

The Antiasthma Effect of Neonatal BCG Vaccination Does Not Depend on the Th17/Th1 but IL-17/IFN- γ Balance in a BALB/c Mouse Asthma Model

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Abstract

Objective This study aimed to determine whether the protective effects of the *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG) vaccination on allergic asthma are associated with the T helper (Th) 17/Th1 balance in a murine asthma model.

Methods BALB/c neonates were vaccinated with BCG on the first day after birth, sensitized with ovalbumin, and then challenged with allergen. The resulting airway inflammation and responsiveness were measured. The levels of IL-17 and interferon (IFN)- γ in BALF and ratio of Th17/Th1 were investigated.

Results We found that although BCG neonatal vaccination inhibited airway hyperresponsiveness and inflammation following allergen challenge in a BALB/c mouse asthma model, reduced levels of Th2 cytokines were not observed. However, BCG neonatal vaccination reduced IL-17 production and increased IFN- γ production in both the bronchoalveolar lavage fluid and the lung lymphocytes in asthmatic mice.

Conclusion The antiasthma effects of neonatal BCG vaccination reversed the IL-17/IFN- γ imbalance in a murine asthma model but did not depend on modifying the Th17/Th1 balance.

Keywords Neonatal BCG vaccination · murine asthma model · Th17/Th1 balance · IL-17/IFN- γ balance

Introduction

Asthma has been defined as a chronic inflammatory disease of immunodysregulation, characterized by airway infiltration of inflammatory cells and airway hyperresponsiveness (AHR). The “hygiene hypothesis” states that a relative lack of infections early in life could promote the development of allergic diseases in genetically predisposed individuals [1]. Based on this hypothesis, the induction of Th1 cytokines such as interferon- γ (IFN- γ), and thus the reversion of the imbalance between Th1/Th2, has been proposed to be a determinant for the development of asthma [2, 3].

Mycobacterium bovis Bacillus Calmette–Guérin (BCG) is a strong inducer of Th1 immune responses and modulates the development of asthma in both animal models and humans [3–9]. The BCG vaccine is a low-cost, safe, and widely used vaccine [10]. Despite the use of this vaccine, there has been an increase in the prevalence of asthma reported. The main mechanism used by BCG to inhibit allergy-induced pathologic changes involves stimulating the production of Th1 cytokines and decreasing Th2 cytokine secretion; however, Th2-mediated allergic lung inflammation coexists with the Th1-mediated responses that are stimulated by diverse forms of antigens [11, 12]. In our previous study, infection with RSV led to a Th1 Ag-specific immune response and could reverse the antiasthma effect of

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neonatal BCG vaccination in BALB/c mice [13]. Although Poly:IC (double-stranded RNA) neonatal vaccination induced IFN- γ expression, protection in BALB/c asthma mice was not developed (data not shown). We therefore concluded that the Th1–Th2 paradigm is unlikely to explain the mechanism by which BCG regulates asthma.

A recent study reported that following ovalbumin (OVA) challenge, T-bet^{−/−} mice displayed increased airway inflammation and enhanced AHR compared to wild-type mice. Interestingly, a marked increase in the levels of the proinflammatory cytokine interleukin (IL)-17 was observed in T-bet^{−/−} mice [14], suggesting that IFN- γ and IL-17 may counterregulate each other during asthma. IL-17 has been suggested as a link between activated T lymphocytes and the recruitment and activation of neutrophils in various types of airway inflammation [15] and is mainly produced by the third Th cell subset (Th17). It has been shown that the level of IL-17 is increased in bronchia alveolus lavage fluid, sputum, and blood from patients with asthma [16–18]. In a mouse model of allergic airway inflammation, systemic blockade of IL-17 inhibited the allergen-induced accumulation of neutrophils and increased eosinophil numbers in the airway [15].

Because of the important role of IL-17/Th17 in asthma and IL-17/IFN- γ counterregulation, we hypothesized that IL-17 might be involved in the effects of BCG on asthma. In the present study, we compared Th17/Th1 function at cell frequencies and related cytokine secretion levels between a BALB/c asthma model and BCG neonatal vaccinated BALB/c asthma model. We aimed to determine if the protective effects on allergic pulmonary inflammation of the BCG vaccination are associated with the Th17/Th1 balance.

Materials and Methods

Animals, Immunization, and Treatment

BALB/c mice, free of specific pathogens and housed in individual filtered cages, were maintained on OVA-free diets. Cages, bedding, food, and water were sterilized before use. Room temperature was maintained at 24°C, and a 12-h light/dark cycle was provided. Pregnant mice went into labor at day 20 of pregnancy, and newborn mice were raised and maintained in the same conditions. The Institutional Animal Care and Research Advisory Committee at the Chongqing Medical University approved this study. All experimental animals were used in accordance with the guidelines issued by the Chinese Council on Animal Care. Experimental groups of mice received the following treatments: (1) sensitizations and airway challenges with ovalbumin (OVA group); (2) treatment with BCG neonatal vaccination plus sensitizations and airway challenges with OVA (BCG + OVA group); and (3) the

control group. On day 1, mice in the BCG group were subcutaneously injected in the back with 20 μ l of BCG (1×10^5 colony-forming units (CFU)) from a 30-gage needle on a tuberculin syringe. Mice in the OVA group and control group were injected with the same amount of BCG diluent in the same area. The mice in the OVA and BCG groups were sensitized intraperitoneally with 100 μ g ovalbumin (Sigma) diluted in 50% aluminum hydroxide (Pierce) to a total volume of 200 μ l on day 28 and day 42. From day 49–56, the mice were exposed to 1% OVA aerosols in a Plexiglas exposure chamber for 30 min each day. The mice in the control group were administered saline solution. In our experiment, each group consisted of 24 mice. Each experiment was repeated three times. Two to three mice were used in every experimental test described hereafter.

