
Inhibition of allergen-induced airway remodeling by neonatal bacillus Calmette-Guerin vaccination is associated with interferon-gamma-producing T cells but not regulatory T cells in mice

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Background: Epidemiological assessments of patients and studies using animal models show that exposure to *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) vaccine in early life prevents asthma development. However, little is known about the potential of neonatal BCG vaccination in preventing the development of airway remodeling of asthma.

Objective: To investigate the effects of multiple BCG vaccinations of neonates on the airway remodeling in mice and the accompanied pulmonary T cell responses.

Methods: BALB/c neonates were vaccinated with BCG 3 times. At 5 and 7 weeks of age, the mice were sensitized and then challenged with aerosolized ovalbumin (OVA) 3 times per week for 8 successive weeks. The extent of airway remodeling and induced pulmonary T cell responses were characterized.

Results: Multiple BCG vaccinations of neonates reduced OVA-induced remodeling events, including levels of peribronchial α -smooth muscle actin, peribronchial fibrosis, and airway epithelial mucin accumulation. The BCG vaccinations also decreased peribronchial cells expression of transforming growth factor beta 1 (TGF- β 1). In contrast, BCG vaccinations increased the frequency of interferon-gamma (IFN- γ)-producing T cells in the lung and IFN- γ level in BALF, with no effects on pulmonary regulatory T cells and IL-17-producing T cells.

Conclusions: Our data suggest that multiple BCG vaccinations of neonates reduced metrics characteristic of allergen-induced airway remodeling. More importantly, this decrease was not associated with an increased number of pulmonary regulatory T cells but instead correlated with an increase of IFN- γ -producing T cells.

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INTRODUCTION

Asthma is a chronic airway inflammatory disease, which is characterized by the presence of increased number of eosinophils and a predominant T helper (Th)2 immune response. The persistent Th2 response in a chronic setting correlates with airway remodeling, characterized by increased goblet cell numbers and subsequent epithelial cell mucin accumulation, smooth muscle hypertrophy/hyperplasia, collagen and elastic fiber deposition, and increased angiogenesis.

Current asthma management strategies principally involve the use of inhaled corticosteroids; however, these therapies

have little effect on inhibiting disease progression or the development of airway remodeling. Therefore, preventive interventions may be a better approach to protect individuals from asthma. Bacillus Calmette-Guerin (BCG) adjuvant therapy as a strong inducer of Th1-type immune response is considered a potential candidate. Recently, a meta-analysis of 23 independent epidemiological studies suggested a protective effect of BCG exposure on childhood asthma occurrence.¹ Many studies demonstrate that BCG adjuvant therapy is effective in preventing or treating asthma in animal models of allergic respiratory inflammation.^{2–5} However, the potential of BCG vaccination in preventing the development of airway remodeling linked with asthma is unclear.

Immunity in newborns is characterized by minimal Th1 function but an excess of Th2 activity,⁶ which suggests that the greatest possibility of modifying asthma risk by inducing a Th1-biased immune response is in early life. Previously, our group has demonstrated that multiple BCG vaccinations of neonates has a long-term effect attenuating allergen-induced airway hyperresponsiveness (AHR) and airway eosinophilia in a mouse model of asthma.⁷ In this study, using a chronic model of ovalbumin (OVA)-induced asthma in mice, we further demonstrate that multiple BCG vaccinations of neo-

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nates are capable of reducing the airway remodeling events associated with chronic allergen challenge.

METHODS

Animals

BALB/c mice were purchased from the Experimental Animal Center of Zhejiang University and housed in specific pathogen-free facility in our campus. All protocols in this study were approved by the Ethical Committee for Animal Studies at Zhejiang University, China.

BCG Vaccination

Freeze-dried living BCG (D2-BP302 strain, Biological Institute of Shanghai, China), was suspended in sterile water. Mice were injected subcutaneously at the base of the tail with 25 μ L of BCG suspension (10^5 infection units) at birth, and boosted twice on days 7 and 14 with the same dose of BCG. The unvaccinated mice were injected with the same volume of sterile water.

Mouse Model of Chronic Asthma

At 5 weeks of age, mice (11–15 mice/group) were immunized on days 0 and 14 via intraperitoneal injection with 20 μ g of OVA (Sigma-Aldrich, St Louis, Missouri) emulsified in 100 μ L Inject Alum (Pierce, Rockford, Illinois). Groups of mice that had been sensitized with OVA were challenged with aerosolized OVA (1% OVA in saline for 40 minutes) 3 times per week for 8 weeks (Fig 1). Age- and sex-matched mice that had been sensitized and challenged with saline at each time point were used as negative controls (SHAM). Mice were killed 48 hours after the final OVA challenge, and bronchoalveolar lavage fluid (BALF) and lung histopathology were analyzed. Lungs from the different experimental groups were processed as a batch for either histological staining or immunohistochemistry under identical conditions. All slides were quantified under identical light microscope conditions. The quantitative histological and image analysis of all slides was performed by research associates blinded to all the slides.

