ORIGINAL RESEARCH

Protective effects of combined *Mycobacterium bovis* BCG and interleukin-12 vaccination on airway inflammation in a murine model of allergic asthma

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Abstract

Purpose. Allergic asthma is characterized by chronic airway inflammation and airway hyperresponsiveness driven by allergen-specific T helper (Th)2 cells. *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccination has been documented to suppress Th2 responses and allergic airway inflammation in animal models. Since interleukin (IL)-12 is capable of inhibiting Th2 responses, we sought to investigate whether IL-12 could function as an adjuvant to increase the efficacy of BCG vaccination against allergic asthma.

Methods. BALB/c neonatal mice (24 mice, 48-72 h old) were randomly divided into 3 subgroups (n = 8 for each group) to be immunized with PBS (control) or BCG with or without DNA plasmid-expressing IL-12. All of the mice were then sensitized and provoked with ovalbumin (OVA) to establish a model of allergic asthma.

Results. Mice vaccinated with BCG alone showed a significant reduction in airway inflammation, percentage of eosinophils in bronchoalveolar lavage (BAL) fluid, and serum OVA-specific immunoglobulin E (IgE) levels in comparison with control animals. The suppressive effects of BCG were substantially augmented by the combination with IL-12. Furthermore, a decreased IL-4 and increased interferon-gamma (IFN- γ) production in BAL fluid were observed in animals inoculated with BCG alone or with IL-12 relative to control animals.

Conclusion. Our data indicate that the combined vaccination with BCG and IL-12 yields a favorable outcome in

prevention of experimental allergic airway inflammation, which is likely mediated through triggering a shift from a Th2 response to a Th1 response.

Allergic asthma is a chronic respiratory disease characterized by reversible airway obstruction, airway hyperresponsiveness, chronic airway inflammation with eosinophilic infiltration, mucus hypersecretion, and elevated serum immunoglobulin E (IgE) levels.¹ Central to the pathogenesis of allergic asthma are T helper (Th)2-mediated immune responses. Th2 cells are the predominant T cell subsets in the asthmatic airway, and become activated upon encountering antigen, consequently releasing numerous proallergic cytokines.²⁻⁴ Among Th2 cytokines, interleukin (IL)-4 plays a pivotal role in the pathogenesis of allergic asthma, which participates in Th2 cytokine responses,⁵ IgE isotype class switching,⁶ and airway remodeling.⁷ IL-4 deficiency in mice was reported to impair repeated allergen exposure-induced airway inflammation and bronchial hyperresponsiveness.⁸ Currently, many efforts have been made to develop drugs targeting allergen-specific Th2 responses in the treat-

ment of asthma. Th1 and Th2 cells can reciprocally regulate each other through their released cytokines,^{9,10} raising the possibility of designing therapeutic modalities that would promote Th1 immune response and thereby antagonize Th2 immune response. Indeed, *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), a potent inducer of Th1 response, has been shown to alleviate allergic airway inflammation in animal models via increased secretion of Th1 cytokines, especially interferon-gamma (IFN- γ), that attenuate allergen-specific Th2 response.¹¹⁻¹³ In addition to enhancing Th1 function, BCG has been shown to propagate regulatory T cells,¹⁴ thereby providing another mechanism for suppression of Th2 allergic reactions.

IL-12 is a heterodimeric cytokine produced mostly by phagocytic cells in response to antigenic stimulation, and to some degree by B lymphocytes.¹⁵ This cytokine holds an essential role in the development of Th1 cells,^{16,17} and therefore has potential implications in the treatment of asthma. Indeed, IL-12 has been reported to impair the development of IL-4-producing Th2 cells in response to *Dermatophagoides pteronyssinus* and *Leishmania major*.¹⁸⁻²⁰ In experimental asthma, systemic administration of IL-12 can abolish airway hyperreactivity and pulmonary eosinophilia.^{21,22} Given the role of IL-12 in the prevention of asthma, we attempted to test whether IL-12 could function as an adjuvant to enhance the efficacy of BCG vaccination against allergic asthma.

