

Original article

Neonatal vaccination with *Bacillus Calmette-Guérin* elicits long-term protection in mouse-allergic responses

Background: *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) vaccination has been shown to inhibit allergic airway inflammation in animal models, associated with the regulation of allergen-specific T-cell immunity. However, little is known about whether neonatal BCG treatment could inhibit allergic inflammation by regulating allergen-specific T-cell response in aged mice. This study was aimed to investigate the impact of neonatal BCG treatment on allergic asthma and possible mechanism(s) underlying the action of BCG in different ages of mice.

Methods: C57BL/6 neonates were vaccinated with BCG on days 1, 7 and 14, sensitized with ovalbumin (OVA) at 5 and 7 weeks of age, and then challenged with allergen at 9 or 45 weeks of age for early- or late-challenged asthma. Their airway inflammation and allergen-specific T-cell responses were characterized.

Results: Following early-challenge, BCG vaccination inhibited airway hyper-responsiveness (AHR), infiltration of eosinophils and mucous overproduction ($P < 0.05$), and shifted OVA-specific predominant Th2- to Th1-type cytokine responses in both the bronchoalveolar lavage fluid and the splenocyte supernatants ($P < 0.05$). In late-challenged mice, neonatal BCG treatment attenuated AHR and eosinophilia ($P < 0.05$), but failed to modulate allergen-specific cytokine responses.

Conclusions: Our data suggest that neonatal BCG vaccination has a long-term effect on inhibiting AHR and eosinophilia, which is associated with the modulation of Th1/Th2 cytokine production in early-, but not in late-challenged mice. Thus, different mechanisms may mediate the long-term protective effect of BCG neonatal vaccination differently in younger adult and aged mice.

H. Shen¹, H. Huang¹, J. Wang¹, S. Ye²,
W. Li¹, K. Wang¹, G. Zhang¹, P. Wang¹

¹Department of Respiratory Disease, Second Hospital of Medical School of Zhejiang University;

²Zhejiang Provincial People's Hospital, Hangzhou, China

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Huahao Shen
Department of Respiratory Medicine
Second Affiliated Hospital
Zhejiang University School of Medicine
88 Jiefang Road
Hangzhou
China

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Asthma is a chronic inflammatory disease, characterized by airway infiltration of inflammatory cells, particularly by eosinophils and T lymphocytes, mucous over-production, and airway hyper-responsiveness (AHR). Asthma is estimated to affect about 300 million persons and its incidence and severity are increasing in the world (1). Currently, many anti-asthmatic medicines, including inhaled corticosteroids, are available for controlling asthmatic attack. However, those treatments are directed at reducing symptoms and have little effect on inhibiting the disease progression. Therefore, preventative interventions may be a better approach to protect individuals from asthma.

Mycobacterium bovis Bacillus Calmette-Guérin (BCG) is a strong inducer for Th1-type immune response. Several studies have demonstrated that BCG can inhibit allergic airway eosinophilia, mucous over-production and AHR in animal models of asthma (2–5). However, little is known about whether neonatal vaccination with BCG could modulate allergic airway inflammation in aged animals. Given that mice at different ages usually have different levels of immune and pulmonary functions as well as varying responses to anti-asthmatic medicines, we

hypothesized that neonatal vaccination with BCG could have different effects on allergic airway inflammation and allergen-specific T-cell immunity between younger adults and aged individuals.

In our study, we vaccinated C57BL/6 neonates with living BCG, sensitized younger adult mice with allergen [ovalbumin (OVA)] and challenged them with OVA at different life stages. Subsequently, we characterized and compared airway inflammation and allergen-specific T-cell responses among the groups of mice, aimed at investigating the impact of neonatal BCG vaccination on allergen-induced airway inflammation at different ages of mice in order to determine the possible mechanism(s) underlying the action of BCG vaccination.

Methods

Animal

C57BL/6 mice were purchased from the Experimental Animal Center of Zhejiang University and housed in specific pathogen-free facility in our campus. Male and female neonates were used and all

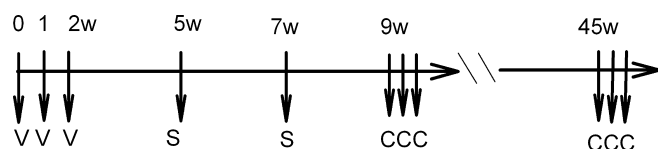


Figure 1. Time line for experimental procedure. V, vaccination; S, sensitization; C, challenge.

protocols in this study were approved by the Ethical Committee for Animal Studies at Zhejiang University, China.

Vaccination

Freeze-dried living BCG (D2-BP302 strain, Biological Institute of Shanghai, Shanghai, China), the commonly used BCG for vaccination in infants, was suspended in phosphate buffer saline (PBS). Mice were injected intradermally with 25 μ l BCG suspension [10^5 infection units (IU)] on the back at birth, and boosted twice on days 7 (10^4 IU) and 14 (10^3 IU). These dosages were the lowest doses that significantly induced protective effects on inhibiting allergic airway inflammation in our preliminary studies. This vaccination procedure was used for the subsequent experiments, illustrated in Fig. 1.