Assessment of Airway Inflammation

Two days after the final OVA challenge, mice were killed with a lethal dose of pentobarbital. Tracheas were cannulated, and BALF was recovered by flushing lungs with 0.8 mL phosphate-buffered saline (PBS). BALF cells were counted and spun onto glass slides using a cytospin and stained with Wright's Giemsa. A total of 400 cells per slide were assessed, and differential cell counts were recorded based on the morphology and staining of the cells.

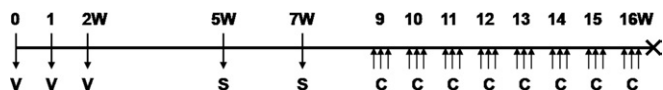


Figure 1. The experimental protocol. V, BCG vaccination; S, OVA/Alum sensitization; C, OVA challenge (3 $\times$ /wk); W, Week; X, Mice sacrifice.

Measurement of Smooth Muscle Layer Thickness and Peribronchial Collagen Deposition

To measure the smooth muscle layer thickness, lung sections were subjected to immunohistochemistry with an anti- α -smooth muscle actin (α -SMA) primary monoclonal antibody (Abcam). Briefly, the lung sections were incubated with the primary monoclonal antibody for 1 hour at room temperature. Immunoreactivity was detected by sequential incubations of lung sections with a biotinylated secondary antibody, followed by chromogen. Masson's trichrome stain was used to detect collagen deposition in the lung tissues. The area of α -SMA staining and Masson trichrome staining were outlined and quantified using a Leica-Qwin image-processing system (Bensheim, Germany). Results are expressed as the area of α -SMA staining or Masson trichrome staining per micrometer length of basement membrane of bronchioles. At least 10 bronchioles with 150 to 250 μ m of internal diameter were counted on each slide.

Quantitative Evaluation of Airway Epithelial Mucin Accumulation

The number of periodic acid-Schiff (PAS)-positive and PAS-negative airway epithelial cells in individual bronchioles was counted as previously described.⁸ At least 10 bronchioles were counted in each slide. Results are expressed as the percentage of PAS-positive cells per bronchiole, which is calculated from the number of PAS-positive epithelial cells per bronchus divided by the total number of epithelial cells of each bronchiole.

Assessment of Airway Hyperresponsiveness

Twenty-four hours after the final OVA challenge, airway hyperresponsiveness (AHR) to inhaled methacholine (Mch, Sigma-Aldrich) was measured. Mice were anesthetized, and the tracheas were cannulated. Each cannula was connected to an inline aerosol administrator and ventilator, which were attached to a preamplifier and computer (Buxco, Wilmington, North Carolina).⁹ Mice were nebulized with PBS followed by increasing doses of Mch (12.5, 25, 50 mg/mL). At each dose, lung resistance was calculated.

Airway hyperresponsiveness also was measured in untrained conscious mice by using barometric plethysmography (Buxco, Troy, New York).^{9,10} Airway hyperresponsiveness is expressed as the fold increase in enhanced pause (Penh) from baseline.

Assessment of TGF- β 1 in BALF and in Lung Tissue

Transforming growth factor beta 1 (TGF- β 1) levels in BALF were assayed by enzyme-linked immunosorbent assay (ELISA) (R&D Systems Inc; sensitivity of assay, 61 pg/mL). Before the TGF- β 1 assay, the samples were treated with 2.5 N acetic acid to activate any latent TGF- β 1.

The level of TGF- β 1 in lung was quantified by immunohistochemistry. As described for lung α -SMA immunohistochemistry, lung sections were processed for TGF- β 1 immunostaining by using an anti-TGF- β 1 monoclonal antibody (Santa Cruz Biotechnology). The number of individual cells staining

positively for TGF- β 1 in the peribronchial space were counted as previously described using a light microscope.⁸ Results are expressed as the number of peribronchial cells staining positively for TGF- β 1 per bronchiole with 150 to 250 μ m of internal diameter. At least 10 bronchioles were counted in each slide.

