



The attenuation of cockroach allergy by DNA vaccine encoding cockroach allergen Bla g 2

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ABSTRACT

Bla g 2 is one of the most potent cockroach allergens. No effective treatment or vaccination strategies are yet available. We evaluated the prophylactic efficacy of Bla g 2 DNA vaccination in a mouse model of allergic airway inflammation. C57/BL6 mice were given Bla g 2 DNA vaccine prior to sensitization with recombinant Bla g 2 (rBla g 2) antigens, followed by nebulized rBla g 2 challenge. Bla g 2 vaccine could express at both transcriptional and translational levels in mammalian cells. Moreover, Bla g 2 vaccine significantly reduced the total inflammatory cell infiltrate and eosinophilia in bronchoalveolar lavage fluid, and markedly decreased allergen-induced inflammatory infiltrates in the lungs and Bla g 2-specific IgE in serum upon challenge with rBla g 2. Importantly, Bla g 2 vaccine could induce the production of antigen-specific IFN- γ and downregulated Th2 pro-inflammatory cytokines IL-4, IL-5, and IL-13. Thus, DNA vaccination showed protective efficacy against a clinically relevant allergen, Bla g 2.

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1. Introduction

The incidence and prevalence of allergic diseases are increasing throughout the world, and their impact on quality of life of children and adults is significant. Theories put forth to try to explain these phenomena include increasing air pollution, increased time spent indoors by children and adults, and decreased rates of infection seen in developed countries (the “hygiene” hypothesis) [1]. Cockroach allergen (Bla g 2) has been recognized as a major indoor allergen, initially in crowded urban settings [2–6] and now in suburban and rural settings as well [2–6]. Indeed, inner city asthmatics often show elevated levels of cockroach-specific IgE, at rates up to 60%. The cockroach species found most commonly in the United States are the American cockroach, *Periplaneta americana*, and the German cockroach, *Blattella germanica* [7].

Bla g 2, a glycoprotein with a molecular weight of 36 kDa, is an inactive aspartic protease with a bilobal structure [2–6]. Moreover, Bla g 2 is the most potent German cockroach allergen that causes the specific IgE responses in ~60% of German cockroach allergic

patients, and exposure levels are 10- to 100-fold lower than those from other common indoor allergens, such as dust mite and cat [2–6]. When conducting observational study on US school children, prompt positive skin test results were in association with the inhalational exposure to median Bla g 2 levels of 0.32 $\mu\text{g/g}$ (range > 0.08–15 $\mu\text{g/g}$); however, comparative studies done on dust mite were 38 $\mu\text{g/g}$ (range 24–150 $\mu\text{g/g}$) [2–6]. Exposure to Bla g 2 results in cross-linking of IgE bound to the surface of mast cells or basophils from sensitized patients and induces release of potent mediators, such as histamine, leukotrienes, and prostaglandins, of allergic reactions [2–6].

Allergic airway inflammation (AAI) is characterized by reduced airflow, airway hyperresponsiveness (AHR), influx of eosinophils into the lung, and hyperplasia of goblet cells with excessive mucus secretion [8,9]. These effects are accompanied, in part, by the overproduction of a variety of cytokines and chemokines that attract inflammatory cells and stimulate a Th2- and IgE-dominated immune response.

The treatment of allergic diseases consists of three complementary measures: avoidance, pharmacotherapy, and specific immunotherapy. Avoidance of cockroaches is difficult. Infestations occur in large apartment buildings, office buildings, and homes, and eradication is difficult, if not impossible. Pharmacotherapy consists of antihistamines to decrease allergic nasal and, to a mild degree, eye symptoms, and inhaled corticosteroids, long-acting β_2 agonists, and leukotriene receptor antagonists in allergic asthma.

Abbreviations: AAI, allergic airway inflammation; AHR, airway hyperresponsiveness; IFN- γ , interferon- γ ; IL, interleukin; PBS, phosphate-buffered saline; rBla g 2, recombinant Bla g 2.

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Treating chronic allergic conditions is made more difficult by the issue of compliance [10]. The third measure, immunotherapy [11,12], offers a great measure of relief to many individuals whose symptoms are not well controlled by avoidance or medications. Traditional immunotherapy includes a long build-up phase, which may take as long as 6–12 months, and a prolonged immunization course, up to 5 years or more [12–14]. The risk of a systemic reaction prevents many of the most needy patients (severe allergic asthmatics) from receiving this beneficial therapy. Many patients, after completing a successful course of immunotherapy, have a return of their symptoms that requires reinstituting allergy desensitization.