Materials and Methods

Animals and immunization

All animal experiments were conducted after approval by the Animal Care Committee of Chongqing Medical University, Chongqing, China. Female and male BALB/c mice, aged 5 weeks, were bred in the Experimental Animal Center of Chongqing Medical University. Neonatal mice, aged 2-3 days, were randomly divided into 3 subgroups (n=8 for each group)

to be immunized with phosphate-buffered saline (PBS) as control or BCG with or without DNA plasmid-expressing IL-12. Each mouse in the BCG group received a one-time subcutaneous inoculation of 5×10^4 colony forming units (CFUs) of live attenuated BCG (0.1 mL; Shanghai Institute of Biological Products, Shanghai, China). Animals in the BCG plus IL-12 group received a subcutaneous injection of an identical dose of BCG, combined with an intramuscular delivery of pcDNA3.1/IL-12 (kindly provided by Dr. Graham Lieschke, Walter and Eliza Hall Institute of Medical Research, Parkville, Australia; 50 μ g/mouse) into the quadriceps and tibia anterior muscles, followed by boosting with the same dose of IL-12 plasmids at weeks 1, 2 and 3. Additionally, control animals without BCG vaccination received administration of PBS (0.1 mL/mouse) into the quadriceps and tibia anterior muscles every week for 4 weeks.

Sensitization and challenges with ovalbumin (OVA)

At 27 and 42 days after the initial inoculation, animals were sensitized by intraperitoneal injections of 50 μ g OVA (Sigma, St. Louis, MO, USA) in 2 mg of aluminum hydroxide (Sigma). On day 15 post-OVA immunization, mice were challenged with a 5% (w/v) OVA/PBS for 20 min in a chamber (20 $\times$ 16 $\times$ 15 cm). The OVA challenge was repeated every day for 6 days. Twenty-four hours after the last OVA challenge, mice were sacrificed, and airway responsiveness and other parameters were examined.

Bronchoalveolar lavage (BAL) fluid

Animals were sacrificed by CO₂ asphyxiation 24 h after the last provocation with OVA aerosols. The trachea was cannulated, and the lung was lavaged three times with 400 μ l of PBS. The BAL fluid was centrifuged at 800 rpm for 10 min at 4°C, and the supernatants were collected for determination of cytokines. The cell pellets were resuspended in 0.5 mL of PBS, and BAL cell differentials were determined on slide preparations stained with Wright's stain (Sigma). At

least 200 cells on each slide were differentiated by light microscopy (1000×) and the mean percentage of eosinophils was calculated.

Histological assessment

After sacrifice, the lungs were collected and fixed with 4% paraformaldehyde-PBS and embedded in paraffin. The tissues were sectioned and stained with hematoxylin and eosin (H&E) according to a standard protocol. Pathological changes in the airway and lung tissues were evaluated by light microscopy.

Enzyme-linked immunosorbent assay (ELISA)

OVA-specific IgE levels in serum were measured by ELISA according to a standard protocol. Briefly, ELISA plates were coated overnight with OVA (10 µg/ml) at 4 °C and blocked with PBS containing 3% BSA. Plates were washed three times and samples were added to the wells after serial dilution in 3% BSA/ PBS. After 1 h incubation at room temperature, wells were washed and incubated with biotinylated rat anti-mouse IgE (1:500 dilution; Jingmei Biotech Co., Ltd., Shenzhen, China) for 1 h at room temperature, followed by incubation with horseradish peroxidase (HRP)-conjugated streptavidin (1:500 dilution; Jingmei Biotech Co., Ltd.) for 30 min. Tetramethylbenzidine was used as substrate, and the OD was determined at 450 nm using an ELISA microtiter plate reader.

The concentrations of IFN-γ and IL-4 in BAL fluid were assessed using commercially available ELISA kits (Jingmei Biotech Co., Ltd., China) following the manufacturers' instructions. The standard curves were generated by the standards of known IL-4 or IFN content provided. The limit of detection was 2 pg/mL for each assay.

Statistical analysis

Values are expressed as mean ± SD. All analyses were done using SAS version 6.12 (SAS Institute, Cary, North Carolina, USA). Differences among groups

were analyzed using an ANOVA together with a Student-Newman-Keuls analysis. *P* values of less than 0.05 were considered statistically significant.

Results

IL-12 augments the protective effects of BCG vaccination against OVA-induced airway inflammation

The OVA-induced allergic asthma model was established in all groups. The animals in the control group showed typical symptoms of asthma, such as dyspnoea, accelerated breathing, wheezing, hyperspasmia and incontinence. The BCG group presented with symptoms that appeared to be milder than those in the control group and the BCG plus IL-12 group presented with the mildest phenotype of asthma.

