

Bacillus Calmette–Guérin-induced interleukin-12 did not additionally improve clinical and immunologic parameters in asthmatic children treated with sublingual immunotherapy

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Summary

Objective To evaluate the effect of bacillus Calmette–Guérin (BCG) as an adjuvant to specific sublingual immunotherapy (SLIT) on the cytokine profile of peripheral blood mononuclear cells (PBMCs) and clinical outcome.

Methods Thirty-two children with asthma and rhinitis allergic to house dust mite (HDM) with negative purified protein derivative (PPD) skin test response were enrolled. After a run-in period of 8 weeks, patients were randomized to receive either SLIT only ($n = 16$) or one dose of BCG immunization before initiation of SLIT ($n = 16$) with a standardized *Dermatophagoides pteronyssinus* (*D. pteronyssinus*) + *D. farinea* 50/50 extract. PPD-negative asthmatics ($n = 5$) allergic to HDM receiving inhaled therapy only were included for comparison of cytokine levels in PBMC cultures. Efficacy was assessed both at the end of run-in and 6 months of treatment periods with criteria including symptom, medication and quality-of-life (QoL) scores, IgE levels, lung function, provocation concentration (PC₂₀), eosinophil count and skin prick tests. IL-4, IL-5, IL-10, IL-12, IL-13 and IFN- γ levels were determined in antigen specifically and polyclonally stimulated PBMC cultures.

Results Both treatment groups showed significant improvement at the end of 6 months for asthma and rhinitis scores and QoL, number of asthma attacks, amount of β_2 -agonists, inhaled and intranasal steroids, blood eosinophil counts and PC₂₀. Interestingly, phytohaemagglutinin (PHA)-stimulated IL-12 and *D. pteronyssinus*-stimulated IFN- γ in PBMC were significantly higher in the treatment groups than controls. In addition, IL-12 levels in response to *D. pteronyssinus* and PHA stimulation were significantly higher in the SLIT+BCG group than the SLIT alone group and controls.

Conclusion The present study demonstrates that successful SLIT is parallel to increased IFN- γ production by PBMC. Although simultaneous BCG vaccination enhanced IL-12 production, it did not additionally improve the clinical outcome.

Keywords bacillus Calmette–Guérin, IFN- γ , IL-12, sublingual immunotherapy

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Introduction

Specific immunotherapy (IT) by the subcutaneous route has been shown to be an effective therapeutic tool for the treatment of respiratory allergic disorders [1–4]. However, subcutaneous IT can be associated with a risk of severe, even fatal systemic reactions [5, 6]. Therefore, alternative routes of administration of allergens involving mucosal absorption have been proposed, and it has been suggested that the natural mechanisms underlying the induction of oral tolerance at mucosal surfaces may be an effective therapeutic

strategy for suppression of ongoing pathologic immune responses in allergic diseases [7].

The precise mechanisms by which oral tolerance is induced remain unclear, but it seems likely that the route of allergen processing and presentation is a critical determinant of the subsequent T cell response [8]. From a theoretic perspective, the mucosal surface has to deal with regular exposure to a wide range of innocuous material, and its default response is set to non-response. Experimental support for this theory is available. It has been shown that locally administered allergen is taken up by mucosal dendritic cells, and at least in non-sensitized mice, the allergen is then presented to T cells, together with IL-12, thereby biasing the response toward a T helper (Th1) profile and away from the IgE-inducing Th2 profile [8–10].

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In contrast to animal models, the immunologic response to SLIT in human studies has not been intensely studied. In a number of studies, some changes have been found in skin sensitivity [11, 12], whereas other studies have not found any change in systemic parameters, such as specific IgE and IgG [13].

The positive results of studies evaluating the effectiveness of specific IT administered by the sublingual route in patients with rhinitis and asthma symptoms due to house dust mite (HDM) or pollen are encouraging [11, 14–17]. It is proposed that using vaccines that induce strong Th1 responses in atopy-prone neonates or young children might protect the children from developing allergen-specific Th2-type cells after exposure to allergen [18]. Animal models have provided clear evidence that the application of live or dead bacteria can inhibit the development of allergic disorders [19–23]. Furthermore, the application of live *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) to mice suppressed allergen-induced airway eosinophilia and the development of airway hyper-responsiveness [24, 25].