Asthma model

After treatment with BCG for three times, the mice, together with age-matched unmanipulated mice, were sensitized by i.p. injecting with 20 μ g chicken OVA (Sigma-Aldrich, St Louis, MO, USA) emulsified in 100 μ l Inject Alum (Pierce, Rockford, IL, USA) at 5 and 7 weeks of age and randomly divided into different groups. Groups of mice that had been sensitized with OVA were challenged with an aerosolized OVA (1% OVA in saline for 40 min) for three consecutive days at 9 weeks of age, representing the early-challenge as described previously (6). Other groups of mice were challenged at 45 weeks of age for the late-challenge, based on our preliminary results that mice sensitized at 5 and 7 weeks of age retained their responses to the allergen with which they were challenged at 45 weeks of age. Additional groups of mice that had been injected with saline at each time point were used as negative controls.

Airway hyper-responsiveness

Two days after challenges, their AHR to inhaled methacholine (MCh) was measured by flow plethysmography (Buxco, Troy, NY, USA) as previously described (7). Enhanced pause (Penh) was normalized by subtracting the baseline values.

Characterization of eosinophilia in Bronchoalveolar lavage fluid (BALF) and Histological examination

Two days after challenges, the mice were anesthetized and their BALF were collected as described previously (6). The collected BALF was centrifuged and the cells were resuspended in 0.2 ml PBS. The cells were stained with the Wright's Giemsa and eosinophils in the BALF were blindly counted in duplicate for each sample under microscope by investigators for determining the total numbers of eosinophils in the BALF.

After being killed, their lungs were fixed in 10% buffered formalin and their tissue sections were stained with hematoxylin/eosin or

Alcian blue/periodic acid-Schiff (AB/PAS), respectively. The severity of peribronchial and perivascular inflammation was scored according to the previous definition (8). Goblet cell hyperplasia ratio (HR) and mucus-occupying ratio (MOR) were determined by Leica-Qwin image-processing system (Leica Imaging Systems, Bensheim, Germany). HR was expressed as the number of goblet cells in basal lamina/mm and MOR was expressed as the percentage of the total area of epithelium occupied by AB/PAS staining.

ELISA assay

Splenic mononuclear cells from experimental and control mice were prepared (9). The cells (2×10^6 cells/ml) were stimulated in triplicate with, or without, OVA (40 μ g/ml) in 10% fetal bovine serum medium in 24-well plates at 37°C and 5% CO₂ for 48 h. The contents of IFN- γ and IL-4 in the supernatants were characterized using the EILSA kits (RD Systems, Minneapolis, MN, USA). The sensitivity of kits was 2 pg/ml. Additional ELISA assays were performed to determine the contents of IFN- γ , IL-4, IL-5, IL-13 and IL-25 in the supernatants of BALF collected from experimental or control mice.

Statistical analysis

Data were expressed as mean $\pm$ SEM. Differences between the groups were analyzed by one-way analysis of variance (ANOVA) or the Mann-Whitney test using spss 13.0 software. *P*-values of <0.05 were considered significant.

Results

Vaccination with BCG-inhibited allergen-induced AHR in both early- and late-challenged mice

To evaluate the effect of neonatal vaccination with BCG on AHR in the mice, challenged with allergen at various ages, their Penh values were assessed by whole-body plethysmography two days after the final challenge with OVA (Fig. 2).

Penh values, in response to different doses of aerosolized MCh, in the mice that had received saline, gradually increased with elevated concentrations of MCh while in early-challenged mice that had not received BCG (OVA group), Penh values, in response to 25 mg/ml aerosolized MCh, were significantly higher than that in saline group ($P < 0.05$) and slightly increased in response to 50 mg/ml MCh. In contrast, Penh values in the mice which received BCG vaccination were paralleled with that of saline controls and were significantly lower than that of OVA group of mice in response to 25 or 50 mg/ml MCh ($P < 0.05$).

Following the late-challenge, the Penh values in OVA group of mice, in response to 25 mg/ml MCh, were lower than that of the mice challenged at early stage, while comparative levels of Penh in response to 50 mg/ml were observed in early- and late-challenged OVA groups of mice. These data suggest that even 40 weeks after sensitization, allergen-challenge induced high levels of AHR in aged mice. Penh values at 50 mg/ml point in

