

Innate and lymphocytic response of birch-allergic patients before and after sublingual immunotherapy

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ABSTRACT

Functional imbalance in Th1/Th2 cell response toward allergens is a recognized hallmark of allergic patients and a major role of dendritic cells (DCs) in redirecting T-cell phenotypes after specific immunotherapy has been suggested. This study investigates the proliferative and cytokine responses of T cells cocultured with monocyte-derived DCs (MoDCs) after allergen stimulation in birch-allergic patients compared with controls and investigates whether sublingual immunotherapy (SLIT) could change the DC-driven immune response. T cells were stimulated with the major birch pollen allergen (nBet v1) and MoDCs from eight birch-allergic patients with seasonal allergic rhinitis and eight nonallergic controls. Proliferation and cytokine production were measured before and after one course of SLIT with birch allergoid. Significantly lower levels of proinflammatory (IL-1 β , $p = 0.027$; IL-6, $p = 0.030$; TNF- α , $p = 0.019$) and Th1 (interferon gamma, $p = 0.032$; IL-12, $p = 0.05$) cytokines were measured in supernatants of T cells and MoDCs cultures from allergic patients compared with nonallergic controls. After SLIT, significant increase in IL-12 ($p = 0.039$), IL-1 β ($p = 0.040$), IL-6 ($p = 0.041$), TNF- α ($p = 0.048$), and IL-10 ($p = 0.048$) and significant decrease in IL-13 ($p = 0.001$) were observed. MoDCs/T-cell cocultures, pulsed with the specific allergen, produced lower quantities of proinflammatory and Th1 cytokines in allergic patients compared with healthy subjects, suggesting an allergen-specific impairment of natural immunity and Th1 immune response. A single course of SLIT was able to enhance allergen-specific innate immunity and to modify lymphocyte response, promoting Th1 and T-cell regulatory activity.

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The proliferative response of peripheral blood mononuclear cells (PBMCs) to allergens and cytokine production after allergen stimulation of PBMCs has been historically used to show differences between atopic and nonatopic subjects in terms of Th1/Th2 profiles^{1,2} and responses to specific immunotherapy.^{3,4}

Optimal activation of T lymphocytes requires a costimulatory signal provided by the interaction of molecules on the surface of T cells with their ligands expressed on dendritic cells (DCs). DCs are pivotal in developing and determining the type of the immune response because DCs govern both the initiation and the polarization of adaptive immunity.⁵ For this reason, T cells elicited by monocyte-derived DCs (MoDCs) after allergen stimulation are particularly useful in the study of the immune response in allergic patients.^{6,7}

Many experimental data show that MoDCs from atopic patients, when fully differentiated by maturing factors (*i.e.*, proinflammatory cytokines or lipopolysaccharide) and exposed to allergens, are able to activate allergen-specific T-helper cells to produce Th2-cytokines.^{8–10} Nevertheless, some observations point to a decreased production of Th1 cytokines by immature MoDCs of atopic subjects compared with healthy subjects.^{11,12} The interaction between T cells and MoDCs, not fully stimulated with maturing factors, may thus reveal impairment of allergen-specific natural immunity and Th1 immune response of allergic patients.

In the present study we investigated the cytokine production from cultures of T cells of allergic patients and healthy controls after allergen stimulation with MoDCs. Our hypothesis was that a decreased Th1 and proinflammatory response would be present in allergic patients compared with controls. We also investigated whether sublingual immunotherapy (SLIT) could change the T-cell immune response. A modification of the oral DC-driven Th1 response has been proposed as one possible mechanism for the efficacy of SLIT.¹³ To investigate this hypothesis, the proliferative response and cytokine production of T cells stimulated by the major birch pollen allergen (nBet v1) in the presence of MoDCs were investigated in birch-allergic patients before and after SLIT.

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Table 1 Patients' clinical characteristics

Patient	Age (yr)	Gender	Asthma	Birch IgE (kU/L)
1	46	F	No	5.71
2	47	M	No	26.40
3	48	F	Yes	20.50
4	37	M	Yes	100.00
5	49	F	No	2.27
6	50	F	No	9.22
7	39	F	No	6.21
8	31	F	No	31.17

MATERIALS AND METHODS

Subjects

Eight birch-allergic patients were selected from our outpatient clinic. All patients were sensitized to birch pollen and reported at least 1 year of respiratory symptoms (rhinitis and/or asthma) coincident with birch pollen season. Skin-prick tests for a panel of inhalant standard allergens (ALK Abello, Hørsholm, Denmark) were performed in all included patients. Positive test was defined as a wheal diameter ≥ 3 mm in the presence of expected results in control solutions (histamine dihydrochloride at 10 mg/mL as a positive and solvent as a negative control). Three patients were monosensitized to birch pollen. Rhinitis and/or asthma were diagnosed according to the Allergic Rhinitis and Its Impact on Asthma and the Global Initiative for Asthma guidelines.^{14,15} Birch-specific IgE was measured in all the patients by ELISA (Lofarma S.P.A., Milano, Italy). Exclusion criteria were sensitized to other pollens overlapping seasonally with birch pollen (*i.e.*, olive and grass pollen), previous immunotherapy, chronic therapy with corticosteroids, and autoimmune diseases. Clinical characteristic and specific IgE values are reported in Table 1.

