

Prevention of Der p2-induced allergic airway inflammation by *Mycobacterium-bacillus Calmette Guerin*

Jaw-Ji Tsai^{1,3}, Yi-Hsia Liu¹, Horng-Der Shen⁴, Shih-Hung Huang², Shou-Hwa Han³

Section of Allergy and Immunology, Cathay General Hospital; Department of ¹Internal Medicine and ²Pathology, Cathay General Hospital; ³Center of Immunology, National Yang-Ming Medical University; and ⁴Department of Medical Research, Taipei Veterans General Hospital Taipei, Taiwan. ROC

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Epidemiologic studies suggest an inverse correlation between infection and development of allergy. The purpose of this study was to test the hypothesis whether a preexisting T helper 1(Th1)-type immune response elicited by *Mycobacterium bovis-bacillus Calmette-Guerin* (BCG) immunization could suppress allergic airway inflammation induced by the mite allergen *Dermatophagoides pteronyssinus* group 2 (Der p2) in an animal model. C57BL/6 mice were immunized with subcutaneous injection of BCG, then intraperitoneal Der p2 emulsified in alum. Der p2-specific immunoglobulin G1 and cytokine production from splenocytes were measured after Der p2 sensitization, and pulmonary function and airway inflammation were determined after inhalation challenge with Der p2. The intraperitoneal Der p2 with alum injection was able to induce Der p2-specific immunoglobulin G1 production, which could be downregulated by the pretreatment with BCG + Der p2. The inoculation of BCG + Der p2 caused splenocytes to produce more interferon- γ , and this level was higher than that elicited by Der p2 or buffer alone. The positive interferon- γ -staining CD4 cells were also increased after activation by phorbol myristate acetate and ionomycin. Lung pathology examination found decreased airway inflammation (associated with the best pulmonary function and least airway desquamation) in the mice inoculated with BCG + Der p2. In this Der p2-induced allergy model, BCG inoculation with Der p2 can cause a Th1-type immune response that hinders Der p2-induced allergic sensitization and the development of airway inflammation.

Key words: Allergic reaction, *Dermatophagoides pteronyssinus* group 2, *Mycobacterium bovis-bacillus Calmette Guerin*

Allergic disorders affect at least 20% of the population of developed countries. They include hay fever, asthma, and atopic dermatitis. Their symptoms are associated with high levels of serum allergen-specific immunoglobulin (Ig)E and eosinophilia [1-4] and are dependent upon interleukin (IL)-4 and IL-5 released from allergen-specific CD4 cells expressing the T-helper 2 (Th2) cytokine profile [5-7]. Recent advances in the understanding of the inflammatory process in allergic diseases led to the hypothesis that a shift in polarization of cytokine production from a Th2 to a Th1 cytokine profile by allergen-specific T cells may allow for more specific therapy. The basis of this strategy is the supposed mutual opposition between Th1 and Th2, where Th1 cytokines downregulate Th2 function, and so would be expected to reduce levels of IgE [8,9]. Infection may skew the cytokine microenvironment

toward preferential proliferation of Th1 cells and protect against the development of Th2-dependent allergic disease. Recent reports showed an inverse correlation between the frequency and intensity of the response to viral and bacterial infection and the development and prevalence of allergic diseases [10-12]. Immunization with the *Mycobacterium bovis-bacillus Calmette-Guerin* (BCG) provided protection against atopy and asthma in Japanese school children [12]. In these studies, positive tuberculin responses predicted a lower incidence of asthma.

The house dust mites, *Dermatophagoides pteronyssinus* (Dp), are clinically the most important allergens [13]. The group II allergens (Der p2) are believed to be the major allergenic components of dust mites on the basis of the stability of Der p2 and the prevalence of Der p2-specific IgE antibodies in mite allergic patients [14-16].

The purpose of this study was to examine whether an attenuated mycobacterial preparation derived from mycobacteria, BCG, can downregulate an ongoing Der

Correspondence: Dr. Jaw-Ji Tsai, Section of Allergy and Immunology, Cathay General Hospital, 280, Section 4, Jen Ai Road Taipei, 106, Taiwan, ROC. E mail: jawji@cgh.org.tw

p2-specific Th2 immune response and allergic airway inflammation.