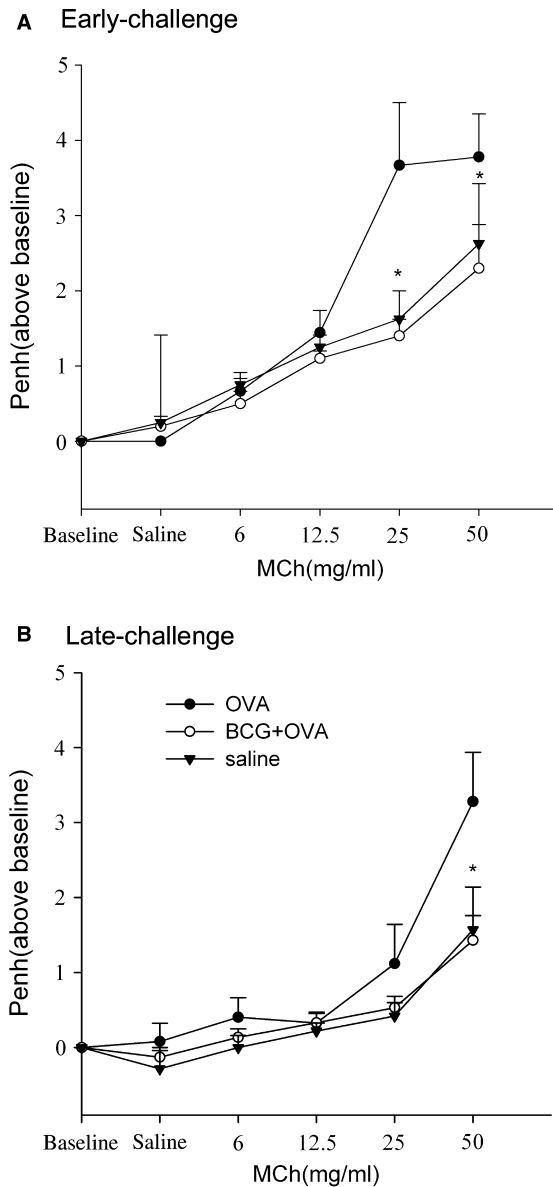


Figure 2. BCG vaccination inhibits allergen-induced airway hyperresponsiveness (AHR) to methacholine (MCh) inhaled. Groups of mice were sensitized at 5 and 7 weeks of age and they were challenged with allergen at 9 or 45 weeks of age for the early- or late-challenge, respectively. Two days after the final challenge, together with negative control mice injected with saline, their AHR (Penh) in response to increased doses of nebulized MCh was assessed by whole-body plethysmograph. (A) Early-challenged mice. (B) Late-challenged mice. Data are presented as the mean $\pm$ SEM of Penh from experimental or control groups of mice ($n = 10$ –12 per group). * $P < 0.05$ vs. OVA group.

BCG + OVA group of mice, similar to that of saline group, significantly decreased when compared with that of OVA group of mice ($P < 0.05$). Therefore, it is observed that neonatal vaccination with BCG significantly reduced AHR in both early- and late-challenged mice.

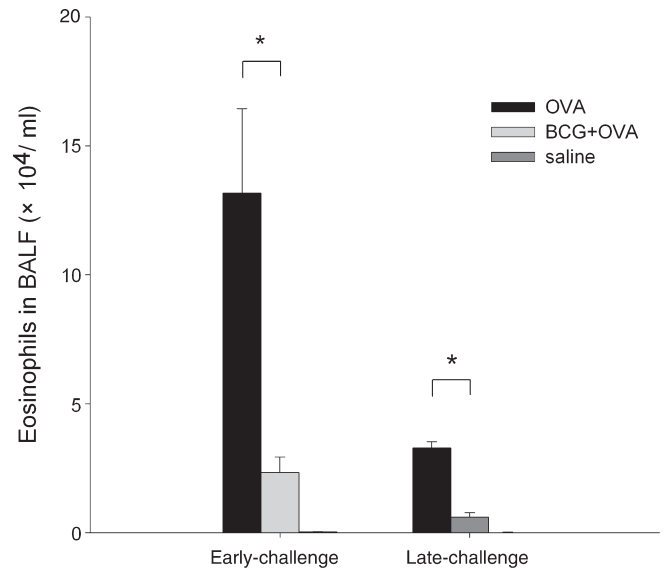


Figure 3. BCG vaccination reduces eosinophilia. Groups of mice were sensitized and challenged with the allergen. Their BALF were collected 2 days after the final challenge and then the number of eosinophils was counted based on the Wright's Giemsa staining. Data are expressed as the mean $\pm$ SEM of the number of eosinophils in each group of mice ($n = 8$ –9 per group). * $P < 0.05$.

BCG vaccination inhibited airway eosinophilia in early- and late-challenged mice

To determine whether BCG vaccination could inhibit eosinophilia in asthmatic mice, following challenge with OVA, their BALF were collected and the number of eosinophils were counted. The mice injected with saline showed few eosinophils regardless of their ages (Fig. 3). Following challenge with OVA at 9 weeks of age, tremendous numbers of eosinophils were observed in BALF. In contrast, the number of eosinophils was dramatically reduced in the mice that had been treated with BCG ($P < 0.05$). A similar pattern of eosinophilia was observed in the late-challenged mice although the total number of eosinophils in BALF of OVA group of mice was reduced in late-challenged mice as compared with that in early-OVA-challenged mice. Therefore, vaccination with BCG inhibited eosinophilia in both early- and late-challenged mice.