ELISA Analysis of Cytokines in BALF and OVA-Specific Immunoglobulin E in Sera

Levels of selected cytokines and chemokines (IFN- γ , interleukin [IL]-5, IL-17, IL-13, eotaxin-1) were assayed in BALF using an IFN- γ (Biosource), IL-5 (Biosource), IL-17 (R&D Systems Inc), IL-13 (ExCell Biology, Shanghai, China), and eotaxin-1 (R&D Systems Inc) ELISA kit. Horseradish peroxidase conjugated goat anti-mouse immunoglobulin E (IgE) antibody (Serotec) was used to test OVA-specific IgE in sera.

FLOW Cytometric Analysis of Pulmonary T Cell Populations

Lungs were cleared of blood by perfusion with 10 mL PBS through the right ventricle, cut into small pieces, and digested in 5 mL Roswell Park Memorial Institute (RPMI) 1640 medium containing 1 mg/mL collagenase (Sigma-Aldrich), 150 μ g/mL DNase I (Sigma-Aldrich), and 1% fetal calf serum at 37°C for 60 minutes. After incubation, single cells were obtained by passing the lung digest through a 40- μ m cell strainer. The resulting cell suspension was centrifuged, and the cell pellet was resuspended in 5 mL lymphocyte separating medium (EZ-Sep Mouse 1X, Dakewe Bio) covered with 200 μ L RPMI 1640, and centrifuged (800g, 30 minutes). Lymphocytes were isolated at the interphase between layers of RPMI 1640 and lymphocyte separating medium. The isolated lymphocytes were stimulated for 5 hours with 10 ng/mL phorbol-12-myristate-13-acetate and 500 ng/mL ionomycin (ALEXIS, Enzo Life Sciences, Lausen, Switzerland) in the presence of a protein transport inhibitor (Brefeldin-A, 10 μ g/mL, ALEXIS). After stimulation, cells were harvested, fixed, permeabilized (fixation and permeabilization buffer, eBioscience), and stained with the following antibodies purchased from eBioscience: PE-Cy5-CD3e, FITC-

CD8a, PE-IFN- γ , PE-IL-4, PE-IL-17A, FITC-CD4, APC-CD25, PE-Foxp3, PE-IL-10. Cell surface staining (ie, CD3, CD4, CD8, and CD25) was performed before fixation/permeabilization. Data were collected and analyzed using a FACScan (Becton Dickinson).

Analysis of Gene Expression in the Lung by Real-Time PCR

The expression of IFN- γ , IL-17A, and Foxp3 genes in lungs were analyzed by real-time polymerase chain reaction (PCR). Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. Complementary DNA was generated from 1 μ g of total RNA using the high-capacity cDNA Reverse Transcription Kit (SuperScript III First-Strand Synthesis System, Invitrogen). The primers of IFN- γ , IL-17A, and Foxp3 were purchased from Applied Biosystems. Quantitative gene expression analysis was performed using a TaqMan MicroRNA assay system (Applied Biosystems). The PCR cycling parameters were 50°C for 2 minutes, 95°C for 10 minutes, and 49 cycles of 95°C for 15 seconds followed by 60°C for 1 minutes. Relative gene expression levels were reported as fold change (FC) using the $\Delta\Delta C_t$ method where $FC = 2^{-\Delta\Delta C_t}$.

Statistical Analysis

All data were analyzed using GraphPad InStat software (version 3.0). Analysis of variance was used to determine the levels of difference between all groups. Comparisons of all pairs were performed by Tukey-Kramer significant difference test. Values for all measurements are presented as means $\pm$ standard error of the mean. Values of $P < .05$ were considered statistically significant.

RESULTS

Multiple BCG Vaccinations Decreased Eosinophil Numbers in the BALF of Mouse after Chronic OVA Provocation

Exposure of mice to chronic OVA challenges induced a significant increase in the numbers of total cells and eosino-

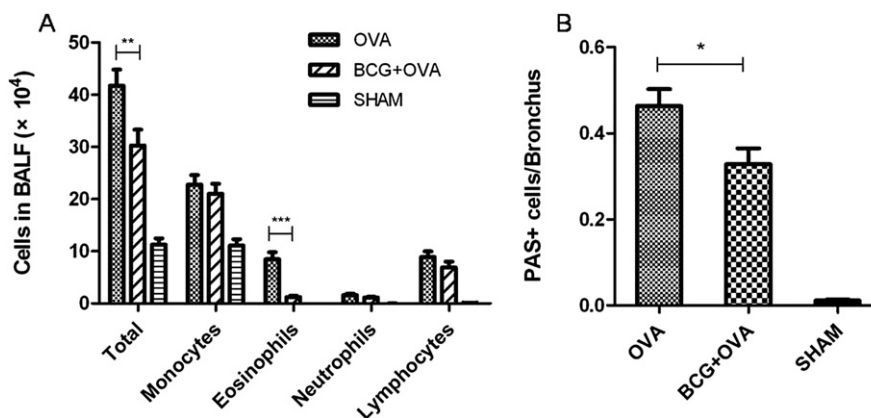


Figure 2. BCG vaccination reduced airway inflammation and mucus production. A, BCG vaccination reduced total cells and eosinophils in BALF ($n = 15$ mice/group). B, Mouse lungs were stained with PAS. The results are shown as the percentage of bronchial epithelial cells staining positively for PAS. * $P < .05$, ** $P < .01$, *** $P < .001$.