It can be proposed that the use of attenuated bacteria or their products as adjuvant in conjunction with allergens can protect humans from the development of atopic disorders [18]. The goal of these vaccinations could be to induce the production of IL-12 and IFN- γ at the site of allergen-specific Th cell priming, leading to a long-lasting Th1 memory response against the allergen.

In this study, we investigated the effect of sublingual immunotherapy (SLIT) on clinical, laboratory and immunologic parameters in asthmatic children allergic to HDM. More importantly, we evaluated the administration of BCG as an adjunct to specific SLIT on clinical and immunologic parameters.

Material and methods

Patients

Thirty-two children (17 girls, 15 boys, mean $\pm$ SD age, 6.7 ± 2.6 years) with persistent asthma sensitized to HDM followed by the outpatient clinic of the Paediatric Allergy and Immunology Division of Marmara University Hospital (Istanbul, Turkey) were included. None of the patients had received IT before. Parents of all patients signed informed consent forms and the study was approved by the ethics committee of Marmara University.

The inclusion criteria were: (a) a positive skin prick test (SPT) to *Dermatophagoides farinea* and *D. pteronyssinus*, and a negative response to all other aeroallergens tested, (b) older than 4 years of age, (c) ongoing respiratory symptoms in spite of mite avoidance measures and appropriate inhaled corticosteroid (ICS) treatment, (d) have a negative response to purified protein derivative (PPD) of *M. bovis* skin test.

Controls

Five children (four girls, one boy, mean $\pm$ SD age, 6.9 ± 3.9 years) with intermittent to persistent asthma allergic to HDM, who fulfilled the inclusion criteria listed above (except

for criteria (c)) were included as controls for comparison of *in vitro* studies with the treatment groups.

Study design

After enrollment, the patients underwent a run-in period of 8 weeks, and parents were asked to maintain daily diary cards throughout this period for assessment of the frequency and severity of asthma/rhinitis symptoms, dose of inhaled and intranasal steroids needed to control symptoms and the frequency of need to use β_2 -mimetics for symptom relief. In addition, quality-of-life (QoL) evaluation was performed weekly.

At the end of the run-in period, skin prick, lung function, bronchial challenge tests and nasal smear were performed. Also, a blood sample was drawn for measurement of serum total IgE and total eosinophil count. Patients were then randomized to receive either SLIT alone (F/M = 7/9, age 8.3 ± 2.7 years) or SLIT in addition to one dose of BCG immunization (F/M = 10/6, age 6.6 ± 1.8 years). After initiation of IT, patients were assessed every 4 weeks, for six visits, until the end of the study. At each clinical visit, symptom scores, use of β_2 -mimetics, compliance to inhaled and intranasal steroid treatment were evaluated with the help of symptom-medication scores and QoL assessments by interviewing the child. The compliance of SLIT was assessed with another diary card. For each patient, dose of inhaled and intranasal steroids needed for control of symptoms was adjusted at each clinical visit. At the end of the study, a nasal smear, skin prick, lung function and methacholine bronchial challenge tests were conducted and a blood sample was taken for measurement of serum total IgE level and total eosinophil count. For the purpose of studying for effect of SLIT and SLIT+BCG on the cytokine levels in supernatants of peripheral blood mononuclear cell (PBMC), blood was drawn from six patients from each treatment group (SLIT group F/M 3/3, age 7.3 ± 2.9 years, SLIT+BCG group, F/M 2/4, age 6.8 ± 2.5 years) as well as from a group of controls.

Treatment

Sublingual immunotherapy Treatment consisted of progressively increasing doses of a standardized extract of 50% *D. pteronyssinus*+50% *D. farinea* (Stallergenes, Antony Cedex, Paris, France) followed by maintenance therapy. The strength of standardized allergen extracts was established as the 'index of reactivity' (IR). The extract was graded in four strengths: 0.1, 1, 10 and 100 IR/mL. The in-house reference extract (labelled 100 IR) was defined as the concentration eliciting a weal with a mean diameter of 7 mm by SPT in 30 subjects sensitive to the corresponding allergen. The *D. pteronyssinus* and *D. farinea* contents of 1 mL of the 100 IR allergen extract used in this study were 8 and 14 μ g, respectively.