It has been reported that neonatal vaccination with BCG inhibits OVA-triggered infiltration of eosinophils and airway inflammation in aged mice.¹² Consistent with these previously published data, immunization with BCG prior to OVA priming significantly reduced the percentage of eosinophils in the BAL fluid

FIGURE 1. Analysis of the proportion of eosinophils in bronchoalveolar lavage (BAL) fluid. Neonatal mice treated with PBS (control) or immunized with BCG or BCG plus IL-12 were sensitized and challenged with OVA. After OVA provocation, mice were sacrificed and the percentage of eosinophils in the BAL fluid was determined. **P* < 0.05 versus the control group; #*P* < 0.05 versus the BCG group.

FIGURE 2. Lung histopathology. Neonatal mice treated with PBS (control) or immunized with BCG or BCG plus IL-12 were sensitized and challenged with OVA. After OVA provocation, mice were sacrificed and histological analysis of the lung tissues was performed. Extensive cellular infiltration of peribronchiolar regions were detected in lungs from control mice (a). In contrast, mice immunized with BCG (b) or BCG plus IL-12 (c) showed significantly less lung inflammation. H&E; original magnification, $\times 100$.

by almost 50% in comparison with the OVA-immunized control group (Fig. 1). The combination of BCG plus IL-12 yielded an even more profound reduction in the proportion of BAL eosinophils. Accordingly, histological examination of lung tissues isolated from OVA-immunized mice revealed remarkably less inflammatory infiltrates in the peribronchial and perivascular areas in the BCG group in comparison with the control group (Fig. 2). This inhibition of airway inflammation by BCG vaccination was considerably

augmented by the co-administration of IL-12. These observations suggest that IL-12 can function as an adjuvant to enhance the protective effects of BCG against allergic airway inflammation.

IL-12 reinforces the suppression of OVA-specific IgE production by BCG

The concentrations of serum IgE have been previously shown to be closely associated with the severity of

TABLE 1. Determination of OVA-specific IgE levels in serum by ELISA

Group	n	OVA-specific IgE (OD ₄₅₀)
Control (PBS)	8	0.44 ± 0.09
BCG	8	0.25 ± 0.023*
BCG plus IL-12	8	0.167 ± 0.03†

* $P = 0.025$ versus the control group; † $P = 0.05$ versus the BCG group.

allergic airway inflammation.^{1,23} Measurement of OVA-specific IgE levels in sera from mice after the final OVA provocation (as summarized in Table 1) confirmed that OVA-specific IgE levels were substantially decreased in the BCG group in comparison with the control group (0.25 ± 0.03 OD₄₅₀ versus 0.44 ± 0.09 OD₄₅₀; $P = 0.025$). A further reduction was observed in the BCG plus IL-12 group with the mean value of 0.17 ± 0.03 ($P = 0.05$ versus the BCG group).

Determination of cytokine levels in BAL fluid

Since allergic asthma is known to be accompanied by a predominant Th2 response, the suppressive effects of BCG plus IL-12 on allergic airway inflammation examined to determine whether they could be attributed to a shift from a Th2 response to a Th1 response. IFN- γ and IL-4 serve as the signature cytokines of Th1 and Th2 cells, respectively.²⁴ ELISA analysis of the two cytokines in BAL fluid revealed a considerable increase in IFN- γ levels and a marked decrease in IL-4 levels in the BCG group in comparison with the control group (IFN- γ : 133.87 ± 95.1 versus 82.76 ± 32.2 pg/mL, $P < 0.05$; IL-4: 118.08 ± 38.8 versus 246.7 ± 103.2 pg/mL, $P < 0.05$) (Table 2). Moreover, co-administration of IL-12 significantly enhanced the modulation of Th1/Th2 cytokine production by BCG.. These findings suggest that BCG plus IL-12 alleviate allergic airway inflammation at least partially through the coordination of Th1/Th2 imbalance.