Materials and Methods

Animals

Male C57BL/6 mice were obtained from the National Laboratory Breeding Research Center in Taiwan and were raised in a specific pathogen-free environment. These mice were used between 6 and 8 weeks of age. Groups of 6 mice were caged separately according to their treatment.

Immunization and treatment of mice

Mice were immunized with an attenuated *M. bovis* strain (BCG, Cannaught Laboratory Limited, Willowdale, Ontario, Canada) commonly used for the *in situ* treatment of urinary bladder carcinoma. Viable organisms (5×10^7 organisms) in 0.1 mL of phosphate buffered saline (PBS) were injected in each mouse subcutaneously for immunization. Recombinant Der p2 was prepared and purified as described [16]. Four groups of mice were included in this study. The control mice received a subcutaneous injection of PBS (100 μ L/mouse); BCG mice received a subcutaneous injection of BCG (5×10^7 organisms/100 μ L/mouse); Der p2 mice received a subcutaneous injection of Der p2 (1 μ g/100 μ L/mouse); and BCG + Der p2 mice received a subcutaneous injection of a mixture of Der p2 (1 μ g/50 μ L/mouse) with BCG (5×10^7 organisms/50 μ L/mouse).

Sensitization with Der p2

Mice were sensitized twice (once each on days 0 and 7) with one concentration of Der p2 (0.1, 1, or 10 μ g), and their serum IgG1 concentration was measured on day 21 to determine the optimal Der p2 concentration (sensitizing dose). Seven days after immunization with BCG, all mice were sensitized by repeated intraperitoneal injection of 1 μ g/0.1 mL Der p2 emulsified in alum (aluminum hydroxide Al(OH)₃ [4 mg/0.062 mL]; Whitehall Lab Ltd. Punchbowl, Australia) on days 7, 21, and 35 after BCG immunization. On day 49, mice were intratracheally challenged directly via intratracheal drip infusion of Der p2 (0.5 μ g). Two days later, mice were sacrificed for pulmonary function measurement and splenocyte culture.

Determination of Der p2-specific IgG1 and IgG2a antibodies

Blood was obtained from the retro-orbital venous plexus on days 7, 21, 35, and 51 of the experiment. Serum

IgG1 and IgG2a titer of anti-Der p2 antibodies was determined by using an enzyme-linked immunosorbent assay (ELISA). Microtiter plates (Nunc Lab, IL, US) were coated with 100 μ L of Der p2 overnight at a concentration of 0.5 μ g/mL in a 4°C refrigerator. Plates were washed with PBS-Tween-20 (PBST) 3 times and stored at -70°C before use. After addition of mouse serum (1:100 dilution for IgG1 and 1:25 for IgG2a), plates were incubated at 4°C overnight, and then washed 3 times before the antibody (Horseradish peroxidase-conjugated goat anti-mouse IgG1 or IgG2a Ab [1:2000], Southern Biotech Assoc, Inc, Birmingham, AL, US) was added. Following 1 h of incubation at 37°C and 3 washes with PBST, the enzyme substrate 2,2'-azino-bis[3-ethylbenzothiazolin-6-sulfonic acid] diammonium salt (ABTS; Bio-Rad, US) was added. The reaction was stopped with 50 μ L of 4N H₂SO₄ after 15 min, and the optical density was measured at 415 nm in a multiscan spectrophotometer (model A-5682, SLT Lab Instruments, Salzburg, Austria). Results were expressed as ELISA units (EU). One EU was defined as the reciprocal value of the serum dilution that gave an optical density of 1. This was always within the linear part of the dilution curve. To assure reproducibility, a known serum was run with each test as a standard.