Patients received increasing doses from each vial, starting with one drop from the 0.1 IR/mL vial and increasing to 10 drops on day 7. This process was repeated for the 1 IR/mL (days 8–14) and 10 IR/mL (days 15–21) vials. On days 22–28, 1–20 drops from the fourth vial (100 IR/mL) were given. Once the 20-drop dose was reached, patients were switched to maintenance therapy consisting of 20 drops of 100 IR/mL

every day for 4 weeks and then two times a week for the following 4 months. Drops were taken sublingually in the morning before breakfast, kept under the tongue for at least 2 min and then swallowed. According to this schedule, an average cumulative dose of 7000 IR (equivalent to 0.56 and 0.98 mg of *D. pteronyssinus* and *D. farinea*, respectively) was administered to each patient.

BCG immunization Patients randomized to the SLIT+BCG group received one dose of BCG (Pasteur Merieux, Lyon, France) immunization administered intradermally with the initiation of SLIT.

ICS treatment According to the protocol of our Allergy Outpatient Clinic, for a newly diagnosed patient our initial dose of ICS is 800 µg/day of budesonide. Patients are then seen every month with symptom-medication diary cards. When an improvement is achieved (usually after 4 weeks), the dose of ICS is decreased by 50% until finding the minimum dose, which controls the symptoms. Patients who continued to have respiratory symptoms in spite of this medical treatment were included in this study.

Patient monitoring

Symptom-medication score Parents of all patients were instructed to keep a diary record card throughout the run-in and treatment periods, for the daily evaluation of lung and nasal symptoms, exacerbations and drug intake as previously described [12].

QoL assessment Patients aged 7 years and older were asked to assess their QoL by completing the paediatric asthma QoL questionnaire, standardized version PAQLQ (S) by interview. This questionnaire had been validated for use in patients between 7 and 17 years of age [26].

Nasal smear A nasal secretion specimen was obtained by using a cotton-tipped applicator into the anterior nasal cavity. Adherent mucus was smeared on a glass microscope slide and Wright stain was performed. When 10% or more eosinophils were present, nasal smear examination was judged as positive.

SPTs and serum total IgE levels Serum total IgE levels were measured with the immulite method (Euro/DPC, Llnberis, Wales, UK). SPT was carried out by employing a panel of 24 allergen extracts in addition to positive and negative controls (histamine dihydrochloride and saline) on the volar surface of the forearm. Bidirectional wheal diameter was recorded. A wheal size equal or larger than that obtained with histamine was judged as positive.

Lung function tests (LFTs) All patients performed a spirometry test before and at the end of SLIT treatment. Forced expiratory volume in 1 s (FEV₁), FEV₁/forced vital capacity (FVC), forced expiratory flow (FEF) between 25% and 75% of vital capacity (FEF 25–75) and peak expiratory flow (PEF) rate were measured with a spirometer (Puriton Bennett, Nellcor, CA, USA) according to standardized guidelines. The best of three successful manoeuvres was recorded. The results are presented as the percent of predicted value for gender, age and height. Normal LFT standards of Polgar were used to generate the predicted values [27].

Bronchial provocation test Non-specific bronchial reactivity to methacholine was measured with the tidal breathing method [28]. The results were expressed as provocation concentration (PC₂₀) methacholine, which was obtained from the log dose–response curve by linear interpolation of the last two points.

PPD skin test

Tuberculin PPD (Intradermal Mantoux test) stabilized solution (Intervax Biologicals Ltd, Montreal, Canada) was used. Testing was carried out with 0.1 mL of solution containing five tuberculin units. PPD solution was injected intradermally into the volar surface of the forearm immediately producing a wheal 5–10 mm in diameter. The amount of induration caused by the Mantoux test was measured using the 'ball point pen' method 48–72 h after administration of the injection. The size of induration was recorded in millimetres bidirectionally and the average diameter was calculated. A PPD skin test reaction ≤ 5 × 5 mm was judged as negative.