Determination of Airway Hyperresponsiveness

Airway hyperresponsiveness to methacholine in conscious, spontaneously breathing animals was measured by single-chamber, whole-body plethysmography (Buxco Electronics Inc., Troy, NY, USA) as described earlier. Briefly, mice were exposed to aerosolized PBS (for the baseline measurement) or methacholine (3.125–50 mg/ml) for 3 min, and readings were taken and averaged for 3 min after each nebulization. Data are presented as the enhanced pause (Penh, dimensionless parameter) vs. the methacholine concentration (milligram per milliliter) used to generate the aerosol. Penh is a dimensionless value that represents a function of the ratio of peak expiratory flow to peak inspiratory flow and a function of the timing of expiration, and it correlates with pulmonary airflow resistance ($\text{Penh} = \text{PEP}/\text{PIP} \times \text{Pause}$).

BAL and Cell Counting

Mice were anesthetized with urethane (15 mg/10 g body weight intraperitoneally) 24 h after the last OVA challenge. The trachea was cannulated, and bronchoalveolar lavage fluid (BALF) was collected by three injections of 0.5 ml phosphate-buffered saline into the lung. Total leukocyte numbers were counted using microscopy. Differential cell counts were performed on cytospin slides stained with Wright–Giemsa in a blinded fashion by counting at least 400 cells under immersion oil at $\times 1,000$ magnification. Blood samples for serum were collected from the vena cava. The BAL was centrifuged, and the supernatant was used to test for cytokine production.

Cytokine Analysis

Concentrations of cytokines in BALF were measured with commercial ELISA kits according to the manufacturer's

instructions. Murine IFN- γ and IL-17 ELISA kits were purchased from Bender MedSystems (Vienna, Austria).

Anti-OVA-Specific IgE Levels in Serum

Anti-OVA-specific IgE in the sera was measured by ELISA as described previously [16]. Briefly, the microplate was coated with 100 μ l of OVA (20 g/ml), mice serum was incubated in the antigen-coated wells, and bound IgE was detected with a biotinylated antimouse IgE. Diluted avidin–alkaline phosphatase was added, the bound enzyme was detected with 3,3', 5,5'-tetramethylbenzidine substrate, and the absorbance at 450 nm was determined.

Lung Cell Preparation

The lung was perfused with PBS through the right ventricle before excision from the mice. The excised lung tissue, separated from all the associated lymph nodes, was minced and incubated for 1 h at 37°C in 5 ml of PBS containing 1.0% FBS and 0.2% collagenase I (Sigma-Aldrich). Single-cell suspensions (pulmonary infiltrated (PIF) cells) were prepared by passing tissue through 30-mm stainless steel mesh. To enrich the pulmonary lymphocytes, PIF cells were resuspended in 8 ml of 45% Percoll solution (Amersham Biosciences), overlaid on 5 ml of 67.5% Percoll solution, and centrifuged at 2,200 rpm for 20 min at 20°C. The cells at the interface were collected and used for flow cytometric analysis.

Flow Cytometric Analysis of Intracellular Cytokine Assay

To analyze the IL-17 and IFN- γ expression of the cells from the lung in all groups, lung cells (2×10^6 cells per milliliter) were incubated with PMA (50 ng/ml; Sigma) and ionomycin (500 ng/ml; Sigma) for 4–6 h at 37°C and 5% CO₂ with GolgiPlug-containing brefeldin A (BD Biosciences) in six-well flat-bottom plates (Nalge Nunc International) in 1 ml of RPMI 1640 medium containing 10% FBS. After 4–6 h of culture, the cells were harvested and washed once in PBS containing 1.0% newborn calf serum and 0.1% NaN₃ (staining buffer). Cells were pretreated with Fc blocker and subsequently surface stained with PEcy5-conjugated anti-CD3 mAb and FITC-conjugated anti-CD4 mAb. To detect Th17 and Th1 cells, we used PE-conjugated anti-IL-17 mAb and PE-conjugated anti-IFN- γ mAb. For both intracellular IL-17 and IFN- γ staining, cells were detected by a flow cytometer, FACSCalibur (BD Biosciences). The data were analyzed with CellQuest software (BD Biosciences).

Histopathology

Formalin-fixed lungs were embedded in paraffin, sectioned in 6- μ m thick slices, and stained with hematoxylin and eosin

for routine histology. In a blind analysis, an experienced histologist examined the slides for peribronchiolitis, perivascularitis, alveolitis, and eosinophilia. Lung lesions were scored semiquantitatively as described by other researchers [2, 17]. The severity of inflammation was evaluated by assigning a value of 0 for no inflammation, 1 for mild inflammation, 2 for moderate inflammation, and 3 for severe inflammation. Counting the number of eosinophils within the inflamed peribronchial region and expressing this as a percentage determined eosinophilia.

Statistical Analysis

Results were shown as means + SD. Statistical analysis was performed with the statistical software package SigmaStat (SPSS 13.0, Chicago, IL). All assays were compared using ANOVA followed by least squares difference *t* analysis. Differences were considered statistically significant when the *P* value was <0.05.