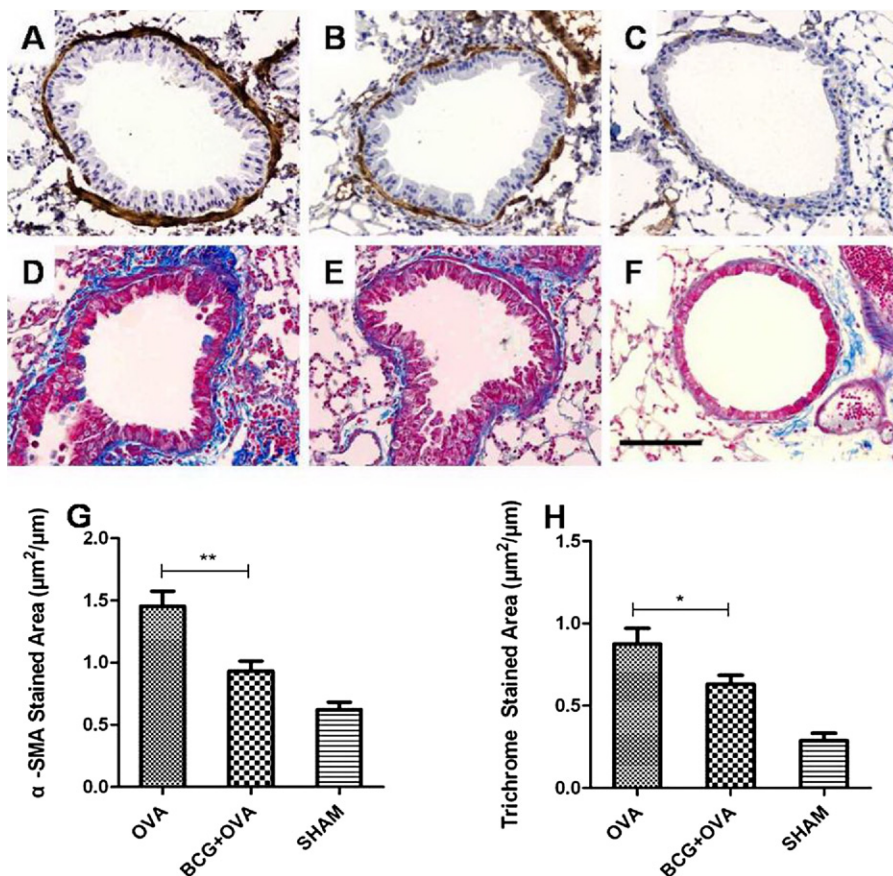


Figure 3. Multiple BCG vaccinations reduced the airway smooth muscle remodeling and peribronchial collagen deposit. Mouse lungs were immunostained with α -SMA Ab (A–C) or stained with trichrome (D–F). A and D, OVA group. B and E, BCG+OVA group. C and F, SHAM group. The results are shown as the area of peribronchial α -SMA (G) or trichrome (H) staining quantitated in squared-per-micrometer length of bronchus by image analysis. * $P < .05$, ** $P < .01$. Bar = 100 μ m.

phils in BALF compared with SHAM mice. However, multiple BCG vaccinations significantly decreased levels of total cells in BALF and eosinophils (Fig 2A). Other cells such as neutrophils, monocytes, and lymphocytes were comparable between the OVA and BCG+OVA groups.

Multiple BCG Vaccinations Decreased the Extent of Airway Epithelial Mucin Accumulation Induced by Chronic OVA Provocation

A significant increase of positive PAS staining in airway epithelium was noted in mice after chronic OVA challenges relative to the saline-challenged controls. Neonatal BCG vaccination significantly decreased the percentage of epithelial PAS staining compared with that in the OVA-challenged group (Fig 2B).

Multiple BCG Vaccinations Decreased the Area of Peribronchial α -SMA Immunostaining and Collagen Deposition in the Model of Chronic Asthma

Compared with saline-exposed mice, repetitive OVA challenge for 8 weeks induced a significant increase in the area of

peribronchial α -SMA immunostaining and trichrome staining, both of which were significantly reduced (Fig 3) in BCG-vaccinated mice.