PBMC isolation and cell culture conditions

PBMC were isolated by Ficoll–Hypaque density gradient centrifugation and then suspended in RPMI 1640 (Sigma Chem Co., St Louis, MO, USA) with 2 mmol/L L-glutamine (Sigma), 100 U/mL penicillin/streptomycin (Sigma) supplemented with 10% fetal calf serum (FCS) (JRH Biosciences, S-00016, Lenexa, KS, USA). 2×10^6 cells were incubated with 15 µg/mL *D. pteronyssinus* (Swiss Institute of Allergy and Asthma Research), 5 µg/mL phytohaemagglutinin (PHA) (Sigma) and 5 µg/mL PPD (Statens Serum Inst., Copenhagen, Denmark) in 200 µL each in quadriplates in 96-well plates (Costar Corp., Cambridge, MA, USA) at 37 °C with 5% CO₂ for 5 and 7 days. The supernatants were collected and stored at –70 °C until tested.

Quantitation of cytokines IL-4, IL-10, IL-12, IL-13 and IFN-γ levels of PBMC culture supernatants were determined by specific solid-phase sandwich ELISA as described previously [29]. The detection limit for IFN-γ was ≤ 10 pg/mL (mAbs and standard of IFN-γ were gifts from Dr S. S. Alkan, Novartis, Basel, Switzerland) and for IL-4 was ≤ 20 pg/mL (mAbs and IL-4 standard were provided by Dr C. H. Heusser, Novartis, Basel, Switzerland). The detection limits for IL-12, IL-13 and IL-10 were ≤ 50, ≤ 100 and 150 pg/mL (Pharmingen, St Louis, MO, USA), respectively.

Statistical analysis Statistical analyses were performed with the SPSS (Release 5.0. 1-SPSS Inc., 1989–1992) package program. Comparisons between groups were made by using unpaired *t*, Mann–Whitney *U*, χ^2 and Kruskal–Wallis tests. Changes within groups were tested for significance with paired *t*, Mc Nemar's χ^2 and Wilcoxon signed-rank tests. A *P*-value of less than 0.05 was considered significant.

Results

All 32 patients completed the study. Sixteen were randomized to the SLIT group and 16 to the SLIT+BCG group at the

end of the run-in period. At baseline, there were no statistically significant differences between those two groups with respect to age, gender, follow-up period, age at onset of respiratory symptoms and duration of disease and ICS therapy (Table 1). Their baseline asthma and rhinitis scores, inhaled drug consumption, QoL scores, serum total IgE levels, blood total eosinophil counts, skin reactivity to *D. pteronyssinus*, bronchial hyper-reactivity (BHR) and lung functions did not show any difference. On the other hand, baseline skin reactivity to *D. farinea*, nasal smear positivity for eosinophils and intranasal drug consumption were found to be significantly high in the SLIT group (Table 2). Furthermore, comparison of characteristics (including age,

gender, age at onset and duration of disease, follow-up period, total IgE, blood eosinophil count, nasal smear positivity and skin test reaction to *D. pteronyssinus* and *D. farinea*) of subjects selected for the cytokine studies in both treatment groups and controls were similar (data not shown).

Symptom scores

There were no statistically significant differences between two groups in the analysis of the daily means for asthma and rhinitis symptom scores at the end of the 6 months of the treatment period. On the other hand, there was a significant decrease at end-point compared with the run-in period in both groups (Table 2). When the two groups were compared based on the number of asthma exacerbations experienced throughout the treatment period, no significant difference was detected between the two groups. On the other hand, the within-group analyses demonstrated a significant decrease in both groups (Table 2).

Medication scores

The mean daily use of β_2 -mimetic drugs at the end of the treatment period decreased significantly compared with baseline in both groups, whereas no significant difference was detected between the two groups at the end of the treatment period (Table 2). At baseline, the mean daily dose of intranasal steroids was significantly higher in the SLIT group. This difference between the two groups disappeared at

Table 1. Characteristics of patients

Characteristics	SLIT (n = 16) mean $\pm$ SD	SLIT+BCG (n = 16) mean $\pm$ SD	P-value
Age (years)	8.3 $\pm$ 2.7	6.6 $\pm$ 1.8	0.69*
Gender (F/M)	7/9	10/6	0.47†
Age at onset of disease (years)	4.6 $\pm$ 2.6	3.5 $\pm$ 2.4	0.33*
Duration of disease (years)	2.3 $\pm$ 1.5	2.8 $\pm$ 1.2	0.52*
Follow-up period (years)	1.4 $\pm$ 1.2	2 $\pm$ 1.4	0.29*
Duration of ICS (months)	14.2 $\pm$ 11.5	17.1 $\pm$ 10.0	0.33*

SLIT, sublingual immunotherapy; BCG, bacillus Calmette–Guerin; ICS, inhaled corticosteroid; M, male; F, female.