Cell culture, immunofluorescence staining, and flow cytometry analysis

Flow cytometric determination of cytokines in activated murine Th cells was assayed according to the method described by Assenmacher *et al* [17]. Two-color staining methods were used to analyze interferon (IFN)- γ and IL-5 expression in CD4 cells. Leukocytes from peripheral blood (PBL) of 6 mice were pooled and stimulated with phorbol myristate acetate (PMA; 50 ng/mL), ionomycin (2 μ M), and GolgiStop (Cytofix/Cytoperm Plus Cat No. 2076, Pharmingen, San Diego, CA, US) for 5 h and then washed twice by PBS. The cells were stained with CD4-fluorescein isothiocyanate (FITC) or IgG1-FITC at room temperature for 30 min and washed. Cells were fixed with cytofix/cytoperm at room temperature for 30 min, stained with anticytokine antibody and IgG2a-PE at room temperature for 30 min, and washed. The IgG1-FITC and IgG2a-phycoerythrin (PE) and anticytokine antibodies (used as negative controls) were purchased from the same manufacturer. Cells were resuspended in 0.5 mL of PBS containing 0.1% w/v sodium azide. Mean fluorescence was measured using a Becton Dickinson flow cytometer (Becton Dickinson, CA, US). A total of 2000 cells were analyzed in each sample. A typical dot plot figure showed that

the separation between the cytokine signal (FL-2H) and the CD4 signal (FL-3H) could be clearly demonstrated and the magnitude of the signals was analyzed simultaneously.

Splenocytes (2×10^6 /mL), after pulmonary function determination, were cultured with RPMI-1640 containing 10% fetal bovine serum and penicillin (100 unit/mL) and streptomycin (100 μ g/mL), and stimulated with or without Der p2 (10 μ g/mL) at 37°C, in a 5% CO₂ incubator for 7 days. The cell free supernatants were collected by centrifugation (400x g, 10 min, 4°C) and stored at -80°C prior to IFN- γ assay. IFN- γ was measured by use of commercially available ELISA kits containing mouse monoclonal antibody recognizing different epitopes of the cytokine molecules. The lowest detectable concentration was 10 pg/mL. For the determination of cytokine expression in CD4 T cells, splenocytes of 3 mice were pooled and stimulated with PMA and ionomycin. Cells were stained and analyzed as for PBL described above.

Pathology of lung and trachea tissue

The lung and trachea tissue from the experimental and control groups of mice were removed and rinsed with PBS pH 7.4 (0.4 mol/L). Tissue sections were fixed with paraformaldehyde and stained with hematoxylin-eosin, and the slides with these sections were mounted with a cover slip.

Pulmonary function determination

Pulmonary function was determined according to the Lai *et al* [18]. Briefly, mice, after anesthesia with pentobarbital sodium (70 mg/kg, intraperitoneal; Sigma Chemical, St. Louis, MO, US), were cannulated with an 18-gauge needle in the trachea and paralyzed with gallamine triethiodide (1 mg/kg; Sigma Chemical). Animals were placed supine inside a whole body plethysmograph (6 cm interdiatometry [ID] and 15.25 cm long; Buxco Electronics, Troy, NY, US). The flow rate was monitored with a Validyne DP 45 differential pressure transducer as the pressure dropped across 3 layers of 325-mesh wire screening in the wall of the plethysmograph. Lung volume (Vt) change was obtained via integration of flow. Airway opening pressure (Pao) was measured using a pressure transducer (DTX/plus, Viggo-Spectramed). Lung volume and its accompanying Pao difference (Δ Pao) were used to calculate dynamic respiratory compliance ($Crs = Vt/\Delta$ Pao).

Statistical analysis

The modulatory effect of BCG on Der p2-induced IgG1

and IgG2a production was compared using a 2-tailed paired Student's *t* test. The differences between mice treated with and without BCG were compared using a non-paired Student's *t* test.

Results

The effect of BCG on Der p2-induced specific IgG1 and IgG2a production

To determine the effect of Der p2 on IgG1 production, different doses of Der p2 were used for intraperitoneal immunization. Results showed that serum IgG1 was elevated 14 days after second immunization. The immunization with 1 μ g of Der p2 was able to induce an IgG1 response (0.31 EU/mL) similar to the one induced by 10 μ g of Der p2 (0.42 EU/mL), 14 days after second immunization. The baseline levels of Der p2-specific IgG1 were undetectable before immunization.