Multiple BCG Vaccinations Prevented AHR in Chronic OVA-Challenged Mice

We used invasive and noninvasive methods to determine the levels of airway responsiveness to methacholine to determine whether the reduced airway inflammation and decreased thickness of smooth muscle were associated with decreased airway hyperresponsiveness. Exposure of mice to chronic OVA challenges induced a significant increase in AHR compared with saline controls (Fig 4). However, repeated BCG vaccinations of BCG-treated mice significantly decreased AHR compared with chronic OVA-challenged mice (Fig 4).

Multiple BCG Vaccinations Decreased Levels of TGF- β 1 in BALF and Reduced OVA-Induced Peribronchial Cell TGF- β 1 Expression

We examined the induced expression of TGF- β 1 in our model as a potential mechanism for the deposition of extra-

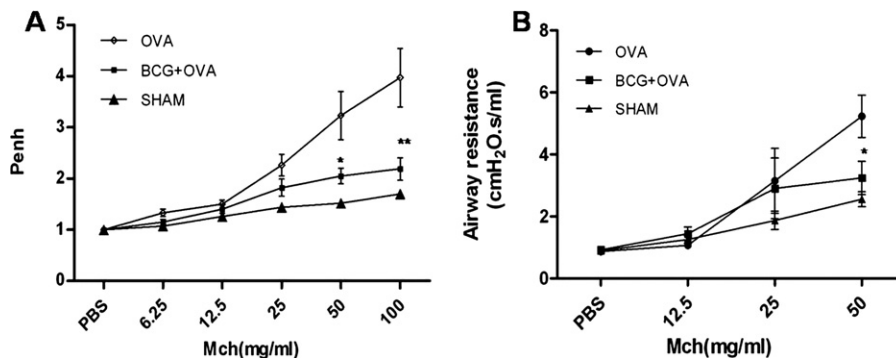


Figure 4. Multiple BCG vaccination decreased AHR in chronic OVA-challenged mice. AHR to Mch was assessed 24 h after the final aerosol challenge. A, Penh values were recorded after increasing doses of Mch. Values were expressed as fold increase in Penh for each dose of Mch and compared with Penh value after PBS challenge ($n = 10/\text{group}$). B, AHR to Mch was assessed in intubated and ventilated mice. Results are expressed as airway resistance in mice exposed to nebulized PBS and Mch (12.5, 25, 50 mg/mL) ($n = 6\text{--}8/\text{group}$). Compared with OVA group, * $P < .05$, ** $P < .01$.

cellular matrix proteins contributing to airway remodeling such as collagen. Exposure of mice to chronic OVA challenge induced a significant increase in the level of TGF- β 1 in BALF and the number of peribronchial TGF- β 1-positive cells compared with SHAM mice. More importantly, repeated BCG vaccination decreased the level of TGF- β 1 in BALF and also reduced the number of peribronchial TGF- β 1-positive cells (Fig 5).

Neonatal BCG Vaccinations Differentially Polarized the Induced Pulmonary Immune Responses to theTh1 Phenotype

Chronic OVA challenge significantly increased levels of BALF IL-5, IL-13, and eotaxin-1, but BALF IFN- γ level was similar to that of saline-challenged mice (Fig 5). Significantly, multiple BCG vaccinations resulted in an increased level of BALF IFN- γ and reduced levels of IL-5 and eotaxin-1 compared with nonvaccinated but chronic OVA-challenged animals (Fig 5). The OVA-specific IgE in sera also decreased in BCG-vaccinated mice (OD_{450} , 0.96 ± 0.12 vs 1.38 ± 0.14 , BCG+OVA vs OVA, $P < .05$). Although BCG-vaccinated mice had lower levels of BALF IL-13, this did not reach statistical significance. The level of IL-17 in BALF was under-detectable in all animals examined.

Neonatal BCG Vaccination Increased IFN- γ -Producing T Cells, But Not T Regulatory (Treg) and IL-17-Producing T Cells in Lung Tissues

Pulmonary T subpopulations were measured using intracellular cytokine staining to detect the effect of multiple BCG vaccinations have on the differentiation/accumulation of pulmonary T cells. The BCG vaccinations increased CD3⁺CD8⁺IFN- γ ⁺ T cells nearly 6-fold in the lungs relative to the nonvaccinated OVA-challenged mice. Compared with SHAM animals, both OVA-challenged BCG-vaccinated and nonvaccinated mice had higher frequency of CD3⁺CD8⁺IL-4⁺ T cells and CD3⁺CD8⁺IL-17⁺ T cells. No significant difference was observed in the frequency of CD4⁺Foxp3⁺ T cells and CD4⁺IL-10⁺ T cells in the lungs of BCG-vaccinated

and non-BCG-vaccinated mice (Fig 6A). The messenger RNA levels of IFN- γ , IL-17A, and Foxp3 in lungs confirmed this phenomenon (Fig 6B).