*Unpaired t-test. † χ^2 test.

Table 2. Changes in clinical measures from baseline to last follow-up visit in the two groups

Characteristics	SLIT (n = 16)		SLIT+BCG (n = 16)	
	Baseline	Last visit	Baseline	Last visit
Lung function				
FVC (%) (mean $\pm$ SD)	104.4 $\pm$ 10.5	112.3 $\pm$ 17.8	104.2 $\pm$ 10.2	110.1 $\pm$ 11.6
FEV ₁ /FVC (%) (mean $\pm$ SD)	93.7 $\pm$ 8.9	93.4 $\pm$ 5.62*	89.4 $\pm$ 3.3	87.6 $\pm$ 4.3*
FEV ₁ (%) (mean $\pm$ SD)	98.1 $\pm$ 10	104.8 $\pm$ 15.5	92.9 $\pm$ 10.0	95.5 $\pm$ 7.7
FEF 25–75 (%) (mean $\pm$ SD)	80.7 $\pm$ 12.0	100.7 $\pm$ 13.1 [#]	72.0 $\pm$ 10.0	77 $\pm$ 9.8*
PEF (%) (mean $\pm$ SD)	86.7 $\pm$ 15.5	92.0 $\pm$ 21.7	84.6 $\pm$ 13.7	82.6 $\pm$ 10.0
PC ₂₀ (mg/mL) (mean $\pm$ SD)	3.0 $\pm$ 4.6	4.3 $\pm$ 5 [#]	1.1 $\pm$ 1.2	1.9 $\pm$ 1.2 [#]
Laboratory				
IgE (IU/mL) (median (range))	711.5 (36.7–2066)	1082 (59–1856)	315.5 (29.4–1623)	650 (2–1416)
Der. f weal diameter (mm) (mean $\pm$ SD)	5.1 $\pm$ 1.9*	5 $\pm$ 2	3.6 $\pm$ 1.8*	4.1 $\pm$ 1.5
Der. p weal diameter (mm) (mean $\pm$ SD)	5.3 $\pm$ 1.8	5.4 $\pm$ 2.4	4.3 $\pm$ 1.2	4.1 $\pm$ 1.1
Blood EØ count (mm ³) (mean $\pm$ SD)	318.3 $\pm$ 198.3	107 $\pm$ 77.8 [#]	251.6 $\pm$ 179.6	85.4 $\pm$ 56.8 [#]
Nasal smear (–/+)	9/7**	12/4**	13/1**	11/3**
Symptoms				
Asthma symptom score (median (range))	0.8 (0.18–2.75)	0.14 (0–0.78) [§]	1.1 (0.31–3.6)	0.19 (0–2.1) [§]
Rhinitis symptom score (median (range))	0.9 (0.04–2.78)	0.07 (0–2) [§]	0.8 (0–1.28)	0.03 (0–0.8) [§]
QoLA (median (range))	75.3 (37–87)	87 (40–91) [§]	83 (58–89)	89.5 (66–91) [§]
No. of asthma attacks (mean $\pm$ SD)	1.4 $\pm$ 1.3	0.6 $\pm$ 0.7 [#]	1.8 $\pm$ 1.2	0.5 $\pm$ 0.6 [#]
Medication				
ICS dose (median (range))	3 (1–4)	0 (0–2) [§]	3 (2–4)	0 (0–3) [§]
Intranasal bud dose (median (range))	3 (2–3)***	0 (0–2) [§]	2.5 (0–3)***	0 (0–3) [§]
β_2 -mimetic use (median (range))	0.2 (0–0.71)	0 (0–0.71) [§]	0.1 (0–1)	0 (0–0.57) [§]

SLIT, sublingual immunotherapy; BCG, bacillus Calmette–Guerin; FVC, forced vital capacity; SD, standard deviation; FEV₁, expiratory volume in 1 s; FEF, forced expiratory flow; PEF, peak expiratory flow; QoL, quality-of-life; ICS, inhaled corticosteroid.