Results

Neonatal BCG Vaccination Inhibited OVA-Induced Airway Neutrophilic and Eosinophilic Inflammation in a BALB/c Mouse Asthma Model

Following challenge with OVA, BALF were collected, and the number of neutrophils and eosinophils was counted. The mice in the control group showed few neutrophils (Fig. 1b) and eosinophils (Fig. 1a). Compared with the control mice, large numbers of neutrophils (Fig. 1b) and eosinophils (Fig. 1a) were observed in BALF of mice in the OVA group. In contrast, the number of neutrophils (Fig. 1b) and eosinophils (Fig. 1a) was dramatically reduced in the mice with BCG neonatal vaccination when comparing to the OVA group. These data indicated that neonatal BCG vaccination inhibited OVA-induced airway neutrophilic and eosinophilic inflammation in a BALB/c mouse asthma model.

Neonatal BCG Vaccination Reduced OVA-Induced Tissue Pathology of BALB/c Mice

In asthmatic mice, airway challenge leads to a dense peribronchiolar and perivascular inflammatory infiltrate of inflammatory cells (Fig. 2b). In BCG neonatal vaccinated mice, tissue inflammation was greatly reduced, with significantly less peribronchial and perivascular cellular infiltration (Fig. 2c). As shown in Fig. 3, the inflammation scores of peribronchial and perivascular regions in mice with BCG neonatal vaccination decreased significantly as compared with that in the OVA group (*P*<0.05). Thus,

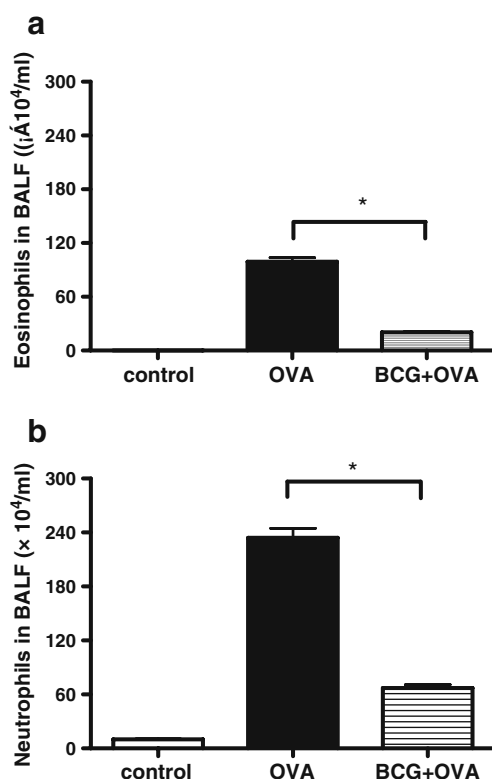


Fig. 1 Neonatal BCG vaccination inhibited OVA-induced airway neutrophilic and eosinophilic inflammation in a BALB/c mouse asthma model. Groups of mice were sensitized and challenged with the allergen. BALF samples were collected 24 h after the final challenge, and then, inflammatory cells were counted based on Wrights–Giemsa staining. **a** Eosinophils in BALF. **b** Neutrophils in BALF. Data were expressed as the mean $\pm$ SD of the number of cells in each group of mice. * $P < 0.05$ vs. OVA group

BCG neonatal vaccination not only inhibited allergen-induced neutrophilia and eosinophilia but also suppressed airway inflammation in asthmatic mice.

Neonatal BCG Vaccination Inhibited OVA-Induced AHR but Had No Effect on OVA-Specific IgE Production in BALB/c Mice

As shown in Fig. 4a, in response to different doses of aerosolized methacholine (MCh), the Penh values of mice that had received OVA without BCG neonatal vaccination (OVA group) gradually increased with elevated concentrations of MCh, whereas the Penh values of mice that had not received OVA (control group) were not significantly increased. Penh values in the mice that received BCG neonatal vaccination were significantly lower than that of the OVA group in response to MCh concentrations ranging from 12.5 to 50 mg/ml ($P < 0.05$).

OVA-specific IgE production was also evaluated in our study. Although very significant increases were seen in

asthmatic mice compared with control mice, no reductions were seen in BCG neonatal vaccinated mice when compared with mice in the OVA group for OVA-specific IgE (Fig. 4b).

Airway Inflammation in Mice with BCG Neonatal Vaccination Did Not Correlate with Th2 Cytokine Production

To investigate whether BCG neonatal vaccination leads to a reduced Th2 response following OVA challenge, cytokine expression in lung cells and BALF was analyzed. As expected, the BALF of OVA-sensitized and challenged animals showed increased levels of Th2-like cytokines, namely IL-5 and IL-13 (Fig. 5a). However, these cytokines were not decreased in BCG neonatal vaccinated asthmatic mice. Intracellular cytokine staining for IL-4 expression was consistent with the level of cytokines in BALF (Fig. 5b). Thus, decreased airway inflammation in mice with BCG neonatal vaccination did not correlate with Th2 cytokine production.