In the past, immunostimulatory DNA-based immunotherapeutics have proven to be a powerful tool to induce protective cellular and humoral immune responses against many parasitic, bacterial, and viral diseases [15–17]. Cramer et al. [16] showed enhanced Th1 response and diminished IgE response to *Escherichia coli* beta galactosidase when administered as a DNA vaccine as opposed to administering beta gal as a solution resulted in enhanced IgE production. Intramuscular or intradermal injection of plasmid DNA-encoding allergens is thought to lead to the recruitment of allergen-specific Th1 cells, which secrete interferon- γ (IFN- γ) and possibly protect against allergic responses by inhibiting the proliferation and development of Th2 cells and IgE production, thereby ameliorating an already ongoing Th2-type response [15–17].

In this study, we examined whether DNA vaccine-encoding Bla g 2 could exert a protective role on cockroach allergen-challenged mice and explored the mechanism(s) of DNA vaccination in immunotherapy of AAI. Indeed, Bla g 2 vaccination prior to sensitization efficiently protected mice from developing AAI. Notably, the suppression of AAI by Bla g 2 vaccine was associated with an induction of Th1 response and a suppression of Th2 reaction.

2. Methods

2.1. Mice

Female C57/BL6 mice, 6 weeks old, purchased from the Jackson Laboratory (Bar Harbor, ME) were used in this study. Mice were maintained in the animal facility at the University of Tennessee Health Science Center according to the institutional guidelines for animal care and use. These studies were approved by the Institutional Animal Care and Use Committee of the University of Tennessee.

2.2. Molecular cloning, expression, and purification of Bla g 2-recombinant plasmid

Total RNA was extracted from German cockroaches with RNA STAT-60 reagent (Promega, Madison, WI) and was reverse-transcribed using oligo dT primers and AMV Reverse Transcriptase (Promega). Bla g 2-encoding fragment (GenBank accession code EF203068) was amplified from the German cockroach cDNA by PCR with Bla g 2-specific primers (5'-CAC CAT GAT TGG CCT AAA GCT AGT G-3', forward, and 5'-AGA AAA TTC AAA ATA TTT CCA GTT T-3', reverse). The sequence of the amplified Bla g 2 was verified by DNA sequencing (Molecular Resource Center, University of Tennessee). After the sequence was confirmed, Bla g 2-encoding fragment was subcloned into pCI-neo Mammalian Expression Vector (Promega). In addition, the expression of Bla g 2 in mammalian cells was verified by transfecting human lung adenocarcinoma epithelial cell line A549 (ATCC, Manassas, VA) with Bla g 2 construct. The cell lysates were analyzed by Western blot for cockroach Bla g 2; Bla g 2 mRNA expression of transfected cells was analyzed by RT-PCR. Large-scale purification of the expression

vectors was conducted with Endo Free Plasmid Maxi kits (Qiagen, Valencia, CA) according to the manufacturer's instructions.

2.3. Sensitization, airway challenge, and vaccination

For sensitization, mice were intraperitoneally injected with 10 μ g of cockroach allergen Bla g 2 (recombinant Bla g 2, Indoor Biotechnologies, Charlottesville, VA) together with 4 mg Al(OH)₃ (Serva, Heidelberg, Germany) in a total volume of 150 μ l sterile phosphate-buffered saline (PBS), and then challenged nasally with rBla g 2. The DNA vaccination was given intramuscularly with 100 μ g of plasmid DNA in a total volume of 150 μ l sterile PBS.

2.4. Experimental design

The study was designed to evaluate the protective effect of DNA vaccination against the development of an allergic Th2-immune response. C57/BL6 mice were given intramuscular injections of plasmid DNA encoding for the cockroach allergen Bla g 2 (pCI-Bla g 2) or pCI-neo blank vector three times at weekly intervals. Two weeks later, mice were sensitized with rBla g 2 three times at weekly intervals and then challenged nasally with rBla g 2. Twenty-four hours after challenge, the mice were euthanized with a lethal dose of ketamine hydrochloride (40 mg/kg) and xylazine (10 mg/kg). The efficacy of vaccination was evaluated by assays for splenocyte recall responses and airway inflammatory responses to rBla g 2 antigen challenges.