*Comparison between groups, $P < 0.05$, unpaired t-test. **Comparison between groups, $P < 0.05$, χ^2 test. ***Comparison between groups, $P < 0.05$, Mann–Whitney U-test. [#]Comparison within groups, $P < 0.05$, paired t-test. [§]Comparison within groups, $P < 0.01$, Wilcoxon signed-rank test.

the end of the treatment period. Within-group comparisons demonstrated that the mean daily dose of inhaled and intranasal steroids significantly decreased in both groups throughout the treatment period (Table 2).

QoL assessment QoL scores were similar in two groups both before initiation and at the end of treatment, whereas the changes from baseline to end-points showed significant improvement in both groups (Table 2).

Lung function and BHR

There were no significant between-group differences in the baseline LFT results, whereas end-point FEF_{25–75} and FEV₁/FVC values of the SLIT group were significantly higher than the SLIT+BCG group. The within-group analyses showed a significant increase in FEF_{25–75} values of the SLIT group. No within-group differences were observed in the SLIT+BCG group (Table 2). When the two groups were compared based on PC₂₀ values, no significant differences were detected between two groups both at baseline and at the end of the study. PC₂₀ values were found to be significantly improved in both groups throughout the study period (Table 2).

Serum total IgE and total blood eosinophil count

There was no significant difference in either between- or within-group comparisons in serum total IgE levels of the two groups. Baseline and end-of-treatment total eosinophil counts were not significantly different between the two groups.

On the other hand, at the end of the treatment the total eosinophil counts diminished significantly in both groups when compared with baseline (Table 2).

Skin sensitivity

Baseline skin sensitivity to *D. farinea* was significantly less in the SLIT+BCG group, whereas end-point wheal diameter obtained with *D. pteronyssinus* and *D. farinea* was not significantly different between the two groups. In within-group comparisons, skin sensitivity to *D. pteronyssinus* and *D. farinea* did not change significantly in either group (Table 2).

Nasal smear

At baseline, nasal smear positivity for eosinophils was significantly more frequent in the SLIT group compared with the SLIT+BCG group, whereas at the end of treatment, no difference was detected between those groups. The within-group analyses did not show any significant difference for the presence of eosinophils in nasal smears at the end of the 6 months of treatment in either groups (Table 2).

PPD skin testing The mean diameter of PPD skin test reaction was found to be 11.1 ± 5.3 mm in subjects, who were immunized with BCG at the end of the study period. All subjects in the SLIT group had negative PPD skin test responses at the end of study evaluation.

Cytokine analyses In the comparison of the treatment and control groups, no significant differences were detected in

IL-4, IL-10 and IL-13 levels in the supernatants of PBMC stimulated with PPD, *D. pteronyssinus* and PHA (data not shown), whereas PHA-stimulated IL-12 and *D. pteronyssinus*-stimulated and -unstimulated IFN- γ production were significantly different between groups (Figs 1 and 2).

Comparison of the SLIT and SLIT+BCG groups revealed that IFN- γ production by PBMC on stimulation with PPD, *D. pteronyssinus*, PHA and without stimulation were not significantly different between the two groups (data not shown). On the other hand, IFN- γ production by PBMC on stimulation with *D. pteronyssinus* and in unstimulated

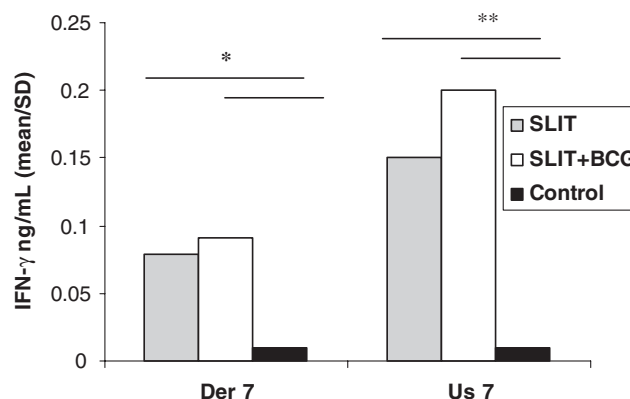


Fig. 1. Comparison of IFN- γ levels of the three groups. IFN- γ levels were measured after 6 months of HDM-SLIT and HDM-SLIT+BCG and in controls in the culture supernatants of PBMC stimulated with *Dermatophagoides pteronyssinus*, PHA, PPD and in unstimulated culture supernatants: *comparison of *D. pteronyssinus* stimulated IFN- γ levels of SLIT group vs. controls $P = 0.008$, SLIT+BCG group vs. controls $P = 0.02$, **comparison of IFN- γ levels of the SLIT group vs. controls ($P = 0.01$) and the SLIT+BCG group vs. controls $P = 0.02$ in unstimulated culture supernatants. HDM, house dust mite; SLIT, sublingual immunotherapy; BCG, bacillus Calmette–Guerin; PBMC, peripheral blood mononuclear cell; PPD, purified protein derivative; PHA, phytohaemagglutinin.