All groups of mice produced high levels of Der p2-specific antibodies, especially in the group receiving Der p2 alone. In the BCG groups, BCG alone was not able to downregulate IgG1 production. Only in the group of BCG + Der p2 mice, a significant down-regulation of IgG1 was detected as compared with that of control (Fig. 1). For the production of IgG2a, there was a significant increase of antibody production only in the BCG + Der p2 group at day 51 (Fig. 1).

The effect of BCG on cytokine production by splenocytes and peripheral blood leukocytes

Peripheral blood leukocytes were cultured with PMA and ionomycin for 5 h, then analyzed for the IL-5- and IFN- γ -producing CD4 cells. The percentage of IL-5-producing CD4 cells was similar in all groups of mice; however, the percentage of IFN- γ -producing CD4 cells was significantly higher in those mice inoculated with BCG (Fig. 2). The ratio of IFN- γ - and IL-5-producing CD4 cells was much higher in the BCG and BCG + Der p2-inoculated mice (Table 1). When splenocytes cultured with PMA and ionomycin were then analyzed for IL-5- and IFN- γ -producing CD4 cells, similar results were obtained. There was a high percentage of IFN- γ -producing CD4 cells and a high ratio of IFN- γ - and IL-5-producing CD4 cells in BCG- and (BCG + Der p2)-inoculated mice (Table 1).

When splenocytes cultured with Der p2 for 7 days and cytokines measured in the harvested supernatants, results showed IFN- γ production by splenocytes of all mice. However, the IFN- γ production became obvious in the splenocyte supernatants from the BCG and BCG plus Der p2-inoculated mice (Table 1).

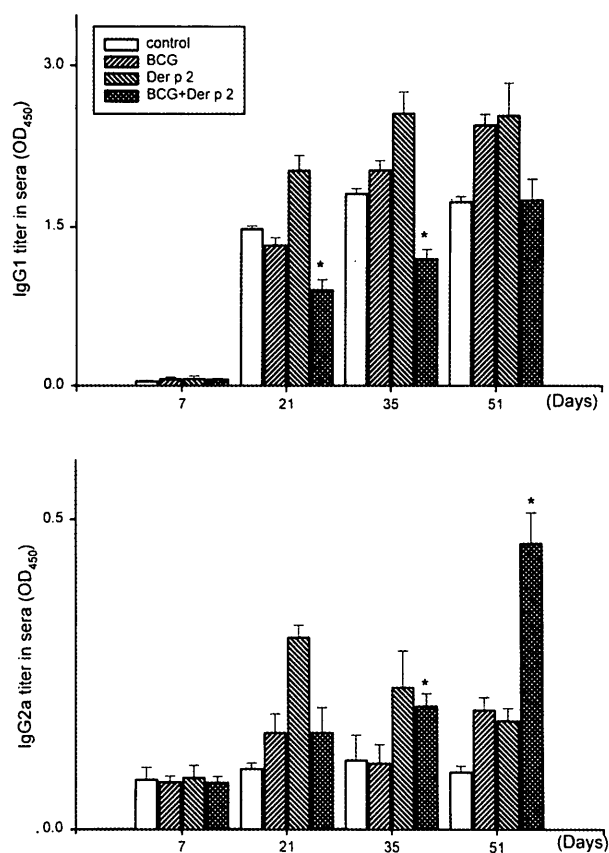


Fig. 1. The effect of BCG on Der p 2-specific IgG1 and IgG2a production. The data represent the means of results from 6 mice. * $p < 0.05$ when BCG + Der p 2 groups are compared with control groups of mice. Two-color analysis of cytokine-producing CD4 cells from PBL.

The effect of BCG on Der p2-induced airway inflammation

C56BL/6 mice were subjected to the same protocol used to generate data presented in Fig. 1. All mice received Der p 2 inhalation challenge on day 49. Lung tissues were removed, fixed, and stained with hematoxylin-eosin 2 days later. Histologic examination of these tissues revealed inflammatory cell infiltration around the trachea with some epithelial desquamation in the control (Group A) and Der p2-inoculated (Group B) groups; however, epithelial damage in the BCG inoculation group (Group C) was only slight, and inflammatory cell infiltration in the BCG plus Der p2-inoculated group (Group D) was sparse (Fig. 3).