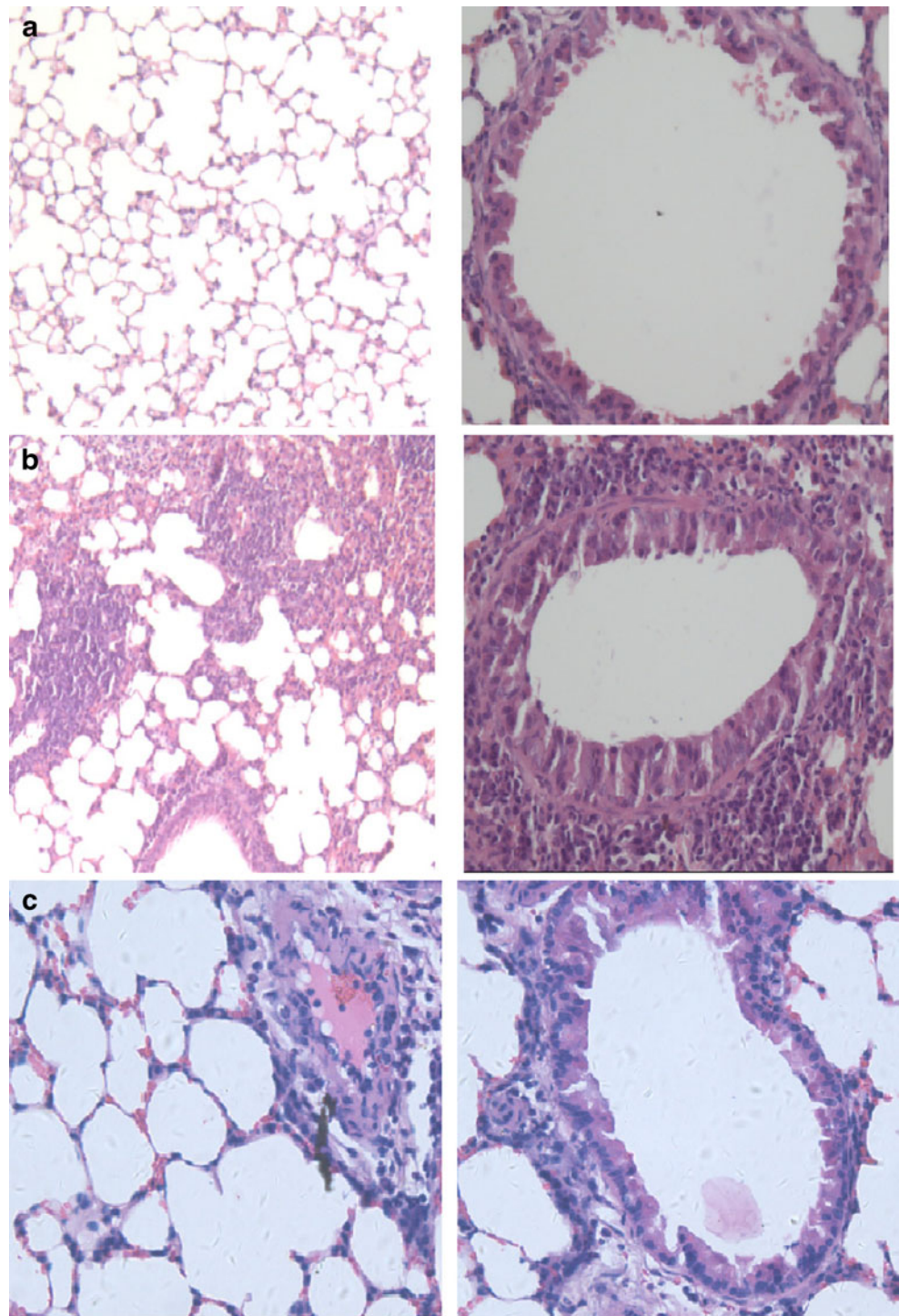
Neonatal BCG Vaccination Inhibited IL-17 Production and Enhanced IFN- γ Production in a BALB/c Asthma Model

Vaccination with BCG usually induces a Th1 response to mediate a protective effect on asthmatic mice. As expected, mice with BCG neonatal vaccination demonstrated an increase in BALF IFN- γ levels compared with mice in the OVA group (Fig. 6c). Interestingly, following OVA-induced allergic lung inflammation, BALF IL-17 levels of mice in the BCG + OVA group were dramatically decreased when compared to those of mice in the OVA group (Fig. 6a). The ratio of IL-17/IFN- γ was significantly decreased in mice with BCG neonatal vaccination when compared with mice in OVA group (Fig. 6e). These data indicate that IL-17 is involved in the effects of BCG on asthma. Furthermore, concentrations of IL-23, a critical cytokine for IL-17 production by Th17, and IL-12, a critical cytokine for IFN- γ production by Th1, were also evaluated. However, differences among the three groups of mice were not observed (Fig. 6b, d).

Neonatal BCG Vaccination Did Not Reverse the Th17/Th1 Imbalance in the BALB/c Asthma Model

Th17 is the main source of IL-17 in allergic inflammation; therefore, we also determined the Th17 cell and Th1 cell levels in mouse lung cells by flow cytometry (Fig. 7a, b). Most of the IL-17-expressing cells isolated from the lungs were found to be CD3⁺CD4⁺ (Fig. 7a). Intracellular cytokine staining of lung lymphocytes demonstrated an increase in IL-17 production after OVA challenge in both

Fig. 2 Neonatal BCG vaccinations reduced OVA-induced tissue pathology of BALB/c mice. Twenty-four hours after the final challenge, mouse lung tissue sections were stained with hematoxylin and eosin (HE). Data are representative of each group of mice. **a** Control group. **b** OVA group. **c** BCG + OVA group. Magnification *left* $\times 100$ and *right* $\times 400$



the OVA group and BCG + OVA group (Fig. 7a). Little if any CD4⁺IL-17⁺ cells were found (Fig. 7a), suggesting a major role for Th17 cells in response to inhaled allergen. However, compared with mice in OVA group, Th17 cells were not significantly decreased in mice with BCG neonatal vaccination (Fig. 7a). However, Th1 cells were increased in mice with BCG neonatal vaccination compared

with the OVA asthmatic mice. Because the total number of inflammatory cells in BALF of mice in BCG + OVA group was significantly decreased, the total number of CD4⁺IL-17⁺ cells in the lung must be lower than in asthmatic mice. However, the ratio of Th17/Th1 was unchanged (Fig. 7c). Thus, neonatal BCG vaccination did not reverse the Th17/Th1 imbalance in the BALB/c asthma model.