Analysis of eosinophilia in the lung tissues revealed that there was almost no eosinophilic infiltration in saline group of mice (Fig. 4A,D), while numerous eosinophils and other inflammatory cells appeared at the peribronchiolar zone in OVA group of mice in both younger and aged stages (Fig. 4B,E), but more so in younger mice (Fig. 4B). In contrast to OVA mice, the mice vaccinated with BCG showed a significantly reduced number of eosinophilia, accompanied by attenuated peribronchial and perivascular inflammation (Fig. 4C,F). As shown in Fig. 5, the inflammation scores of peribronchial

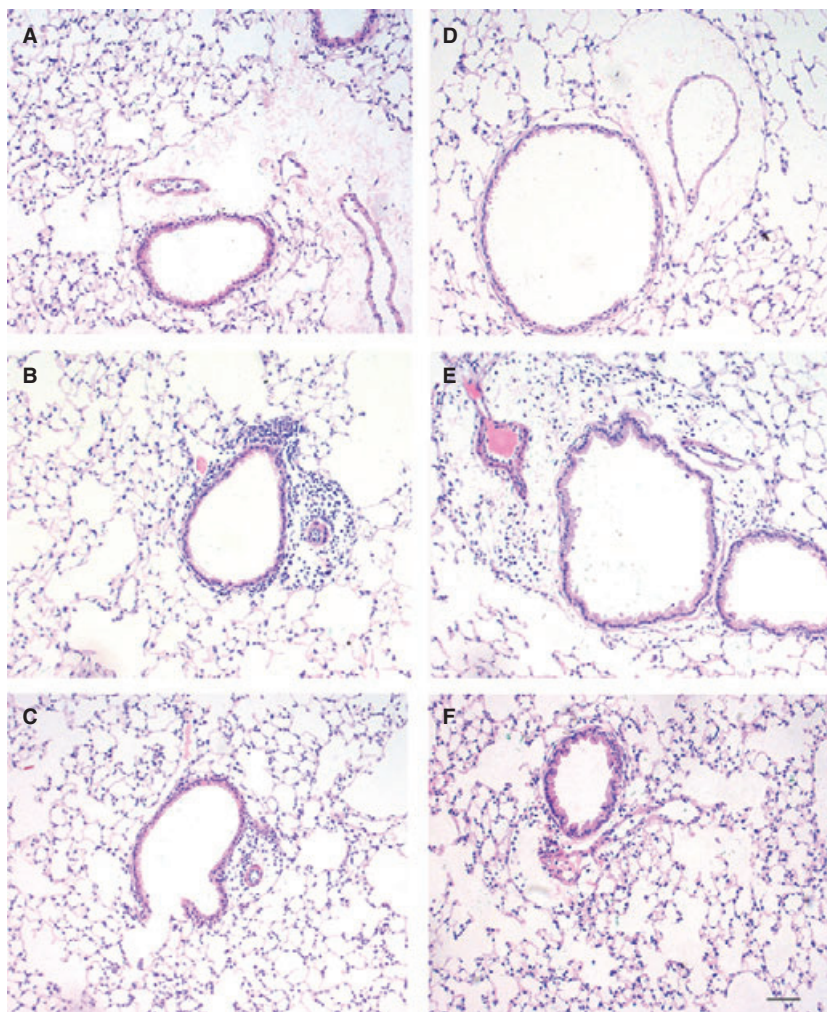


Figure 4. Histological examination of the lung tissues in asthmatic mice. Two days after the final challenge, mouse lung tissue sections were stained with hematoxylin and eosin (HE). Data are representative of each group of mice ($n = 8-9$ per group). A and D, saline group; B and E, OVA group; C and F, BCG + OVA group. A–C, mice challenged at 9 weeks. D–F, mice challenged at 45 weeks (Magnification 200 $\times$). Bars = 100 μ m.

and perivascular regions decreased significantly as compared with that in OVA group of mice at the corresponded time point ($P < 0.05$). Hence, vaccination with BCG not only inhibited allergen-induced eosinophilia, but also suppressed airway inflammation in asthmatic mice.

BCG vaccination inhibited the mucus overproduction in early-challenged mice

To further examine the impact of BCG vaccination on airway inflammation in asthmatic mice, lung sections from experimental and control mice were stained with AB/PAS (Fig. 6) and their airway mucous production was measured by HR and MOR (Fig. 7). Little or no mucus was observed around the airways in saline group of mice (Fig. 6A), while numerous PAS-positive cells

were observed in the airways in OVA group (Fig. 6B) following the early challenge with OVA. BCG immunization inhibited the airway mucous production (Fig. 6C), as PAS-positive cells in BCG + OVA group had remarkably reduced. In late-challenged mice, numerous PAS-positive cells were also observed in the lungs of both OVA and BCG + OVA groups of mice. Apparently, BCG had little effect on inhibiting disease-related mucus overproduction in asthmatic and aged mice.