2.5. In vitro cytokine production and lymphocyte proliferation

Twenty-four hours after challenge, splenocytes from the experimental mice were harvested and processed. After making a single-cell suspension, cells (3×10^5 cells/well) were cultured in 96-well flat-bottomed plates (Costar, Corning, NY) in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS; Gibco, Paisley, UK), 50 μ M 2-mercaptoethanol, 2 mM L-glutamine, and 10 U penicillin/streptomycin (Gibco) and stimulated with rBla g 2 (10 μ g/ml). Positive control wells contained 2 μ g/ml anti-mouse CD3 (BD Biosciences, San Diego, CA, USA), and negative control wells contained only PBS. Supernatants were harvested after 48 h and stored at -80°C for their cytokine measurement. Cells were pulsed with [^3H]-thymidine during the last 18 h of the 72-h assay. Pulsed cells were harvested and counted for incorporation of [^3H]-thymidine (Packard Instrument, Boston, MA). Stored supernatants were assayed in duplicate for the presence of cytokines interleukin (IL)-4, IL-5, IL-13, and IFN- γ by ELISA according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA).

2.6. Histology

After BAL fluid collection, the lung was fixed in 10% formalin and embedded in paraffin. Sections were obtained and stained with H&E. The sections were evaluated by microscopy. The amount of cells that infiltrated was measured with Kwon method [19]. This method is able to distinguish between the control and experiment groups in amount of mononuclear cells on the periphery of the blood vessels (score 0–3), periphery of bronchiole (score 0–3), and amount of patchy cellular infiltration (score 0–3).

2.7. Statistical analysis

Statistical analysis was performed using Student's *t*-test for comparisons between the control DNA and experimental groups.

3. Results

3.1. Expression of Bla g 1 DNA vector

The Bla g 2 DNA fragment (Fig. 1A–2) and the expressed protein of Bla g 2 are shown in Fig. 1. The expression level of the Bla g 2 DNA construct was tested in A549 cells. The expression of Bla g 2 mRNA was detected by RT-PCR (Fig. 1B–1). Culture supernatants and cell lysates from transfected cells were analyzed by Western blotting using Bla g 2-specific antibody. The protein was detected in the culture supernatants (Fig. 1C–2). No Bla g 2 protein was present in control samples transfected with the empty vector (Fig. 1C–1). Therefore, Bla g 2 construct was able to express at both transcriptional and translational levels. Moreover, culture supernatants from transfected cells were collected after 72 h and were tested for cytokines by ELISA. A549 cells transfected with plasmid DNA-Bla g 2 produced significant levels of IFN- γ (64 ± 11.5 pg/ml). However, there was no detectable IFN- γ in the A549 cells transfected with empty vector, indicating that only the expression of Bla g 2 from DNA vaccine was able to induce the production of IFN- γ from A549 cells.

3.2. Bla g 2 DNA vaccine prevented cockroach allergen-induced pulmonary manifestations

To test whether Bla g 2 DNA vaccination prevented cockroach allergen-induced pulmonary manifestations, we administered Bla g 2 vaccine to mice three times at weekly intervals before sensitization with three doses of rBla g 2 antigens plus alum and then challenged nasally with rBla g 2 (Fig. 2A). BAL fluid showed a significantly reduced number of total cells in Bla g 2-vaccinated mice (Fig. 2B) compared to control DNA mice. Specifically, there was no detectable eosinophilic infiltration in the lungs of Bla g 2-vaccinated mice (Fig. 2C). Moreover, Bla g 2 vaccine was able to prevent the induction of Bla g 2-specific IgE (Fig. 2D). In contrast, the levels of Bla g 2-specific IgE were high in the control group. Furthermore, histological analysis of the lungs showed that the cockroach

allergen-challenged mice that received control DNA (Fig. 3B) demonstrated the major features of AAI, including clusters of inflammatory cells surrounding the airways and bronchiolar epithelium showing columnar epithelial cell hyperplasia, while the Bla g 2-vaccinated mice almost completely abrogated these features (Fig. 3A). In addition, the amount of cells that infiltrated on the periphery of central bronchi, alveoli and blood vessels was measured (Table 1). The amount of cellular infiltration on the periphery of central bronchi, alveoli and blood vessels in Bla g 2-vaccinated mice was lowered, when compared to control DNA group ($p < 0.01$). Therefore, these data demonstrated the protective effects of Bla g 2 vaccination.