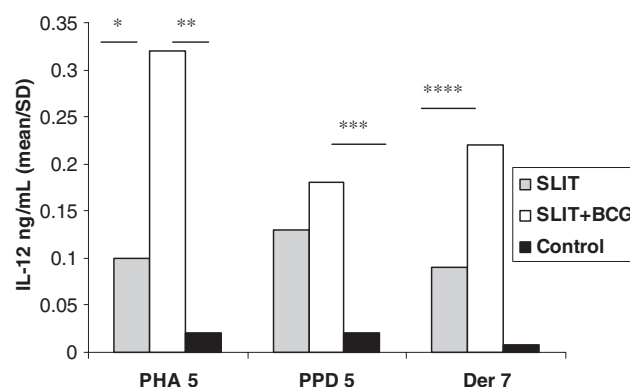


Fig. 2. Increased IL-12 levels in BCG+SLIT. IL-12 levels were measured after 6 months of HDM-SLIT and HDM-SLIT+BCG and in controls in the culture supernatants of PBMC stimulated with *Dermatophagoides pteronyssinus*, PHA, PPD and in unstimulated culture supernatants: *comparison of PHA stimulated IL-12 levels of SLIT group vs. SLIT+BCG group $P = 0.04$, **SLIT+BCG group vs. controls $P = 0.04$. ***Comparison of PPD stimulated IL-12 levels of SLIT+BCG group vs. controls $P = 0.04$. ****Comparison of *D. pteronyssinus* stimulated IL-12 levels of the SLIT group vs. the SLIT+BCG group ($P = 0.04$). BCG, bacillus Calmette–Guerin; SLIT, sublingual immunotherapy; HDM, house dust mite; PBMC, peripheral blood mononuclear cell; PPD, purified protein derivative; PHA, phytohaemagglutinin.

cultures was significantly higher in SLIT and SLIT+BCG groups when compared with controls (Fig. 1). IL-12 production by PBMC on stimulation with PHA and *D. pteronyssinus* was significantly higher in the SLIT+BCG group when compared with the SLIT group. Furthermore, although no significant differences were detected in the comparison of IL-12 levels of the SLIT vs. control groups, IL-12 levels in supernatants of PBMC stimulated with PPD and PHA were significantly higher in the SLIT+BCG group when compared with controls (Fig. 2).

Discussion

This study demonstrates that both SLIT and SLIT following one dose of BCG immunization resulted in a significant improvement in scores for asthma, rhinitis and QoL, number of asthma attacks, amount of medication, blood eosinophil count and BHR.

Today, allergen-specific IT is accepted as the only means that can both alter the outcome of the disease and prevent the formation of new sensitization [30–32]. SLIT, which is based on oral desensitization, is another promising application in terms of reliability and efficacy. A number of controlled studies have demonstrated the good safety profile and efficacy of SLIT in the treatment of allergic disorders [11, 12, 14–17].

In accordance with those studies [9–13, 17], in our study significant improvement was observed both in SLIT and SLIT+BCG groups with respect to asthma and rhinitis symptom scores and drug consumption as early as 6 months. One of the parameters we monitored in this study was the number of acute asthma exacerbations, which showed a significant reduction at the end of the study period in both treatment groups.

QoL is unfavourably affected in chronic diseases like asthma [33]. In our study, children's QoL was evaluated before and 6 months after treatment showing a significant improvement in both treatment groups. Bousquet et al. [17] reported improving QoL score after 2 years of SLIT in 85 patients with asthma due to HDM.