Eight healthy nonatopic subjects of comparable age and gender served as controls for baseline measurements.

All subjects gave their written informed consent to participate in the study, which was approved by our Institutional Research Ethics Committee.

Sublingual Immunotherapy

SLIT was administered with birch monomeric allergoid (Lais Betulaceae; Lofarma S.P.A.) after a pre-seasonal schedule that consisted of a 16-day induction phase and a 10-week maintenance phase (6000 UA/week), starting ~6–8 weeks before the expected birch pollen season.¹⁶ Rescue therapy was provided with second-generation antihistamines, salbutamol and nasal fluticasone.

Blood Sample Collection

Before the pollen season, whole blood samples were collected from the eight birch pollen-allergic patients and from eight nonatopic age-matched subjects. In birch pollen-allergic patients, blood samples were also collected after the first SLIT course (at the end of the pollen season).

MoDCs Generation and T-Cell Stimulation

MoDCs and T cells were obtained from PBMCs of birch-allergic patients, before and after one course of SLIT, and from nonatopic controls.

Monocytes were purified from PBMCs through immunomagnetic separation with CD14 MicroBeads (purity >90%; magnetic activated cell sorting (MACS); Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). To obtain MoDCs, CD14⁺ cells were plated at day 1 and then cultured for 6 days with IL-4 (10 ng/mL) and granulocyte macrophage colony-stimulating factor (100 ng/mL). The CD14 cell aliquot was frozen at -80°C until the coculture preparation.

At day 6, the CD14⁺ aliquots were defrosted, and autologous CD3⁺ T cells were obtained through immunomagnetic separation with CD3 MicroBeads (purity >90%; MACS, PAN T isolation Kit II, MACS; Miltenyi Biotec GmbH). MoDCs were harvested and suspended in medium (RPMI-1640 supplemented with FCS 10%) for the coculture assay.

MoDCs and T cells were plated with a final volume of 100 μL in a 96-well plate in a 1:20 ratio (4000 DCs/80,000 T cells). Cells were stimulated with nBet v1 at a 2- $\mu\text{g}/\text{mL}$ concentration for 5 days. Such concentration was chosen as the best of three different doses (0.4, 2, and 10 $\mu\text{g}/\text{mL}$) in separate experiments. Separate cultures of DCs and T cells were used as negative, non-stimulated controls. Natural Bet v1 was supplied by Lofarma S.P.A as 47 mL of lyophilized protein (purified >90%) 0.1 $\times$ (0.015M) and used in the assay after being resuspended in RPMI-1640 plus with FCS 10% medium at 100 $\mu\text{g}/\text{mL}$. After 5 days, supernatants were collected and stored at -20°C for further cytokine analysis. The same culture conditions were used for all patients and controls, and the experiments were performed in triplicate.

Proliferation Assay

Five days after of allergen stimulation, immediately after supernatant collection, cell cultures were incubated with 0.5 $\mu\text{Ci}/\text{well}$ [³H] thymidine (GBq/mM; Amersham Biosciences, Uppsala, Sweden) for 6 hours and frozen at -20°C . Cells were harvested with a Skatron AS cell harvester (Perkin Elmer, Life Science, Boston, MA), and thymidine incorporation into DNA was measured in a β -counter (1600 CA, TRI-CARB,

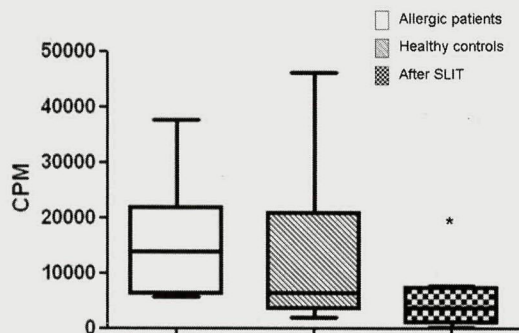


Figure 1. T-cell proliferation assay (expressed as count per minute [cpm]) of birch-allergic patients (white at baseline, checked after sublingual immunotherapy [SLIT]) and healthy controls (striped). $p^* = 0.046$; T-cell proliferation of patients before and after SLIT.

Liquid Scintillation Analyzer, Perkin Elmer, Life Science) in counts per minute (cpm).

Cytokine Assay

Cytokines were measured by multiplex analysis (Bio-plex/Luminex; Bio-Rad Life Science, Hercules, CA) of supernatants obtained from cell cultures that had been pulsed with the nBet v1 dose giving the highest stimulation index in both patient and control samples.