3.3. Induction of Bla g 2 specific immune responses after Bla g 2 DNA vaccine administration

To investigate whether Bla g 2 DNA vaccine induced a protective response, splenocytes were harvested and stimulated *in vitro* with rBla g 2 to study the nature of their recall response to allergen challenge. The supernatants from the stimulated cultures were harvested and tested for the presence of cytokines.

In the prophylactic regimen, the proliferative response of T cells in response to Bla g 2 was significantly elevated 5- to 6-fold in Bla g 2-vaccinated mice in comparison with the control mice (Fig. 4). The result demonstrated the potential of this vaccine to stimulate cellular responses against isolated proteins, an observation that may have important implications for the development of vaccines.

The analysis of the culture supernatants revealed that Bla g 2 vaccine-treated mice produced significant levels of IFN- γ compared with the mice treated with control DNA (Fig. 4). Furthermore, Bla g 2 vaccine resulted in a significant reduction in the concentration of IL-4, IL-5, and IL-13 (Fig. 4), indicating that DNA vaccine profoundly altered the cytokine secretion pattern in cockroach-challenged mice.

Taken together, these findings show that Bla g 2 vaccine protects mice from allergic airway inflammatory disease by inhibiting Th2 cytokines and by increasing the production of Th1 cytokine IFN- γ .

4. Discussion

In the recent past, several studies have shown the immunogenic potentials of DNA vaccine against various diseases including certain allergic inflammatory diseases [15–22]. Some studies suggested that allergen genes delivered via plasmid DNA vectors could induce a protective Th1-mediated immune response against the allergen when the allergen is encountered after DNA vaccine administration [16]. In the present study, we tested the efficacy of a DNA vaccine against a common household cockroach allergen, Bla g 2, involved in exacerbation of allergic airway inflammation. We showed that the DNA vaccine was able to mount a protective immune response against allergic airway inflammation.

The histological evidence of Bla g 2 vaccine's ability to abrogate lung inflammation is noteworthy. In contrast, the mice that received control DNA demonstrated a significant influx of leukocytes around the airways and epithelial cell hyperplasia. This reduced cellular infiltration corresponded to significantly low levels of cytokines IL-4, IL-5, and IL-13 in the DNA-vaccinated mice compared to control mice. It is possible that the DNA vaccination prior to rBla g 2 challenge induced an immune response that prevented the induction of otherwise normal Th2-biased immune response against rBla g 2 and thereby prevented the development of airway inflammation induced by Bla g 2.

IL-4, derived initially from activated Th2 cells in the lung and subsequently from effector cells, including mast cells and baso-

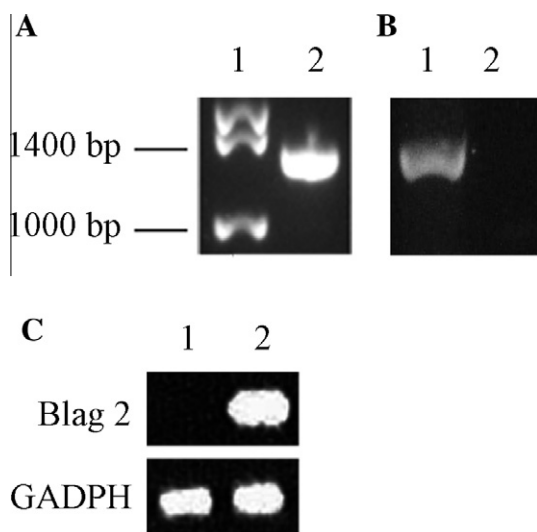


Fig. 1. Construction and expression of Bla g 2 DNA vaccine. (A) Production of Bla g 2 by PCR. (1) DNA marker; (2) Bla g 2 DNA fragment. (B) RT-PCR analysis to detect the expression Bla g 2 mRNA. A549 cells transfected with either pCI-neo-Bla g 2 (1) or empty vector (2). (C) Western blot analysis using antibody to Bla g 2. The culture supernatants of A549 cells transfected with either empty vector (1) or pCI-neo-Bla g 2 (2) were run on a 15% polyacrylamide gel. The proteins were transferred onto a nitrocellulose membrane. The membranes were probed with mouse anti-cockroach-Bla g 2 monoclonal antibody, followed by sheep anti-mouse peroxidase-conjugated antibody and enhanced chemiluminescence detection; GAPDH served as loading control.