Pulmonary function determination

The pulmonary functions (including forced expiratory volume [FEV] 0.1 and Crs) were compared between the 4 groups of mice. The results showed no statistically significant differences between groups. The FEV_{0.1} in

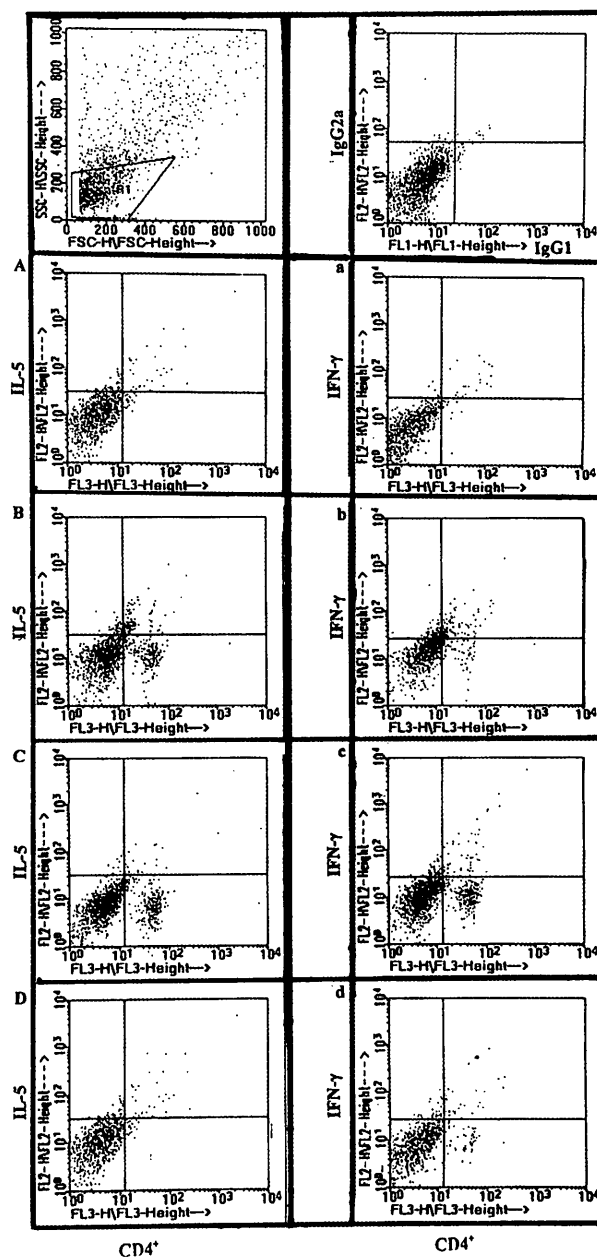


Fig. 2. (A) to (D) represent cells stained with anti-CD4 and anti-IL-5. (a) to (d) represent cells stained with anti-CD4 and anti-IFN- γ . A and a: Control mice; B and b: BCG-inoculated mice; C and c: Der p2-inoculated mice; and D and d: BCG plus Der p2-inoculated mice.

the group of Der p2-inoculated mice was reduced when compared with control mice, but this reduction in FEV_{0.1} was abolished only in the BCG + Der p2 mice (Table 2).

Discussion

Mycobacterial products have been used as potent immunoadjuvants for more than a century as they are components of commonly used adjuvants such as