Paralleled with the pathologic manifestation, both HR and MOR in early-challenged OVA group of mice were significantly higher than those in saline group (Fig. 7). In contrast, HR and MOR values decreased significantly in the early-challenged BCG + OVA group when compared to that in OVA group ($P < 0.05$). However, HR and MOR of the BCG + OVA group of aged mice just slightly decreased, but insignificantly, as compared with

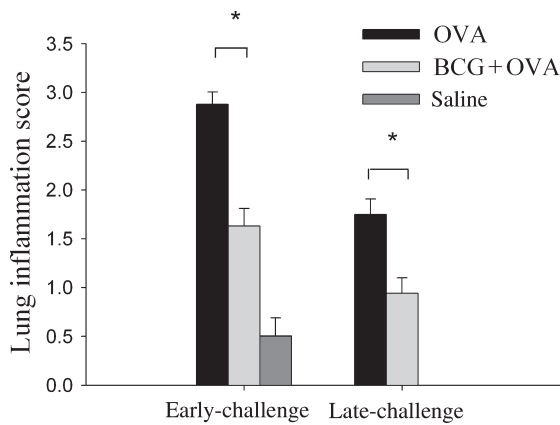


Figure 5. BCG vaccination decreases the peribronchial and perivascular inflammation. Two days after the final challenge, the peribronchial and perivascular inflammation of experimental and control mice was scored. Data are expressed as mean score $\pm$ SEM of each group ($n = 8-9$ per group). * $P < 0.05$.

that of OVA group ($P > 0.05$), unlike in younger mice. Together, BCG vaccination suppressed the mucus production predominately in younger asthmatic mice.

The effect of BCG on Th1/Th2 cytokine production in early- and late-challenged mice

Vaccination with BCG usually induces Th1 response, which has been thought to down-regulate allergen-induced Th2 response, mediating a protective effect on asthmatic mice. To evaluate the association of BCG vaccination and OVA-specific T-cell responses in asthmatic mice, the levels of cytokines in BALF and OVA-stimulated splenocyte supernatants were measured (Fig. 8). In early-challenged mice, Th2 response was predominant in OVA group, as shown in Fig. 8. Higher levels of IL-4, IL-5 and lower levels of IFN- γ were observed in BALF of OVA group of mice. Furthermore, such dominant Th2 responses were further demonstrated