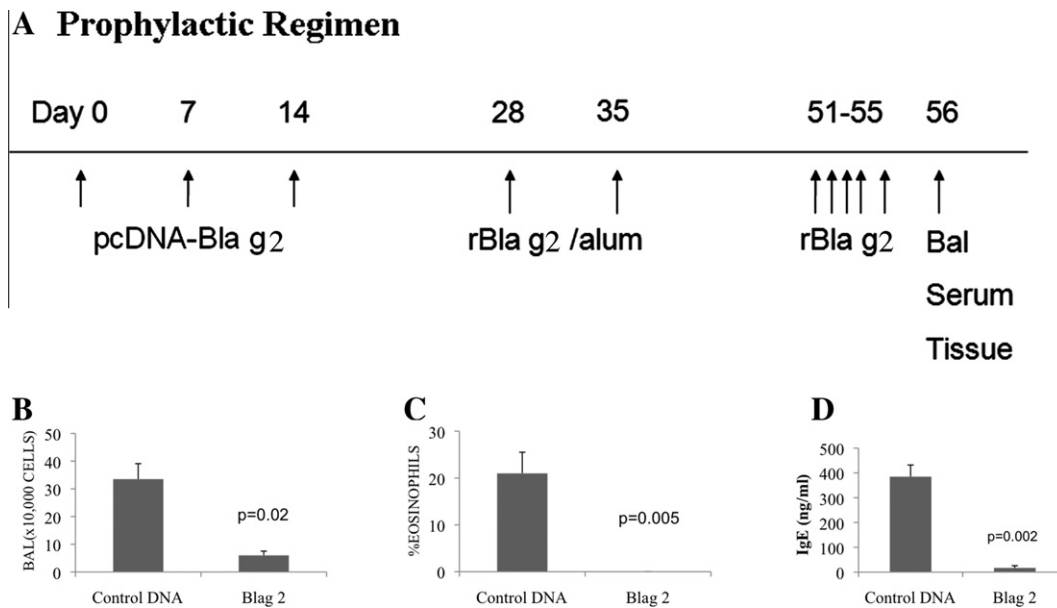


Fig. 2. Prophylactic effects of DNA vaccine on rBla g 2-induced AAI. (A) Prophylactic DNA vaccination scheme. C57/BL6 mice were vaccinated with plasmid DNA coding for the allergen Bla g 2 or pCI-neo blank vector three times at weekly intervals. Two weeks later, mice were sensitized with rBla g 2 three times at weekly intervals and then challenged nasally with rBla g 2. Mice were sacrificed 24 h after challenge, and spleens and BAL fluids were collected for cytokine measurements and cellular analysis. BAL fluid was collected and centrifuged. Cells were resuspended to count total cells (B), and eosinophils (C) were determined with Wright-Giemsa stain. Serum Bla g 2-specific IgE (D) was determined by ELISA (modified BD OptEIA ELISA kit; BD Pharmingen, San Jose, CA, USA). The data represent the means $\pm$ SE from five mice/group. *P* value versus control DNA mice.

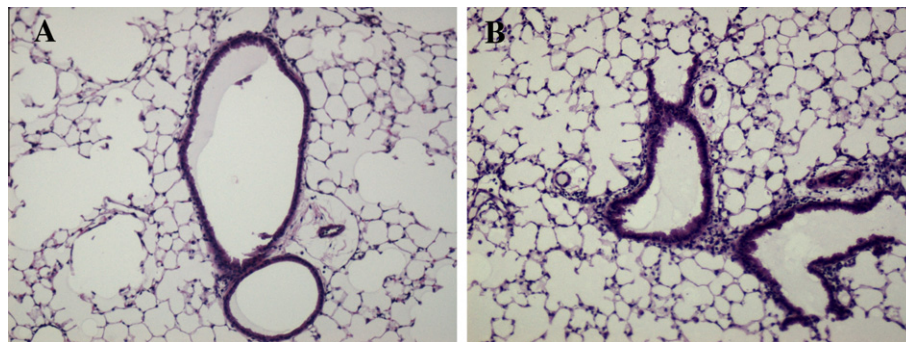


Fig. 3. Histological examination of allergen-challenged mice demonstrates reduced inflammation in Bla g 2-vaccinated mice. 24 h after challenge, lungs from all mice were fixed in formalin; sections were stained with H&E and examined by light microscopy. Data shown are representative of C57/BL6 mice receiving Bla g 2 vaccine (A) and C57/BL6 mice receiving control DNA (B).

Table 1

Bla g 2 DNA vaccination reduced allergen-induced inflammatory infiltrates in the lungs.