BHR is the main characteristic of the disease [34, 35] and accepted as the most important parameter that reflects the severity. The effect of specific IT on respiratory function tests and BHR is still on debate. This study demonstrated a favourable effect of SLIT on BHR in both the treatment groups. Furthermore, a significant improvement was achieved in FEF 25–75 values in the SLIT group. There are limited number of studies that investigate the effect of SLIT on BHR and respiratory function tests. Tari et al. [14] showed the reduction of specific and non-specific BHR in children after 12 months of treatment. Another report, in which respiratory function and asthma symptom scores significantly improved, displayed 1.75 times increase in provocation dose (PD₂₀) values after 24 months of SLIT treatment [17].

In the current study, we also demonstrated skin reactivity to *D. pteronyssinus* and *D. farinea*, and serum total IgE levels did not show any significant change throughout the study period, whereas the total eosinophil count decreased significantly in both treatment groups.

Although the clinical efficacy and safety of SLIT has been established by a number of controlled trials, the mechanism of action of SLIT is not well known. One of the current

hypotheses is induction of tolerance by Langerhans' cells that internalize the antigens in the sublingual mucosa before migrating to the regional lymph nodes, where they act as antigen-presenting cells. This may result in T cell response modifications responsible for oral tolerance [8–10]. The tolerogenicity of the oral route has been clearly demonstrated in animal models. It seems to involve mainly Th1/Th2 differentiation and IgE production regulation: the oral administration of the antigen appears able to induce a selective Th1 cell proliferation and subsequently, a protective effect [8].

Although the exact mechanism by which mucosal vaccines induce tolerance against allergens remains unclear, application of high doses of allergens by mucosal route seems to be associated with energy and/or deletion of allergen-specific Th2 cells, whereas the low doses of allergen appears to result in active suppression by Tr cells [36].

Adjuvants such as killed bacteria or components derived from them, CPGoDNs or pDNA that induce Th1 responses, are readily available and have been shown to inhibit the development of allergic responses in animals [19, 20, 22–25, 37–41]. By administering the allergen in conjunction with the adjuvant, the goal is to induce selectively long-lived allergen-specific memory Th1 cells. It is hoped that, upon re-encountering allergen, these cells will secrete IFN- γ , thereby suppressing the development of allergen-specific Th2 cells.

In the light of these data, one of the aims of our study was to test whether the administration of BCG as an adjuvant to allergen-specific SLIT has any additive effect on the cytokine profile in children with asthma allergic to HDM. For this purpose, we selected children with a negative response to PPD skin testing. According to the practice in our country, BCG immunization is routinely performed at birth. Our study design is in compliance with the recommendations of Ministry of Health; since according to their recommendation, documentation of a negative PPD response warrants another BCG vaccination.

Our results also showed a higher *in vitro* production of IFN- γ from PBMC of asthmatic children in the treatment groups compared with controls who never received IT. Although BCG immunization in addition to SLIT enhanced IL-12 production, it did not result in additional clinical benefit.

As allergen-specific CD4⁺ T cells play a crucial role in the allergic immune response, specific inactivation of these Th2 cells or the selective induction of Th1 cytokines might directly interfere with the disease process that may be of potential therapeutic benefit for asthma and allergic diseases.

Mycobacteria are known to be very potent inducers of Th1 immune response, which is characterized by IL-12 and IFN- γ release [42–44]. IFN- γ , a major cytokine from Th1 cells, directly suppresses IgE synthesis and inhibits the development of atopic disease [45].

IL-12 is known to be the key cytokine involved in regulating the balance between Th1 and Th2 cells [46, 47]. Furthermore, IL-12 inhibits airway hyper-responsiveness and airway eosinophilia after antigen challenge in several animals with allergen sensitization [48, 49].

Barnes et al. demonstrated that IL-12 lowers the numbers of blood and sputum eosinophils, whereas it has no significant effect on airway hyper-reactivity or the late asthmatic

reaction [50]. The authors suggested that IL-12 might have to be given in early childhood in order to suppress subsequent allergic responses. They also pointed out that IL-12 is potentially best used as an adjuvant in conjunction with allergen [51].

In our study, we demonstrated that IFN- γ levels increased significantly both in SLIT and SLIT+BCG receiving groups. This increase was well correlated with the improvement in BHR, clinical parameters and decrease in eosinophil counts in both groups. On the other hand, although the IL-12 levels were significantly higher in the BCG receiving group, this difference did not result in any further improvement in clinical and laboratory parameters. These results question the role of BCG-induced IL-12 in the treatment of allergic disorders.

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