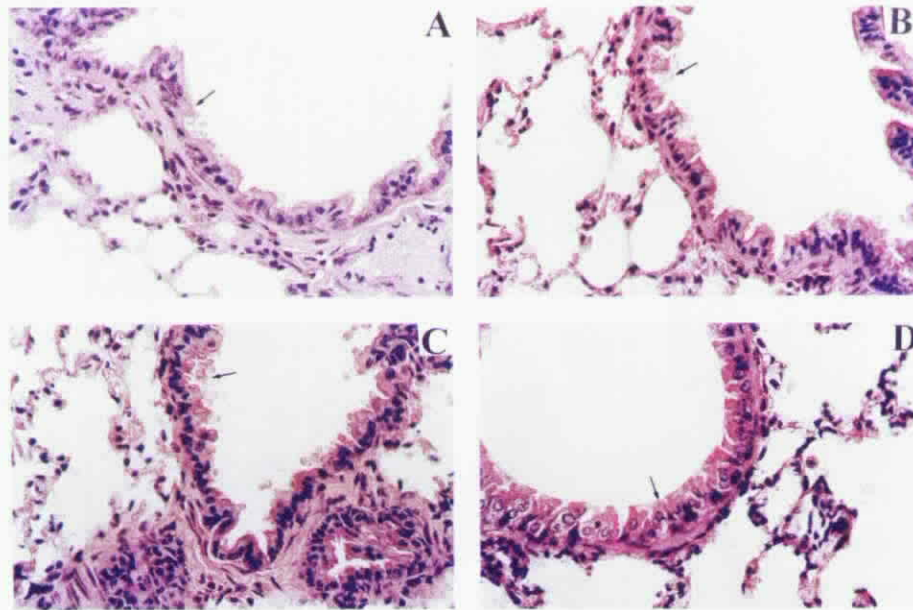


Fig. 3. The effect of BCG on Der p2-induced airway inflammation. All mice received Der p2 intratracheal challenge. The lung tissues were obtained 2 days after Der p2 exposure from (A) control mice; (B) BCG-inoculated mice; (C) Der p2-inoculated mice; and (D) BCG plus Der p2-inoculated mice (HE stain, 400X).

complete Freund's adjuvant. Although these agents have been used for some time to elicit potent Th-1 like responses, the potential therapeutic benefit of adjuvants of bacterial origin for the treatment of allergic asthma has only recently been examined.

Epidemiologic studies have suggested that either environmental exposure or immunization with the *M. bovis*-BCG provides protection against atopy and asthma in Japanese school children [12]. In that study, positive tuberculin response predicted a lower incidence

of asthma, lower serum IgE levels, and cytokine profiles biased toward a type-1 profile. These results suggested that exposure and response to BCG may, by modification of immune cytokine profiles, inhibit atopic disorders. However, it is not clear from these studies whether a decline in *M. tuberculosis* infection rates or whether immunization with the potent bacterial vaccine (BCG) is responsible for the apparent protection from development of atopy. This study demonstrated that BCG + Der p2, not BCG alone, was able to down-

Table 1. Positive cytokine-staining cells among CD4⁺ lymphocytes from PBLs and splenocytes, and concentrations of IFN- γ in the culture supernatants (pg/mL)

	Control	BCG	Der p 2	BCG + Der p 2
PBL				
IL-5/CD4 ⁺ (%)	3.71	5.5	3.71	3.97
IFN- γ /CD4 ⁺ (%)	3.15	10.2	3.76	6.95
IFN- γ /CD4 ⁺ :IL-5-CD4 ⁺	0.85:1	1.85:1	1.01:1	1.75:1
Splenocytes				
IL-5/CD4 ⁺ (%)	0	0.5	1.7	1.2
IFN- γ /CD4 ⁺ (%)	0	2.3	0.7	4.0
IFN- γ /CD4 ⁺ :IL-5-CD4 ⁺	0:0	4.6:1	0.4:1	3.3:1
Supernatant IFN-γ level				
Unstimulated	<10	24.6	147	228
Der p 2-stimulated	1855	2928	1312	3669

Abbreviations: BCG = *Mycobacterium bovis*-bacillus Calmette-Guerin; Der p 2 = *Dermatophagoides pteronyssinus* group 2; PBL = Peripheral blood leukocyte; IL = Interleukin; IFN- γ = Interferon- γ

Note: Splenocytes were cultured with or without Der p 2 for 7 days, and the culture supernatants were collected and analyzed using ELISA.