	Bronchioli	Blood vessels	Patchy
Bla g 2 vaccine	1.26 $\pm$ 0.22*	1.07 $\pm$ 0.23*	0.83 $\pm$ 0.12*
Control DNA	2.05 $\pm$ 0.31	2.42 $\pm$ 0.36	2.31 $\pm$ 0.33

The data represent the means $\pm$ SD (*n* = 5).

* *P* < 0.01 versus control DNA.

phils, is critical for the initial induction of Th2-dependent allergic responses and recruitment of effector cells [23]. Eosinophils play a central role in the pathogenesis of AAI. Their recruitment, activation, differentiation, and survival are critically dependent on the initial production of IL-5 by Th2 cells that migrate to the airways following allergen exposure [24,25]. Eosinophils may further exacerbate the inflammatory response, both through further production of IL-5 and possibly as antigen-presenting cells expressing MHC class II molecules and costimulatory molecules on activation

[26]. Thus, the reductions in IL-5 in the DNA-vaccinated mice may partially account for the amelioration of the AAI phenotype.

IL-13 has a great influence on bronchial hyperreactivity, inflammation, and airway remodeling. Moreover, this cytokine drives many cellular responses relevant in asthma, including epithelial cell maturation and mucus production, synthesis of extracellular matrix proteins, and enhanced contractility of airway smooth muscle cells. Thus, targeting IL-13 and its associated receptors may be a therapeutic approach to treating asthma and/or allergy [27,28].

Allergic diseases are all characterized by elevated levels of serum IgE, and total and specific IgE shows a positive close relationship with clinical symptoms. Suppression of IgE has been targeted by several approaches such as synthetic peptides and T-cell epitope vaccine. Bla g 2 vaccination has the ability to suppress production of IgE, which is associated with the decrease in levels of the Th2-derived cytokines IL-4 and IL-13.

In summary, the present study demonstrated that Bla g 2 allergen DNA vaccine displayed a protective potential, suggesting that DNA vaccines may provide a novel therapeutic approach for

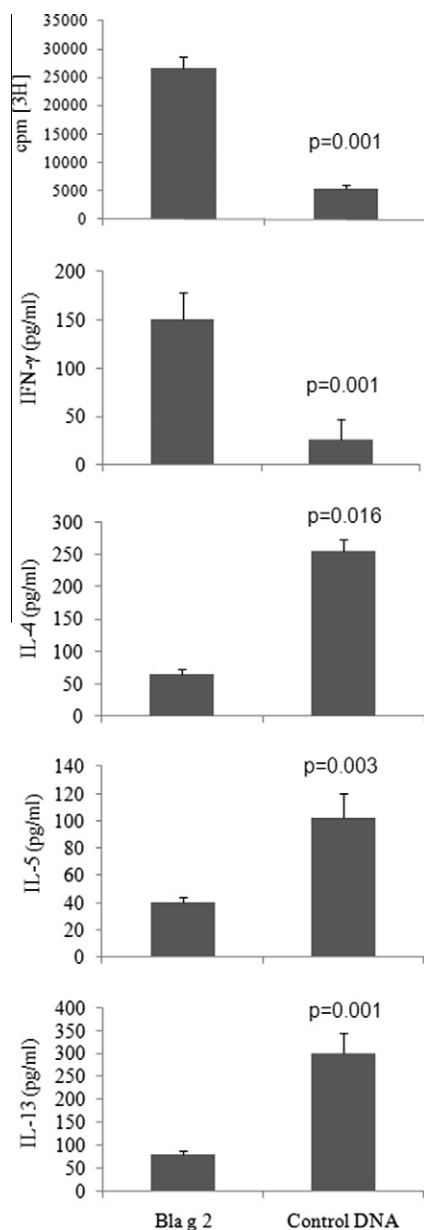


Fig. 4. Induction of Bla g 2-specific immune responses after Bla g 2 vaccination. 24 h after challenge, splenocytes were harvested and restimulated *in vitro* with rBla g 2. Cells were pulsed with [³H]-thymidine after 3 days. The supernatants were collected after 72 h and were tested for cytokines by ELISA. The data depict the means ± SE from five mice/group.

allergic diseases. Mechanistically, our results indicate that the Bla g 2 vaccine might be inducing a Th1 response and suppressing Th2 reaction.

Disclosure of potential conflict of interest

The authors declare that they have no conflict of interest.

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