DISCUSSION

Our previous studies have shown that multiple BCG vaccinations of neonates elicited long-term protection by inhibiting allergic airway inflammation.⁷ In this study, we have extended these earlier studies, demonstrating that multiple BCG vaccinations of neonates reduced the airway remodeling, including reduction of peribronchial α -SMA and collagen, associated with chronic allergen provocation.

In a chronic OVA model of allergic respiratory inflammation, we found that neonatal BCG vaccinations significantly inhibited the eosinophil accumulation occurring in the lungs of OVA-challenged mice. Studies in mice deficient in eosinophils have demonstrated that airway eosinophils are important to the development of AHR^{11,12} and also contribute to the thickening of the peribronchial smooth muscle and airway remodeling.^{8,13} Kanda and colleagues¹⁴ further demonstrated that adoptively transferred eosinophils induced lung fibrosis and collagen deposition. Collectively, these studies suggest that eosinophils play an important role in the pathophysiology of airways remodeling. In this study, repeated BCG vaccinations of neonates not only significantly decreased numbers of eosinophils compared with non-BCG-vaccinated mice but also led to a concomitant reduction in airway remodeling events after chronic OVA challenge.

Bacillus Calmette-Guerin vaccination of neonatal mice also decreased levels of TGF- β 1 in BALF and reduced accumulation of TGF- β 1⁺ cells in the peribronchial spaces, suggesting a possible mechanism responsible for the decreased remodeling events in BCG-vaccinated mice. This decrease in TGF- β 1 is likely linked to the decreased peribronchial inflammatory cell infiltrate associated with BCG-vaccinated mice. For example, Cho and colleagues⁸ found that eosinophils (approximately 63%) and macrophages (approximately 35%) were the major peribronchial inflammatory