Discussion

Allergic asthma is the most common form of asthma, affecting over 50% of the 20 million asthma

TABLE 2. Determination of IFN- γ and IL-4 levels in bronchoalveolar lavage fluid by ELISA

Group	n	Cytokines (pg/mL)	
		IFN- γ	IL-4
Control (PBS)	8	82.76 ± 32.2	246.7 ± 103.2
BCG	8	133.87 ± 95.1*	118.08 ± 38.8*
BCG plus IL-12	8	385.59 ± 111.9†	49.10 ± 20.13†

* $P < 0.05$ versus the control group; † $P < 0.05$ versus the BCG group.

sufferers.²⁵ This allergic disorder usually develops as a consequence of predominant Th2 immune responses. BCG, a potent Th1 response inducer, has been widely used to prevent the development of allergic diseases.^{26,27} A recent single-blind, randomized study examining the protective effects of BCG vaccination early in life against development of allergic diseases have shown some conflicting results; i.e., the prevalence of allergic disease was not significantly reduced by BCG inoculation.²⁸ Therefore, development of new modalities is necessary. Indeed, genetically modified BCG and the combination with other agents have been explored in the prevention of experimental asthma.^{11,13,29,30} IL-12 is an immunomodulatory cytokine that plays an important role in promoting Th1 immune responses and cell-mediated immunity.¹⁵⁻¹⁷ The protective role of IL-12 in allergic asthma has been documented in many previous studies.^{21,22,31} Local administration of IL-12 has been reported to inhibit airway hyper-responsiveness and pulmonary eosinophilia,²¹ while IL-12 deficiency abolishes the suppression of airway inflammation by Th1 directing vaccines such as BCG or oligodeoxynucleotide.³¹ Here, we investigated whether IL-12 could function as an adjuvant to enhance the protective effects of BCG vaccination on allergic asthma. Our data demonstrated that co-administration of IL-12 significantly reinforced the inhibition of OVA-induced airway inflammation by neonatal vaccination with BCG in mice. This amelioration was accompanied by the reduction of eosinophil infiltration and allergic-specific IgE levels. These observations suggest an enhanced role for IL-12 in the prevention of allergic diseases by BCG immunization.

Skewing the immune response towards a Th1 phenotype has been proposed as one important mechanism for the protective role of BCG vaccination.^{11,12} BCG is thought to increase the production of Th1 cytokines, especially IFN- γ that can directly suppress IL-4 gene expression and attenuate Th2 responses,²⁴ and subsequently impair Th2-mediated allergic airway inflammation. Consistent with this hypothesis, we also found that immunization of neonatal mice with BCG resulted in a shift from Th2 response to Th1 response, as evidenced by increased IFN- γ and decreased IL-4 levels in BAL fluid. Most important, this shift towards Th1 response was substantially enhanced by co-administration of IL-12, suggesting the additive role of IL-12 in the prevention of allergic diseases may be associated with the promotion of Th1 cytokine release. Indeed, IL-12 has been demonstrated to be involved in the induction of IFN- γ by BCG.³² Moreover, several studies have reported that IFN- γ is required for the inhibition of IL-12 on allergic airway inflammation.^{33,34} In wild type mice, administration of IL-12 can inhibit OVA-induced IgE elevation; however, in IFN- γ -deficient mice, IL-12 has little influence on the production of OVA-specific IgE.³³ These findings suggest that the beneficial effects of IL-12 in the inhibition of allergic airway inflammation may be at least partially mediated through the promotion of IFN- γ production.

Although our data have shown improved protective effects achieved with the combination of BCG and IL-12 in comparison with BCG alone, there are still some unresolved issues. First, it is unclear to what extent IL-12 contributes to the enhancement of vaccine potency. Indeed, we can not exclude the possibility that IL-12 was primarily responsible for this enhancement, since IL-12 alone has exhibited abilities to prevent antigen-induced airway hyper-responsiveness.²¹ The addition of another control group, with IL-12 alone and blockage of IL-12 using neutralized antibody, would provide more insight into the IL-12 function in the combined vaccine regimen. Second, it remains uncertain whether the improvement in vac-

cine efficacy is specific to IL-12, but the inclusion of other study groups to control for nonspecific effects would strengthen the conclusion about the enhanced role of IL-12 in BCG vaccination.

In summary, our data demonstrate that neonatal vaccination of BCG combined with IL-12 offers beneficial effects against the development of allergic asthma in mice. These protective effects may be mediated via the promotion of Th1 cytokines, especially IFN- γ , which subsequently compromise the predominant Th2 responses induced by allergen.

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