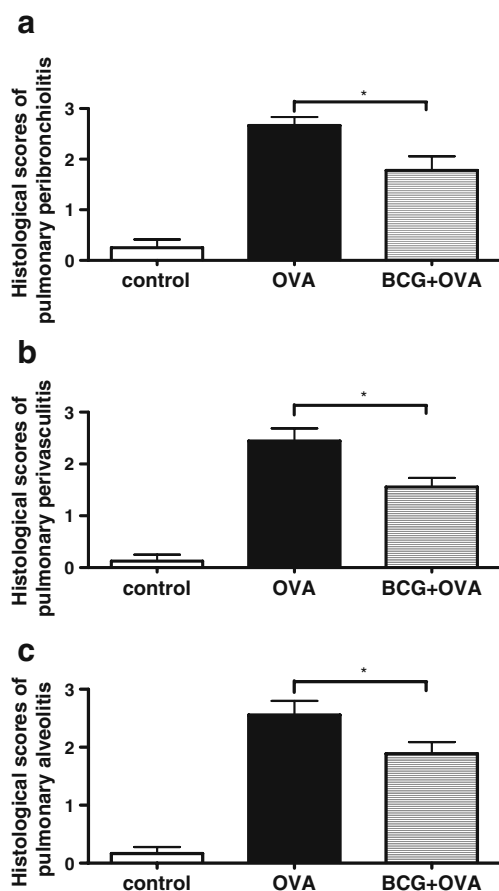


Fig. 3 Histological scores of pulmonary peribronchiolitis, perivascularitis, and alveolitis. HE-stained lung sections were scored for levels of peribronchial, perivascularitis, and alveolitis inflammation. BCG vaccination decreased peribronchial, perivascular, and alveolitis inflammation. Data are expressed as mean score $\pm$ SD of each group. $*P < 0.05$ vs. OVA group. **a** Histological scores of pulmonary peribronchiolitis. **b** Histological scores of pulmonary perivascularitis. **c** Histological scores of pulmonary alveolitis

Discussion

Previous studies have demonstrated that BCG vaccination can suppress airway eosinophilia and AHR in animal experiments [2, 18–20]; however, a recent meta-analysis of 23 studies concluded that early BCG vaccination was associated with a small reduction in the risk of asthma (OR = 0.86; 95% CI 0.79–0.93) [21]. In a randomized controlled study in humans in which multiple intramuscular injections of BCG were used, BCG vaccination was found to have a limited effect on asthma and allergic rhinitis [22]; however, another study reported an association between BCG vaccination in the neonatal period and a reduction in the prevalence of asthma in individuals with a family history of rhinitis or eczema [23]. Moreover, neonatal vaccination with BCG in murine asthma models elicits long-term protection in mouse-allergic responses [24]. Our results show that a neonatal, subcutaneous injection of BCG had a

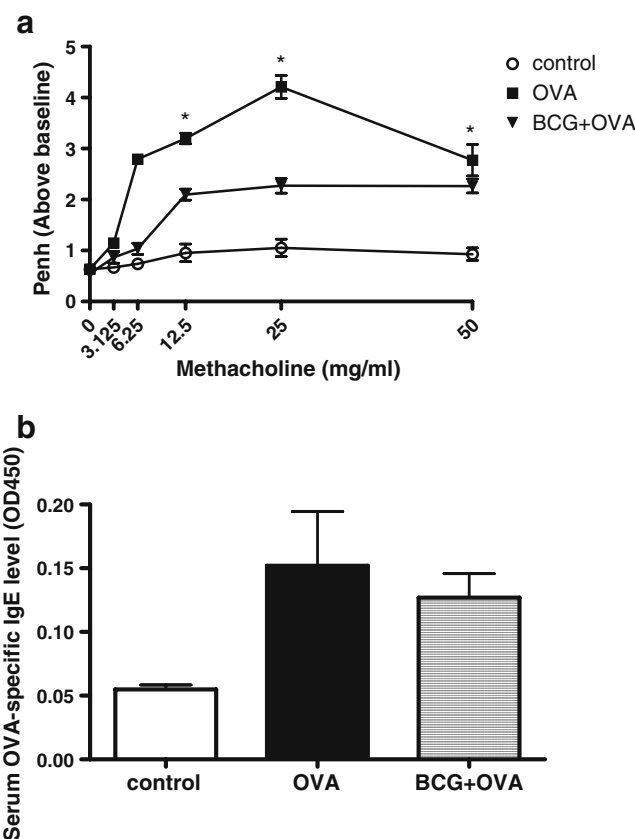


Fig. 4 Neonatal BCG vaccination inhibited OVA-induced AHR but had no effect on OVA-specific IgE production in BALB/c mice. **a** Groups of mice were sensitized at 4 and 6 weeks of age, and they were challenged with allergen at 7 weeks of age. Twenty-four hours after the final challenge, AHR (Penh) in response to increased doses of nebulized MCh was assessed by a whole-body plethysmograph in the experimental groups and negative control mice injected with saline. Data are presented as the mean $\pm$ SEM of Penh from experimental or control groups of mice. $*P < 0.05$ vs. OVA group. **b** Serum OVA-specific IgE level showed that neonatal BCG vaccination had no effect on IgE production. Data are expressed as mean score $\pm$ SD of each group

potential protective role following specific allergen provocation, as demonstrated by a decrease in granulocytic cellular infiltration into the airways, specifically, eosinophils, neutrophils, and the suppression of bronchial responsiveness. Thus, our data confirmed the notion that neonatal vaccination with BCG inhibits allergen-induced airway inflammation and AHR in asthmatic mice.