Table 2. The effect of BCG on pulmonary function of Der p 2-sensitized mice

	Control	BCG	Der p 2	BCG + Der p 2
Crs (mL/cm-H ₂ O)	0.102 ± 0.01	0.10 ± 0.0	0.078 ± 0.01	0.111 ± 0.005
FEV _{0.1} (mL/s)	5.97 ± 0.9	3.70 ± 0.7	4.18 ± 0.3	6.45 ± 0.07

Abbreviations: BCG = *Mycobacterium bovis*-bacillus Calmette-Guerin; Der p 2 = *Dermatophagoides pteronyssinus* group 2; Crs = dynamic respiratory compliance; FEV_{0.1} = forced expiratory volume in 0.1 second

regulate Der p2-induced specific IgG1, upregulate IgG2a production, enhance the production of IFN- γ , and increase IFN- γ -producing CD4 cells in the spleen and PBL. These results indicate that the atopy prevention by BCG might be achieved cooperatively with allergen through the activation of IFN- γ -producing CD4 cells. Similar results have been reported for coimmunization with ovalbumin (OVA) and *M. tuberculosis* bacilli and suggested to the authors of this report that both components cooperatively polarize anti-OVA Th cell development toward a Th1-dominant phenotype and ameliorate murine tracheal eosinophilia [19].

Despite the various studies using several animal models of asthma to study airway inflammation, there are still no reports using the major allergen of the house dust mite Der p2 as the sensitization allergen. In this study, Der p2 sensitization (followed by intratracheal challenge) was able to induce airway inflammation in mice. Recently, it has been reported that in mice, BCG immunization may provide protection from the development of an OVA-induced airway response [20, 21]. Erb *et al* [20] reported that BCG infection of mice prior to subsequent sensitization and challenge with OVA prevented the development of airway eosinophilia. The protective effect of BCG appeared to be dependent upon IFN- γ signaling, as it was lost in IFN- γ -R^{-/-}-mice. However, in this study, BCG inoculation did not downregulate IgG1 production or prevent the reduction of pulmonary function and airway inflammation. These discrepancies may be due to different allergens and be specific for the mice used for this study. In this study, BCG + Der p2 inoculation followed by a schedule of 3 intraperitoneal injections of Der p2 was able to prevent the deterioration of pulmonary function and airway inflammation when the mice were intratracheally challenged with Der p2. These results suggested that the reduction in the Der p2-induced airway inflammatory response in the lung by inoculation with BCG + Der p2 was Der p2-specific, and that this effect may be due to BCG and Der p2 cooperatively altering the cytokine environment so that a subsequent exposure to Der p2 is less capable of causing an inflammatory response.

Allergic asthma is characterized by airway hyperresponsiveness to specific stimuli, chronic

eosinophilic inflammation, elevated serum IgE levels, and excessive mucus production. The inflammatory component of this disease is characterized by increased numbers of activated Th lymphocytes, mast cells, and eosinophils within the airway lumen and bronchial mucosa [22,23]. Recent studies have shown a strong correlation between the level of activated CD4⁺ T cells and disease severity [23,24]. The type of cytokines released by activated CD4⁺ T cells from asthmatic patients appear to fit one of the 2 basic patterns of cytokine expression described in human and murine CD4 Th clones [25,26]. In this paradigm, Th1 cells are characterized by elevated secretion of IL-2 and IFN- γ and are important in the development of cell-mediated immunity. On the other hand, the Th2 subset of CD4⁺ T cells preferentially secretes IL-4, IL-5, IL-6, IL-9, and IL-13 and is important in the generation of the humoral immune response [27,28]. Studies in animal models of disease support a pivotal role for CD4⁺ Th2 cytokine-producing cells in the development of allergic airway responses [29,30]. This study demonstrated that BCG + Der p2 inoculation resulted in the decrease of CD4⁺ Th2 cytokine-producing cells and increase of CD4⁺ Th1 cytokine-producing cells. Thus, it was likely that BCG + Der p2 could be redirecting the type of Der p2-induced immune response from a Th2-dominant (cellular) response to a Th1-dominant (humoral) response.

In conclusion, BCG + Der p2 inoculation can modulate the Der p 2-induced Th2 immune response and protect Der p 2-induced airway inflammation. Although there is no direct evidence that IFN- γ plays a role in the modulatory effect of Der p 2-induced allergic inflammation, this modulation may be partially due to the BCG-induced IFN- γ production from the Der p2-specific CD4 cells. This point cannot be clarified until further studies using anti-IFN- γ monoclonal antibody has been performed. Notwithstanding, this study may provide an alternative effective way for the prevention of mite-induced allergic disease in humans.

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