). The control group consisted of nonmanipulated animals. The experiments were performed 24 hours after the last OVA challenge. Total numbers of (A) cells, (B) eosinophils, (C) IL-5 secretion, and (D) IL-13 secretion in the BAL fluid of WT and IL-12 KO mice were determined. Results are expressed as means $\pm$ SEM for groups of five mice and are representative of two experiments. Significant differences ($P < 0.05$) when compared with the PBS/OVA group.

rBCG-S1PT is superior in preventing Th2-driven allergic airway disease to BCG.

One proposed mechanisms for mycobacterial interference with the Th2-driven lung pathology would be the induction of T-regulatory mechanisms and the secretion of suppressive cytokines (18). It has been shown that a killed *M. vaccae*-suspension induced allergen-specific Treg cells that protected against airway inflammation by an IL-10- and TGF- β -mediated mechanism (18). However, in our model, suppressive cytokines such as IL-10 and TGF- β were not increased in rBCG-S1PT- or BCG-treated animals. Moreover, we found a reduction in the total number of CD4⁺CD25⁺ T cells and CD4⁺Foxp3⁺ Treg cells in the BAL of these groups. These findings argue against the notion that regulatory mechanisms exerted by Treg and suppressive cytokines are operating in lung. Another argument for not considering the involvement of Treg in our model is the

fact that Treg cells exert a systemic inhibitory effect and that the inhibition of allergy was restricted to the lung but not to the peritoneal cavity.

It has been previously shown that rBCG expressing a specific allergen is efficient in inhibiting allergen-specific IgE response when compared with animals that received WT BCG and were allergen sensitized and challenged (20). However, this work did not investigate the allergen-specific lung immune responses to ascertain whether the lung is spared from allergen-induced inflammation. Our approach was different because we used rBCG strains expressing unrelated antigens, which have adjuvant activities, and evaluated their effects on allergen-specific immune responses.

It is not clear how the immunomodulation of BCG is modified by the expression of the toxin derivatives, but it is possible that these toxin fragments engage different pattern

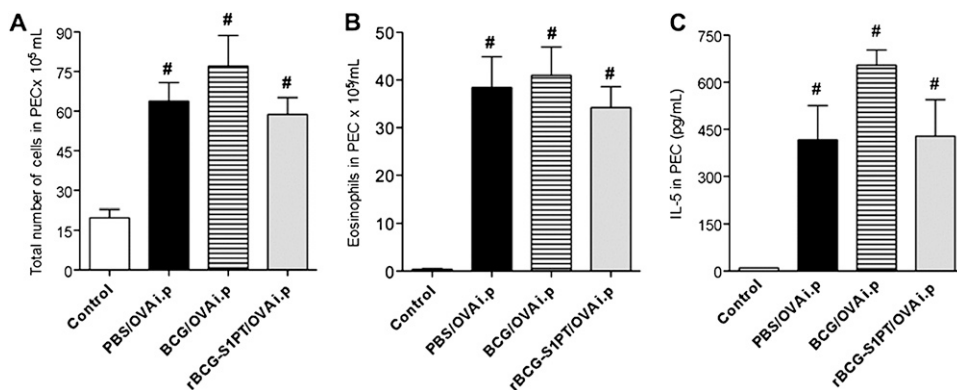


Figure 7. BCG or rBCG-S1PT does not prevent the peritoneal inflammatory reaction induced by OVA. BALB/c mice received intranasal BCG (BCG/OVA group), rBCG-S1PT (rBCG-S1PT/OVA group), or PBS (PBS/OVA group) 30 days before OVA/alum sensitizations and OVA challenges. The control group consisted of nonmanipulated animals. The experiments were performed 24 hours after the last intraperitoneal OVA challenge. (A) Total peritoneal exudate cell (PEC) counts. (B) Eosinophils in PECs. (C) IL-5 secretion in PECs. Results are expressed as means $\pm$ SEM for groups of six mice and are representative of two experiments. #Significant differences ($P < 0.05$) when compared with control group.

recognition receptors. Indeed, it was shown that, similar to LPS, PT triggers the TLR4-signaling pathway, resulting in the translocation of NF- κ B (34, 35). It remains to be determined whether the nontoxic fragment, S1PT, is also a TLR4 agonist.