The ability of BCG or mycobacteria (which stimulate Th1 lymphocytes) to modulate the Th2 response has been documented in vitro and in various models of asthma [4, 25]. In our murine asthma model, IL-5 and IL-13 levels in BALF were upregulated in mice following allergen challenge. Compared with mice in the OVA group, BCG-vaccinated mice showed reduced airway inflammation and hyperreactivity with increased IFN- γ expression. We did not observe a correlation between Th2 cytokine production

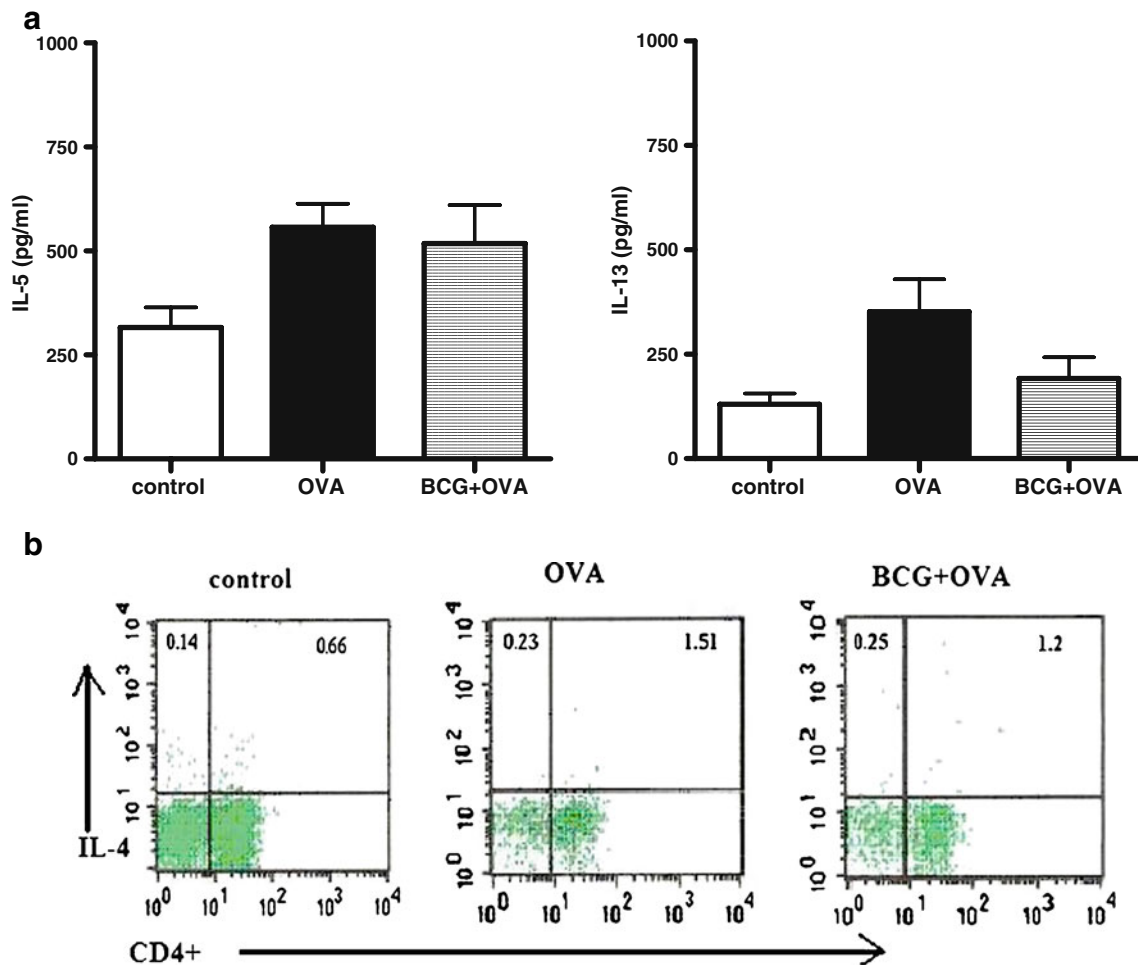


Fig. 5 Airway inflammation in mice with BCG neonatal vaccination did not correlate with Th2 cytokine production. IL-5 and IL-13 cytokine levels in the BALF of mice sensitized and challenged with OVA or saline. One day after a 7-day OVA challenge, BALF were collected, and cytokine levels were measured by ELISA. The data

represent means $\pm$ SD. **b** Flow cytometric detection of IL-4-producing cells in lung lymphocyte preparations from control and OVA-sensitized mice following a 7-day OVA challenge. The data represent mean percentages of positively stained cells within the lymphocyte gate of six mice per group

and reduced inflammation in BCG neonatal vaccinated mice following a 7-day inhalation challenge with OVA. Previous studies reported that the levels of Th1 cytokines (IFN- γ) increased in the inflammatory process of asthma, indicating that activated Th1 and Th2 responses might coexist [26, 27]. In addition, there is evidence that the induction of a Th1 response by the mycobacterium is insufficient to modulate allergic responses [28]. Thus, our results suggest that mice with BCG neonatal vaccination do not develop severe airway inflammation solely through reduction of Th2 cytokines or increase of Th1 cytokines.