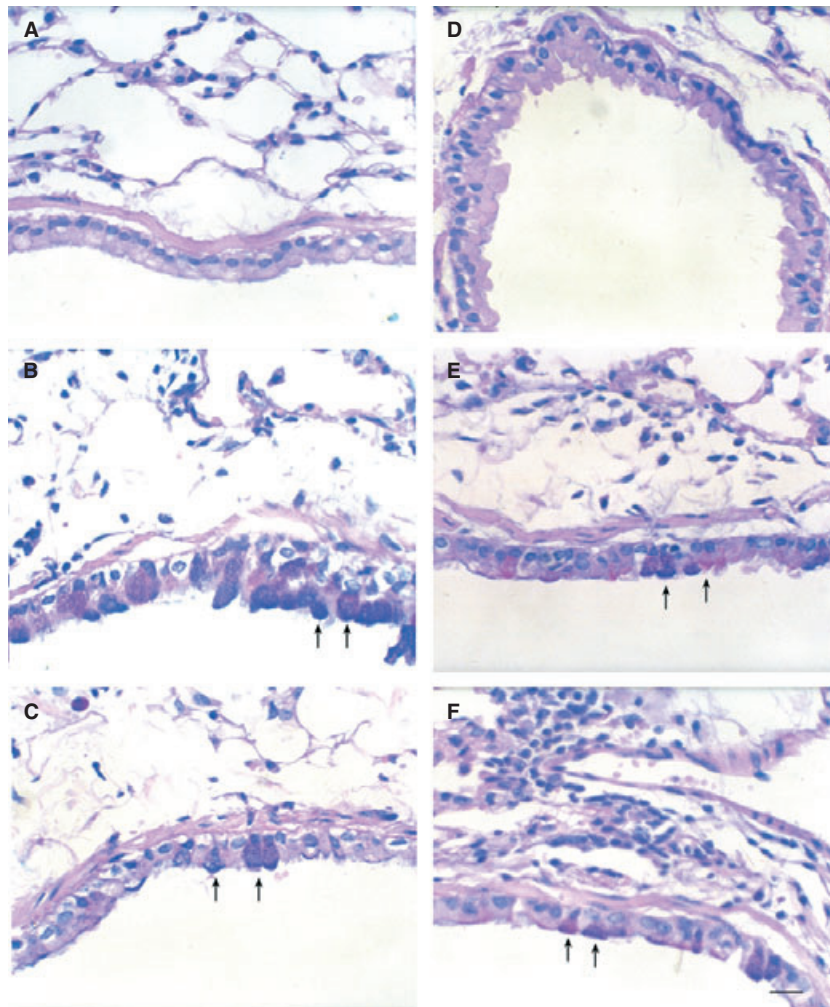


Figure 6. Analysis of lung tissue sections by AB/PAS staining. Data are representative of each group of mice ($n = 8-9$ per group). The goblet cells displayed with purplish red staining. A and D, saline group; B and E, OVA group; C and F, BCG + OVA group. A-C, mice challenged at 9 weeks. D-F, Mice challenged at 45 weeks (magnification 600 $\times$). Bar = 33.3 μ m. The arrow represents PAS-positive cells.

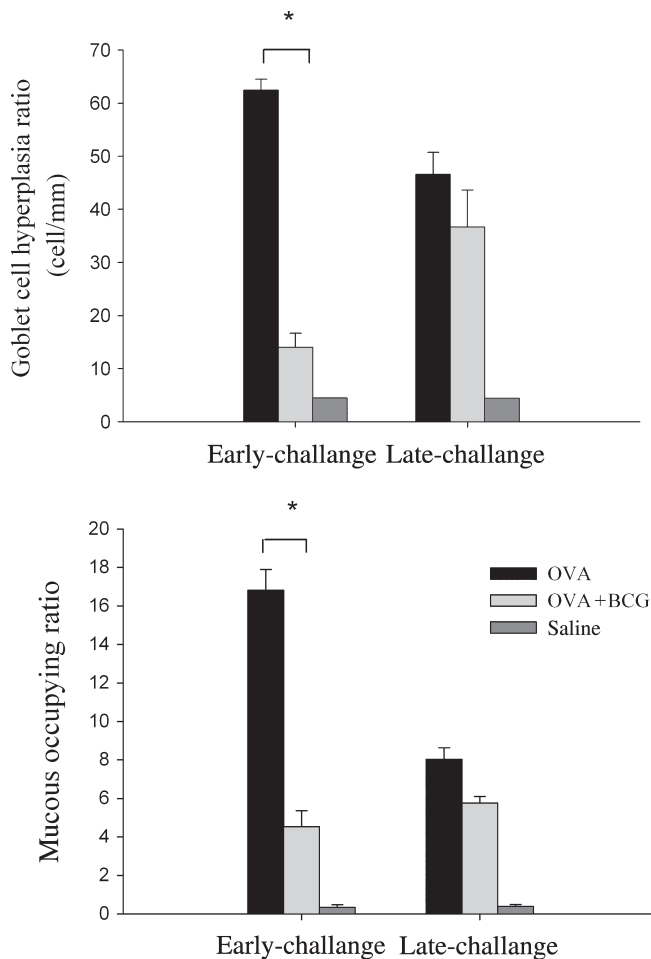


Figure 7. Effect of BCG on the mucous overproduction in asthmatic mice. The values are presented as mean $\pm$ SEM of 2–5 secretions per mouse from each group ($n = 8$ –9 per group). * $P < 0.05$.

in OVA-stimulated splenocyte supernatants, evidenced by higher levels of IL-4 and lower levels of IFN- γ in OVA group. Importantly, BCG immunization shifted the dominant OVA-specific Th2 responses to Th1 responses in early-challenged mice. The levels of IFN- γ in BALF and splenocyte supernatants significantly increased, accompanied by dramatically reduced levels of IL-4 and IL-5 in BALF and IL-4 in splenocyte supernatants in BCG + OVA group of mice, as compared with that of the OVA group ($P < 0.05$). Surprisingly, similar levels of IL-13 and IL-25 were detected in BALF in different groups of early-challenged mice (data not shown) although IL-13 and IL-25 were crucial for allergic inflammation (10, 11). However, in late-challenged mice, very low levels of IFN- γ , IL-4, IL-5, IL-13 and IL-25 in BALF and low levels of IFN- γ and IL-4 in splenocyte supernatants were observed in aged mice, regardless of the mice that had been challenged with, or without, OVA. Thus, vaccination with BCG shifted allergen-specific

Th2-dominated responses to Th1 type of responses in younger, but not in aged asthmatic mice.

Discussion

The primary aim of this study was to evaluate the effect of neonatal vaccination with BCG on the airway inflammation in asthmatic mice induced with allergen at various times. Previous studies have demonstrated that BCG vaccination can suppress airway eosinophilia, mucus overproduction and AHR in the animal experiments (5, 12–14). We found that neonatal BCG vaccination inhibited the allergen-induced AHR, eosinophilia and mucus overproduction in asthmatic mice, challenged with allergen at younger adults (9 weeks old). This protective effect of BCG vaccination was associated with elevated IFN- γ response, accompanied by significantly reduced IL-4 and IL-5 responses. Our data are consistent with the notion that neonatal vaccination with BCG induces Th1 responses, which subsequently inhibits allergen-specific Th2 responses, leading to the attenuation of allergic airway inflammation.