Because TLR4 agonists are strong Th1 adjuvants (36, 37), one possibility for the inhibitory effect of rBCG-S1PT on Th2 immunity is that, during allergen sensitization, rBCG-S1PT acts as a Th1 adjuvant and therefore shifts the allergen-specific responses toward a Th1 pattern, with the production of type 1 cytokines such as IFN- γ (19). In our model, inhibition of allergic responses by BCG or rBCG-S1PT was positively correlated with the number of cells secreting IFN- γ or with the expression of T-bet, a master transcription factors of Th1 immunity, in lung (38) (see Figure 5). In addition, the treatment with BCG or rBCG-S1PT decreased the number of cells secreting IL-4 or the expression of GATA-3, a master regulator of Th2 immunity (39), in the lung (see Figure 5). However, we could not test directly this hypothesis because, when IFN- γ -deficient mice received BCG, the animals became severely sick, confirming previous reports showing that IFN- γ KO mice cannot control mycobacterial infections (40). As an alternative, we used IL-12-deficient mice, which were shown to be more resistant than IFN- γ -deficient mice to mycobacterial infection (40). We confirmed these findings by showing that upon BCG administration, the animals did not succumb to infection with BCG or rBCG-S1PT. However, in the absence of IL-12, a fundamental cytokine for robust IFN- γ production (32), the degree of inhibition of allergic lung responses by rBCG-S1PT was only partial (Figure 6). We favor the involvement of IFN- γ and not IL-12 by itself or IL-17 that is also impaired in our IL-12-deficient mice in the inhibition of allergic inflammation because we could not find an increase in IL-12 or IL-17 levels in BALB/c animals that received BCG or rBCG (data not shown).

Altogether, our data indicate an important role for IL-12 in the prevention of allergic responses probably via IFN- γ production. It is known that B6 animals are more Th1-prone than BALB/c animals; therefore, it is of note that the inhibition of allergic inflammation was much more pronounced in WT B6 animals than in WT BALB/c animals, a finding that is line with our assumption that enhanced Th1 immunity is the main mechanism to the attenuation of allergic airway disease. It is conceivable that rBCG-S1PT acts as a stronger Th1 adjuvant than the other BCG strains and therefore shifts the allergen-specific responses toward a Th1 pattern. However, we do not favor this notion because the production of OVA-specific IgE antibodies, a Th2-affiliated isotype, was still robust in BCG-treated animals (see Figure 2C). The notion that allergen-specific Th1 responses antagonize allergen-specific Th2 immunity is an oversimplification because experimental and clinical evidence indicates that asthma phenotypes could be also categorized as allergen-specific Th1 or Th1/Th2 phenotypes (41). Thus, a potential risk of *Mycobacteria*-based allergen delivery systems is the development of a type 1 asthma phenotype (42, 43). In our model, the lack of lung neutrophilic inflammation in animals treated with rBCG argues against allergen-driven Th1 responses (41). To further clarify this point, we used the same protocol of allergen sensitization and rBCG treatment, but instead of giving the OVA challenge via the intranasal route, we challenged the animals by the intraperitoneal route. Under these circumstances, rBCG-S1PT administration failed to inhibit eosinophilic inflammation and type 2 cytokine production in the peritoneum (Figure 7). We conclude that treatment with rBCG did not lead to a systemic deficiency of allergen-specific Th2 effector cells. This finding indicates that an allergen-independent mechanism is controlling the lung allergic responses. In line with this assumption,

we found that rBCG was also able to attenuate an established airway allergic disease (unpublished observation).

A plausible explanation for the inhibition of allergic lung responses by rBCG is that local immune responses due to the presence of rBCG-S1PT create a milieu in the lung that impairs Th2 effector functions directly or indirectly by inhibiting Th2 cell recruitment. Because this inhibition is partially dependent of IL-12, it follows that local Th1 lung immunity triggered by rBCG-S1PT (as revealed by the increased number of IFN- γ -secreting cells and the augmented expression of Tbet) appears to be the main mechanism involved in the suppression of lung allergy. Although our data support this assumption, there are limitations that should be addressed in future work, such as differences in Th2 responses among mouse strains and direct comparison of isolated T cells from lung, draining lymph node, and spleen.

According to the classical view of the hygiene hypothesis, infections during childhood can affect the susceptibility to develop allergic diseases by inducing systemic allergen-specific Th1 immunity or Treg cell activity (1, 3, 4, 6, 7, 9, 18). Our data offer a nonclassical interpretation for the hygiene hypothesis highlighting the role of regional rather than systemic immunity in the protection against asthma and provide evidence that rBCG, besides inducing specific immunity against various microbial products, might have an additional local therapeutic effect.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgments: The authors thank Paulo Albe for assistance in histology and Eliane Gomes for technical assistance.

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