One recent study showed that following OVA challenge, T-bet^{-/-} mice displayed increased histological inflammation in the lungs as well as greater thickening of the bronchiole linings, increased numbers of eosinophils and neutrophils in the lung, and enhanced airway hyperresponsiveness as compared to WT mice. However, the production of Th2 cytokines in T-bet^{-/-} mice did not

appear to be significantly greater than in WT mice. Interestingly, a marked increase in the levels of the proinflammatory cytokine IL-17 was observed in T-bet^{-/-} mice [15]. These results indicated that the Th1 and Th17 cytokines might need to achieve a balance in the asthmatic immune response. In our study, the IL-17 level in BALF was upregulated after allergen challenge, whereas the level of IFN- γ was decreased; however, neonatal BCG vaccination inhibited IL-17 production and enhanced IFN- γ production in a BALB/c asthma model when compared with mice in OVA group. These data suggest that IL-17 is involved in the effects of BCG on asthma and BCG neonatal vaccination regulates the IL-17/IFN- γ imbalance in the BALB/c asthma model.

According to our results, neonatal BCG vaccination inhibits OVA-induced airway inflammation and tissue pathology in a BALB/c mouse asthma model. The total number of inflammatory cells in BALF of mice in BCG + OVA group

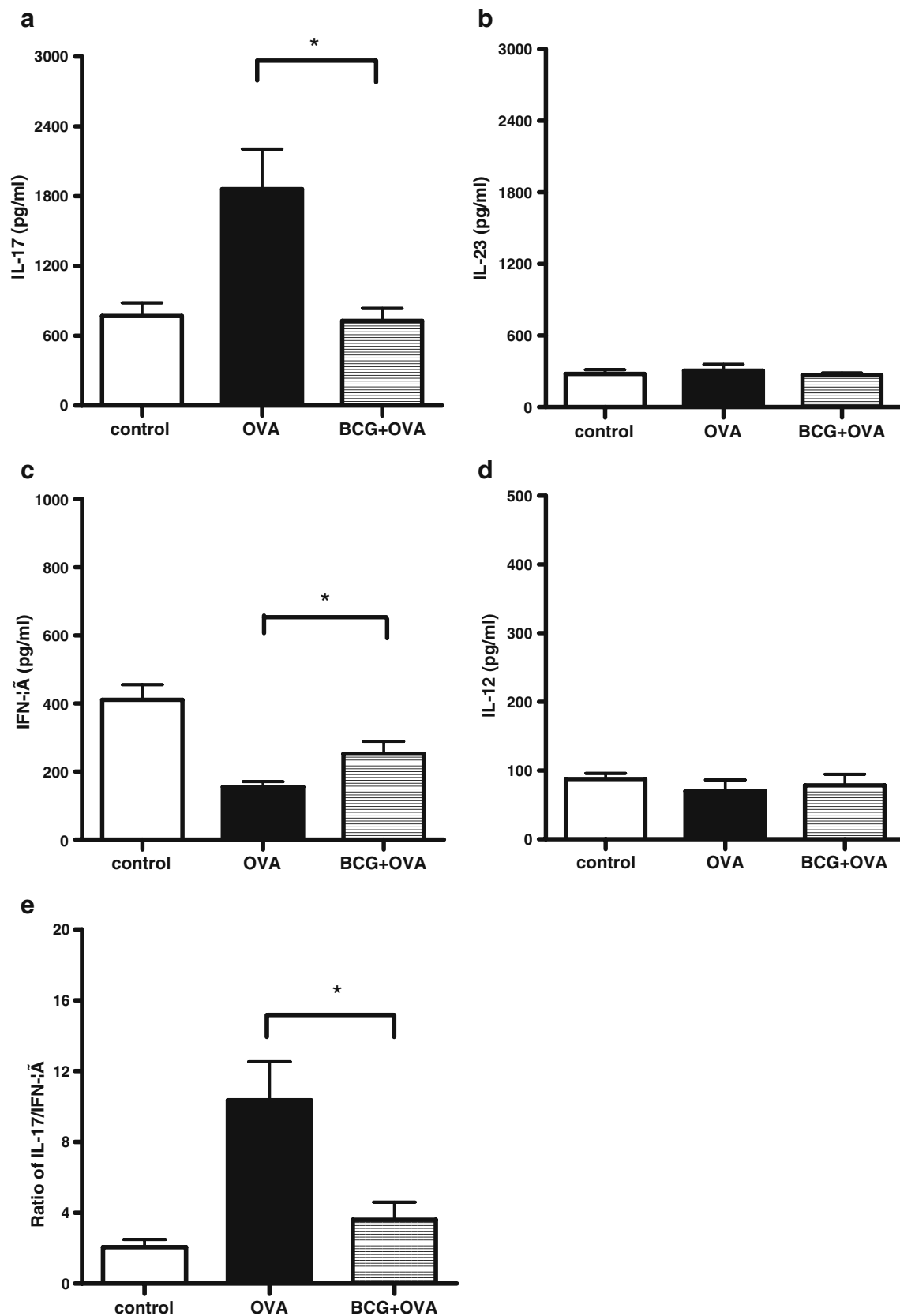


Fig. 6 Neonatal BCG vaccination inhibited IL-17 production and enhanced IFN- γ production in a BALB/c asthma model. IL-17, IL-23, IFN- γ , and IL-12 cytokine levels in the BALF of mice sensitized and

challenged with OVA or saline. One day after a 7-day OVA challenge, BALF were collected and cytokine levels were measured by ELISA. The data represent means $\pm$ SD