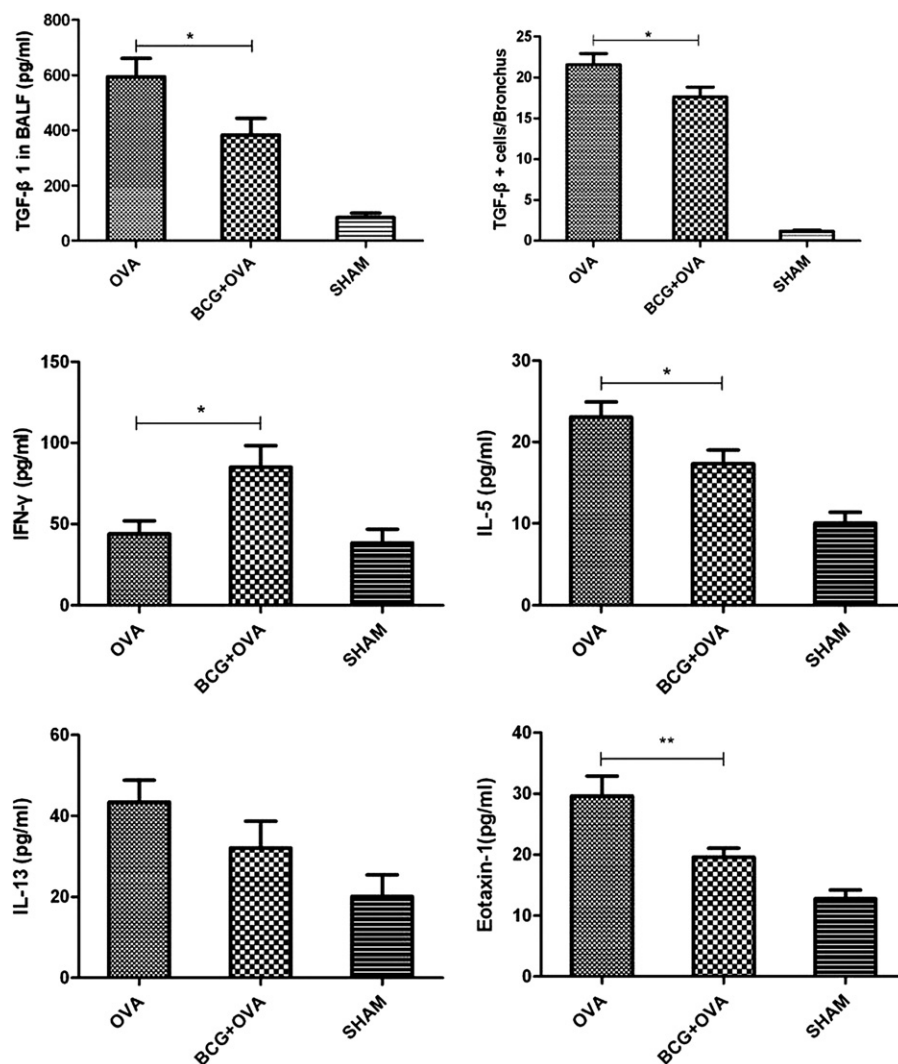


Figure 5. Effect of neonatal BCG vaccination on the levels of cytokines in BALF and TGF- β 1 in lung. The contents of IFN- γ , IL-5, IL-13, IL-17, eotaxin-1, and TGF- β 1 in BALF were analyzed by ELISA ($n = \sim 7$ –10 per group). The level of IL-17 in BALF was undetectable. The level of TGF- β 1 in lung was quantitated by immunohistochemistry. The number of individual cells staining positively for TGF- β 1 in the peribronchial space were counted. * $P < .05$, ** $P < .01$.

cellular sources of TGF- β 1 after repetitive OVA challenge for 3 months.⁸ In our study, the decreased levels of TGF- β 1 in lung may be related to the reduced eosinophils induced by the BCG vaccinations and, in turn, the reduced levels of remodeling observed in vaccinated mice, which would have to be confirmed by subsequent studies.

Bacillus Calmette-Guerin is a strong inducer of Th1-type immune responses. Previous studies have shown that BCG inoculation elicits the protective effect against the development of asthma by changing the Th1/Th2 balance. In particular, Erb and colleagues⁴ demonstrated that the BCG-induced inhibition of airway eosinophilia was mediated by interferon-gamma (IFN- γ).⁴ Our previous study showed that the inhibition of airway eosinophilia by neonatal BCG vaccination was

associated with the modulation of Th1/Th2 cytokine production in early-, but not in late-challenged mice (45 weeks of age).⁷ Other studies suggested that BCG or killed Mycobacterium conferred protection against airway inflammation by giving rise to allergen-specific regulatory T cells.^{15–17} However, these previous studies used acute allergen challenge models, which are somewhat different from the chronic model of asthma used in the current study, providing an explanation for the difference in T cell responses observed here. Specifically, we found that Th1 cells in the lungs of BCG-vaccinated mice increased significantly compared with non-BCG-vaccinated mice. However, Tregs (whether expressing Foxp3+ or producing IL-10) were similar between BCG-vaccinated and nonvaccinated mice. These results dem-