Notably, many aged people develop allergic asthma, responding to allergens that they were exposed to in childhood. To determine whether neonatal vaccination with BCG could modulate allergic asthma at the late life stage, groups of mice that had been neonatally vaccinated with BCG and sensitized with allergen at younger age were challenged with the allergen at the late life stage and their airway inflammation was characterized. We found that neonatal vaccination with BCG also reduced AHR and eosinophilia in the late-challenged aged mice, but insignificant in reducing mucus production when compared with that of the OVA group of mice that had not been treated with BCG. Interestingly, BCG vaccination failed to modulate allergen-specific cytokine responses in aged asthmatic mice, suggesting that this protective effect was not associated with the elevation of Th1 cytokine production. This novel finding suggests that BCG vaccination has a long term of protective effect on inhibiting allergic airway inflammation in our experimental model. Notably, the results from epidemiological studies on the association of *Mycobacterium tuberculosis* infection or BCG vaccination with asthma development in human are elusive and controversial (15). Accordingly, whether neonatal BCG vaccination could inhibit and prevent asthmatic inflammation in human remains to be further investigated.

We observed that the protective effect of BCG vaccination on allergic inflammation lasted much longer, when compared to the previous findings (4, 16), even at a very late life (at 45 weeks of age). In our experimental protocol, unlike most other studies with single dose of BCG immunization, the mice received three dosages of BCG. Indeed, data from a recent study have shown that repeated BCG vaccinations improved the lung function and increased the IFN- γ /IL-4 ratio in the peripheral