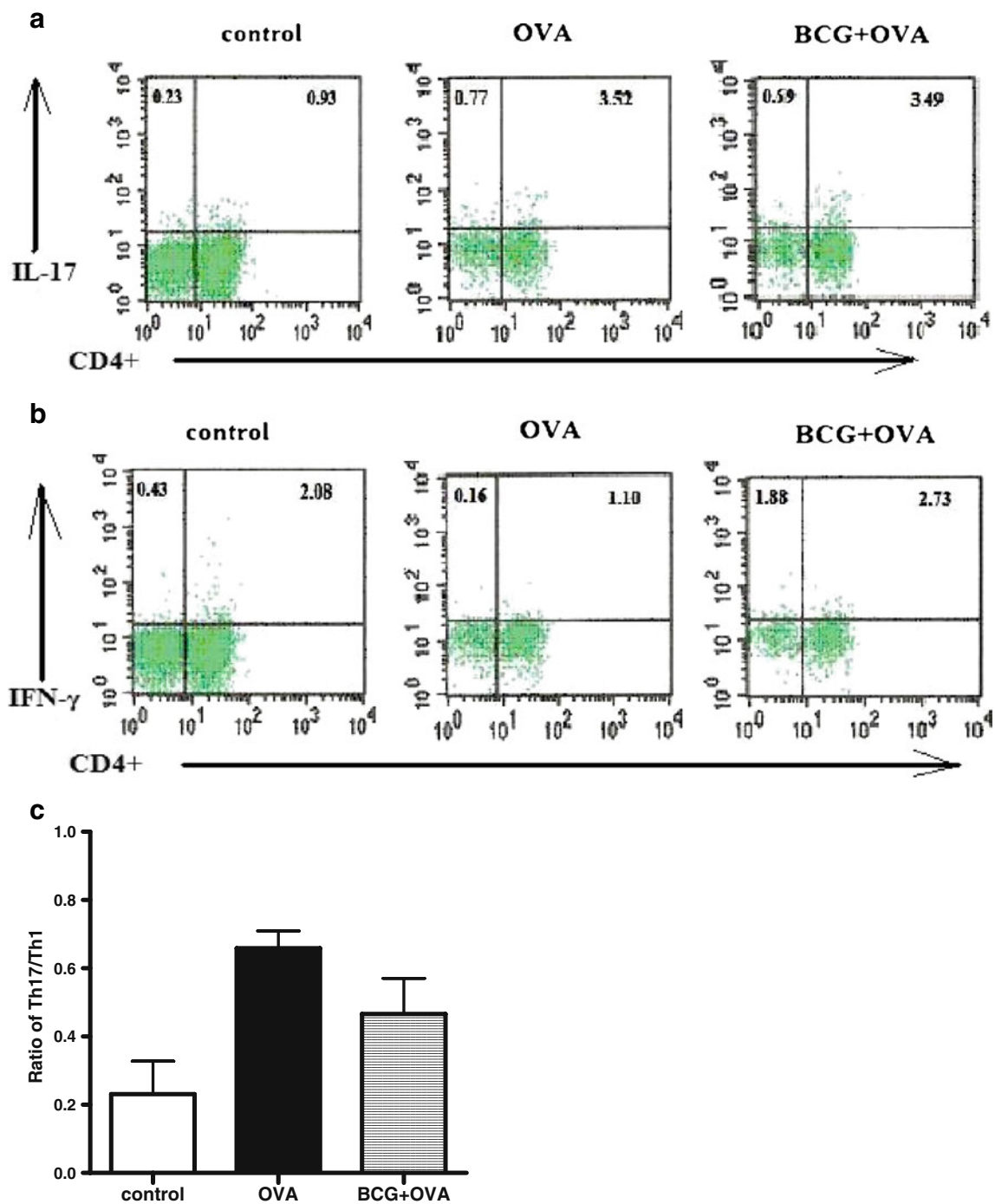


Fig. 7 Neonatal BCG vaccination did not inhibit recruitment of Th17 or reverse the Th17/Th1 imbalance in BALB/c asthma model. Flow cytometric detection of IL-17-producing cells and IFN- γ -producing

cells in lung lymphocyte preparations from naïve and OVA-sensitized mice following a 7-day OVA challenge. The data represent mean percentages of positively stained cells within the lymphocyte gate

was significantly decreased; therefore, the total number of CD4⁺IL17⁺ cells in the lungs of the mice in the BCG + OVA group must be lower than in asthmatic mice, but the ratio of Th17/Th1 was not decreased. Furthermore, BALF concentrations of IL-23, a critical cytokine for IL-17 production by Th17, and IL-12, a critical cytokine for IFN- γ production by

Th1, were not different between mice in the OVA group and BCG + OVA group. These data indicate that neonatal vaccination with BCG elicits protection in mouse allergic responses mainly through the regulation of IL-17/IFN- γ balance but does not depend on reversing the Th17/Th1 imbalance.

Conclusion

In summary, our study confirmed that neonatal BCG vaccination plays a protective role in the prevention of asthma and that this role is associated with reduction of IL-17. We also observed that mice with BCG neonatal vaccination did not develop severe airway inflammation solely through reduction of Th2 cytokines or increase of Th1 cytokines. The IL-17/IFN- γ ratio, but not the Th17/Th1 imbalance, played a key role in the impact of neonatal BCG vaccination in a BALB/c mouse asthma model. Therefore, the antiasthma effect of BCG could be reversed by factors that have effects on the balance of IL-17/IFN- γ , such as bacterial colonization/infection or virus infection in the airway [29]. These findings provide a basis for the design of new strategies for the prevention and treatment of human asthma.

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Conflict of Interest The authors declare that there is no conflict of interest.

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