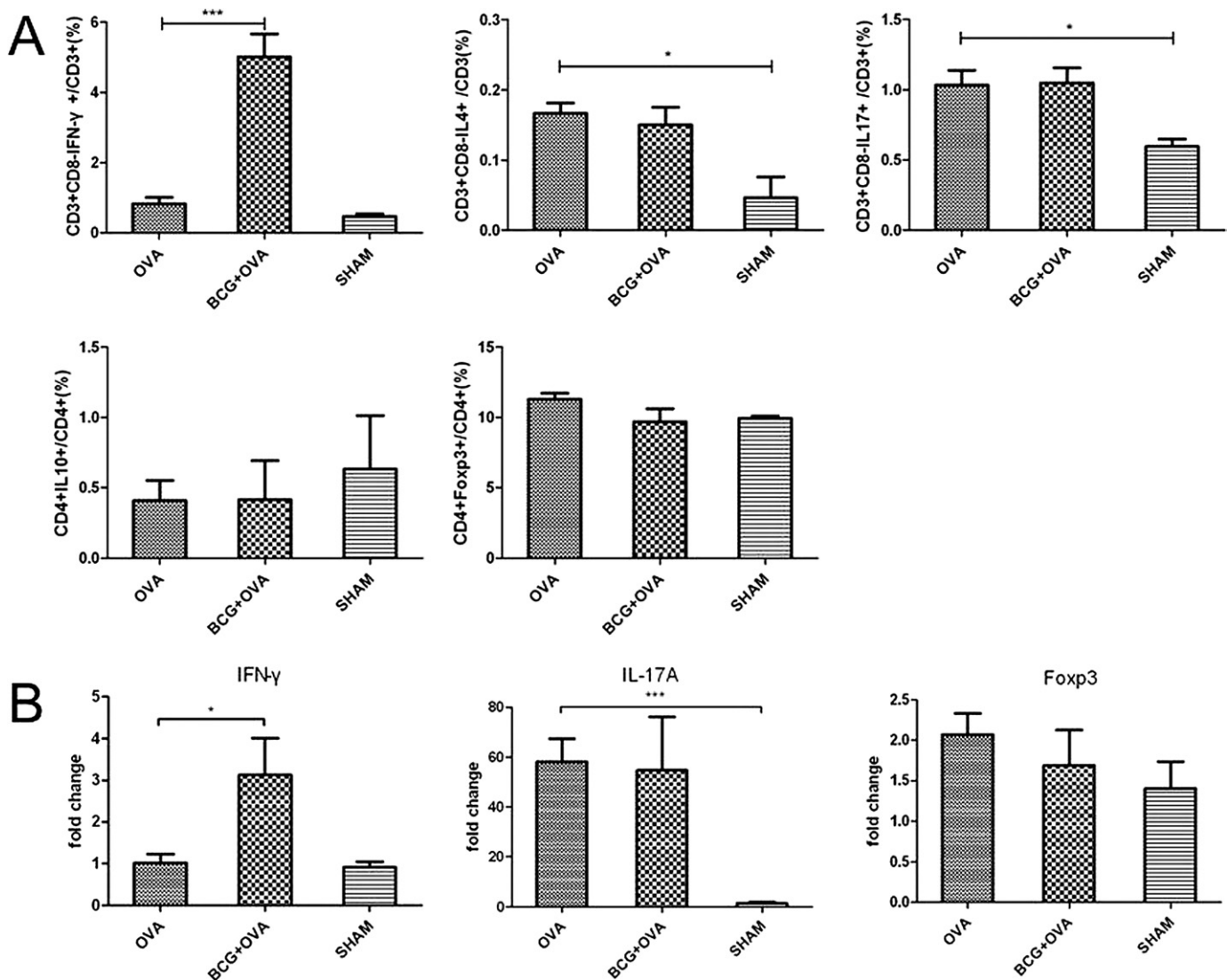


Figure 6. Neonatal BCG vaccination influenced pulmonary T cell responses in a chronic asthma model of mice. A, Flow cytometric detection of T cell subpopulations in the lungs using intracellular cytokine staining ($n = 4$ per group). Results gated on CD3 (IFN- γ , IL-17, and IL-4) and CD4 (Foxp3, IL-10). B, Expression of IFN- γ , IL-17A, and Foxp3 genes in whole lung homogenates was tested by real-time PCR. * $P < .05$; *** $P < .001$.

onstrated that the inhibition of allergic lung inflammation by multiple BCG vaccinations was not associated with an increased number of pulmonary regulatory T cells but was positively correlated with the increase in IFN- γ -producing T cells in the lung.

Th17 is the newest member of the Th cell family. Its function in asthma, especially in eosinophilic asthma, is still largely unknown and controversial. In our chronic model of asthma, we found that Th17 cells increased after OVA sensitization and challenge in the lungs compared with SHAM mice. But the percentage of Th17 in the lungs of BCG-vaccinated mice was similar to that from nonvaccinated mice. Umemura et al¹⁸ reported that in adult mice inoculated intratracheally with BCG, both IL-17 and IFN- γ expression in the BCG-infected lung

increased.¹⁸ Furthermore lung-infiltrating T cells of IL-17-deficient mice produced less IFN- γ in comparison with those from wild-type mice, which indicated that IL-17 is an important cytokine in the induction of optimal Th1 response after pulmonary BCG infection. In our study, however, we used subcutaneous BCG inoculation of neonates, not intratracheal inoculation. This experimental difference may have led to our observed increase in IFN- γ expression in lung tissue without changes in Th17 cell numbers and IL-17 levels.

The mechanism of AHR in asthma is still not fully understood. Previous studies demonstrate that airway eosinophils are important to the development of AHR.^{11,12} In addition, AHR is associated with the level of expression of smooth muscle contractile protein, such as α -SMA. A higher level of

α -SMA expression may indicate a more elastic behavior and therefore may increase AHR.¹⁹ In this study, we found that neonatal BCG inoculations significantly decreased the eosinophilic infiltrate in the airway and reduced the peribronchial α -SMA area. Both the decreased peribronchial eosinophils and decreased α -SMA are likely to contribute to the decreased AHR.

In summary, we have demonstrated that multiple BCG vaccinations of neonates reduced levels of allergen-induced airway responsiveness and airway remodeling. This inhibition of asthma was not associated with an increased number of pulmonary regulatory T cells but was positively correlated with the increase in IFN- γ -producing T cells.

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