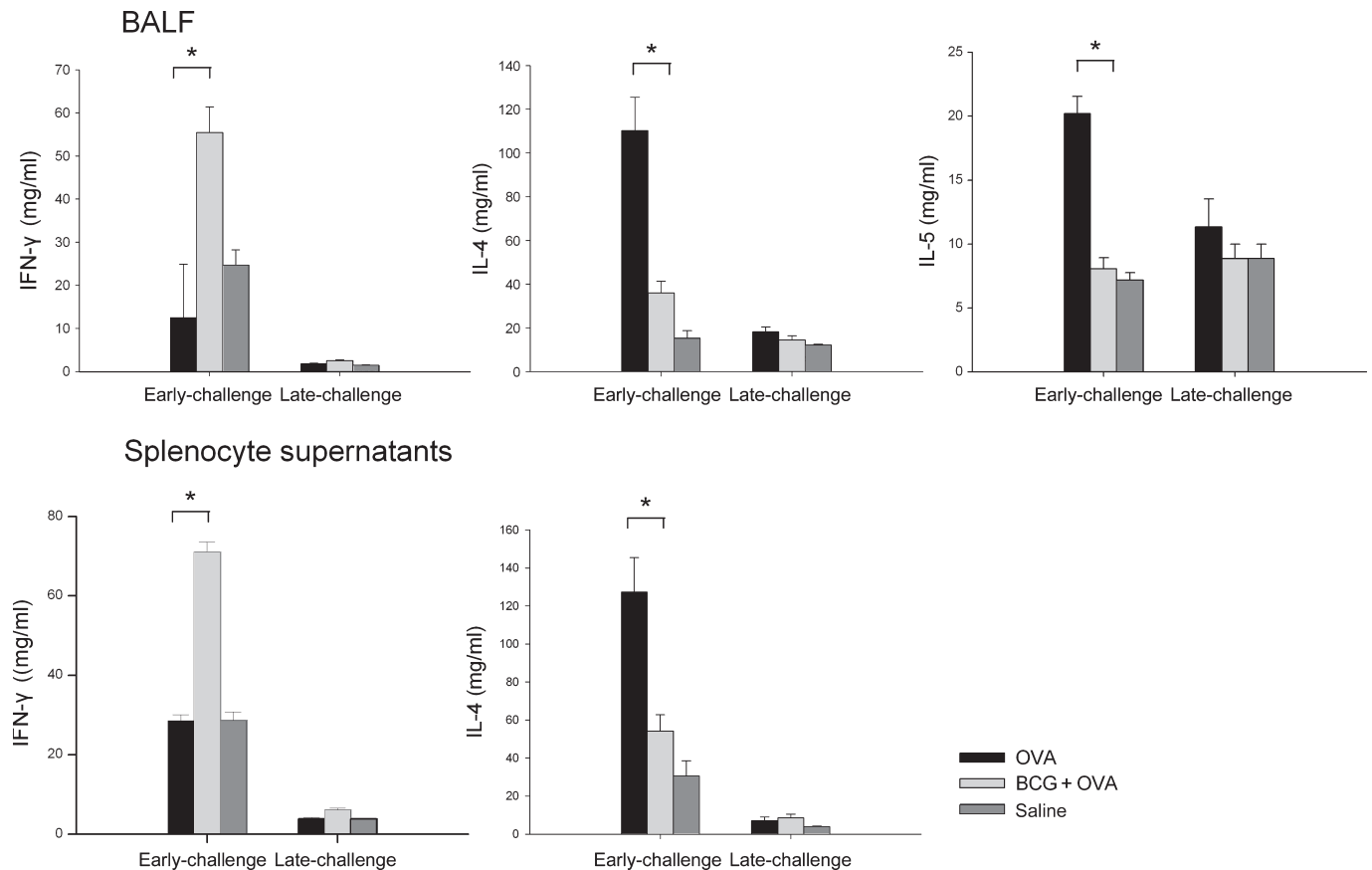


Figure 8. The effect of BCG on Th1 and Th2 cytokine production in early- and late-challenged mice. The contents of IFN- γ , IL-4, and IL-5 in BALF were quantitatively analyzed by ELISA. Splenic mononuclear cells at 2×10^6 /well from individual mouse were stimulated with 40 μ g/ml OVA for 48 h and their culture supernatants were collected. The levels of IFN- γ and IL-4 were quantitatively analyzed by ELISA. The values are presented as mean $\pm$ SEM of each group ($n = 6$ per group). * $P < 0.05$.

blood in asthmatic patients (17). However, the efficacy and mechanism of BCG revaccination and whether the revaccination is necessary to elicit the long protective effect we observed remain to be further examined. Furthermore, neonatal immunization with antigen in complete adjuvant or vaccination with BCG induces unipolar antigen-specific Th1 responses (18, 19). The induced Th1 responses should effectively shift Th2-biased immune responses in neonates to a Th1-type of response as memory and effective T-cell immunity affect the subsequently-induced T-cell responses (20). We also found that neonatal vaccination with BCG elicited long-lasting protection from allergic airway inflammation, which was associated with the induction of OVA-specific Th1 responses in BCG-treated mice. Finally, C57BL/6 mice are a Th1-biased strain and respond to BCG by a strong Th1 response (21). In comparison with Th2-biased BP2 or BALB/C mice, in which allergen-challenge usually induces higher levels of AHR and IgE titers (21, 22), the strong Th1 response induced by BCG vaccination may inhibit the development of Th2-dependent airway inflammation more efficiently in C57BL/6 mice.

Notably, following the early challenge, higher levels of Th2 cytokine (IL-4 and IL-5) responses in OVA group of mice and predominant IFN- γ responses in BCG-treated mice were observed. However, very low levels of cytokines were detected in aged mice following the late-challenge regardless of BCG treatment. Apparently, the OVA-specific T-cell responses established by sensitization at younger adult were attenuated with aging. The plain cytokine response may be attributed to poor responses of T cells and the unique kinetics of cytokine production in aged mice (23–26). Therefore, we should be cautious in using the levels of allergen-specific cytokine responses as accurate surrogate measures for allergic responses in aged individuals.

Interestingly, we observed that neonatal BCG vaccination induced a strong Th1-type response, which down-regulated allergen-specific Th2 response in early-challenged asthmatic model, consistent with previous findings (13, 14, 27, 28). However, we surprisingly found that neonatal BCG vaccination significantly inhibited allergic inflammation, but did not significantly change IFN- γ , IL-4 and IL-5 responses in late-challenged mice.

This failure in inducing allergen-specific IFN- γ response suggest that the long-term protection of neonatal BCG treatment in aged asthmatic mice is independent of IFN- γ , which supports previous finding (16), but is in disagreement with the other reports (3, 5). Moreover, it suggests that other mechanism(s) may underlie the action of BCG in aged mice. Allergen-specific regulatory T cells have been shown to mediate long-lasting protection in asthmatic models (29) and increased frequency of CD4⁺ CD25⁻ Foxp3⁺ regulatory cells are associated with hyporesponse in aged mice (30). We are interested to investigate the precise effect of those regulatory T cells on allergic inflammation in asthmatic aged mice, which may provide a basis for the design of new strategies for the prevention of allergic airway inflammation in human.

In summary, we examined the effect of neonatal BCG vaccination on allergic airway inflammation in both early- or late-challenged asthmatic mice and found that neonatal vaccination with BCG inhibited allergic eosinophilia, AHR and mucus overproduction in early-challenged mice, associated with shifting allergic Th2 responses to IFN- γ response. Furthermore, neonatal

vaccination with BCG inhibited allergic eosinophilia and AHR, but not mucous overproduction in late-challenged mice, which was not associated with elevated levels of IFN- γ response to the allergen. Therefore, neonatal vaccination with BCG elicits long-term protection by inhibiting allergic airway inflammation in both younger adult and aged mice. These novel findings suggest that other mechanism(s) may underlie the long-lasting protection of BCG vaccination in aged mice. Our findings provide a basis for the design of new strategies for the prevention and intervention in human asthma.

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