Statistical Analysis

Data were analyzed with the SPSS Statistical Software Package (Version 13.0 for Windows; SPSS, Inc., Chicago, IL). Normal distribution of variables was assessed according to Kolmogorov-Smirnov's test of normality. In cases of non-normal distribution, the variables were computed as logarithmic. Comparisons between continuous variables were estimated with the unpaired and paired Student's *t*-test or the Mann-Whitney test, depending on the distribution of the variables. Results are expressed as mean $\pm$ SEM.

RESULTS

T-Cell Proliferation after Exposure to nBet v1

Significantly decreased proliferation of T cells from allergic patients was observed after SLIT (from 13,986, CI 95%, 6330–25,280 cpm, to 3936, CI 95%, 628–7300; $p = 0.046$; Fig. 1), and the baseline (before SLIT) proliferative response of T cells stimulated with nBet v1 was not significantly different between patients and controls.

Cytokine Production of T Cells Stimulated with nBet v1 and MoDCs

The coefficient of variation in Bio-Plex cytokines assays for replicate samples was $<10\%$. Compared with healthy subjects, supernatants of T cells and MoDCs

Table 2 Cytokine production (pg/mL) of supernatants from cultures of T cells stimulated with nBet v1 and MoDCs in birch-allergic patients and healthy controls

Cytokine	Patients	Controls	<i>p</i> Value
IL-1 β	3.69 $\pm$ 0.82	40.65 $\pm$ 17.20	0.027
IL-4	30.2 $\pm$ 17.7	17.1 $\pm$ 6.3	0.553
IL-5	225.6 $\pm$ 110.5	102.2 $\pm$ 78.2	0.412
IL-6	292.5 $\pm$ 138.5	1616.8 $\pm$ 598.8	0.030
IL-9	99.3 $\pm$ 36.2	162.8 $\pm$ 88.5	0.442
IL-10	13.6 $\pm$ 4.6	331.0 $\pm$ 157.5	0.036
IL-12	2.4 $\pm$ 1	14.9 $\pm$ 9.0	0.050
IL-13	157.2 $\pm$ 29.8	180 $\pm$ 67.9	0.741
IL-17	59.15 $\pm$ 18.2	133.4 $\pm$ 19.1	0.017
IFN- γ	420.6 $\pm$ 343.6	4014.3 $\pm$ 1667.0	0.032
TNF- α	79.9 $\pm$ 25	4053.1 $\pm$ 1714.6	0.019

Results are expressed as mean $\pm$ SEM. Statistically significant values are highlighted (bold font).

IFN- γ = interferon γ ; MoDCs = monocyte-derived dendritic cells.

cultures of birch-allergic patients (evaluated out of pollen season) stimulated with nBet v1 produced significantly lower levels of proinflammatory (IL-1 β : 3.69 $\pm$ 0.82 pg/mL versus 40.65 $\pm$ 17.20 pg/mL, $p = 0.027$; IL-6: 292.5 $\pm$ 138.5 pg/mL versus 1616.8 $\pm$ 598.8 pg/mL, $p = 0.030$; TNF- α : 79.9 $\pm$ 25 pg/mL versus 4053.1 $\pm$ 1714.6, $p = 0.019$), Th1 (interferon [IFN] γ : 420.6 $\pm$ 343.6 pg/mL versus 4014.3 $\pm$ 1667.0 pg/mL, $p = 0.032$; IL-12: 2.4 $\pm$ 1 pg/mL, versus 14.9 $\pm$ 9.0 pg/mL, $p = 0.05$) and T-regulatory (IL-10: 13.6 $\pm$ 4.6 pg/mL versus 331.0 $\pm$ 157.5 pg/mL, $p = 0.036$) cytokines compared with controls (see Table 2). No difference was observed in Th2 cytokines levels (IL-13, IL-4, and IL-5).

After SLIT, a significant increase in IL-12 (from 1.75 $\pm$ 0.54 to 5.0 $\pm$ 1.34 pg/mL, $p = 0.039$), IL-10 (from 12.9 $\pm$ 3.8 to 39.8 $\pm$ 13.3 pg/mL, $p = 0.048$), IL-1 β (from 4.1 $\pm$ 1.0 to 24.1 $\pm$ 6.3 pg/mL, $p = 0.040$), IL-6 (from 259 $\pm$ 163.9 to 1222 $\pm$ 309.8 pg/mL, $p = 0.041$), and TNF- α (from 90.2 $\pm$ 31.9 to 1917.4 $\pm$ 727 pg/mL, $p = 0.048$) was observed (Fig. 2, A–E). A significant decrease in IL-13 (from 172.2 $\pm$ 38.3 to 90.5 $\pm$ 31.4 pg/mL, $p = 0.001$; Fig. 2 F), but no significant differences in IL-4, IL-5, IL-17, and INF- γ levels, were observed after SLIT.

DISCUSSION

The main result of our study was that cultures of T cells of birch-allergic patients, out of the pollen season, when stimulated with nBet v1 in the presence of MoDCs, produced significantly lower levels of proinflammatory (IL-1 β , IL-6, and TNF- α) and Th1 (IL-12 and IFN- γ) cytokines compared with healthy subjects.

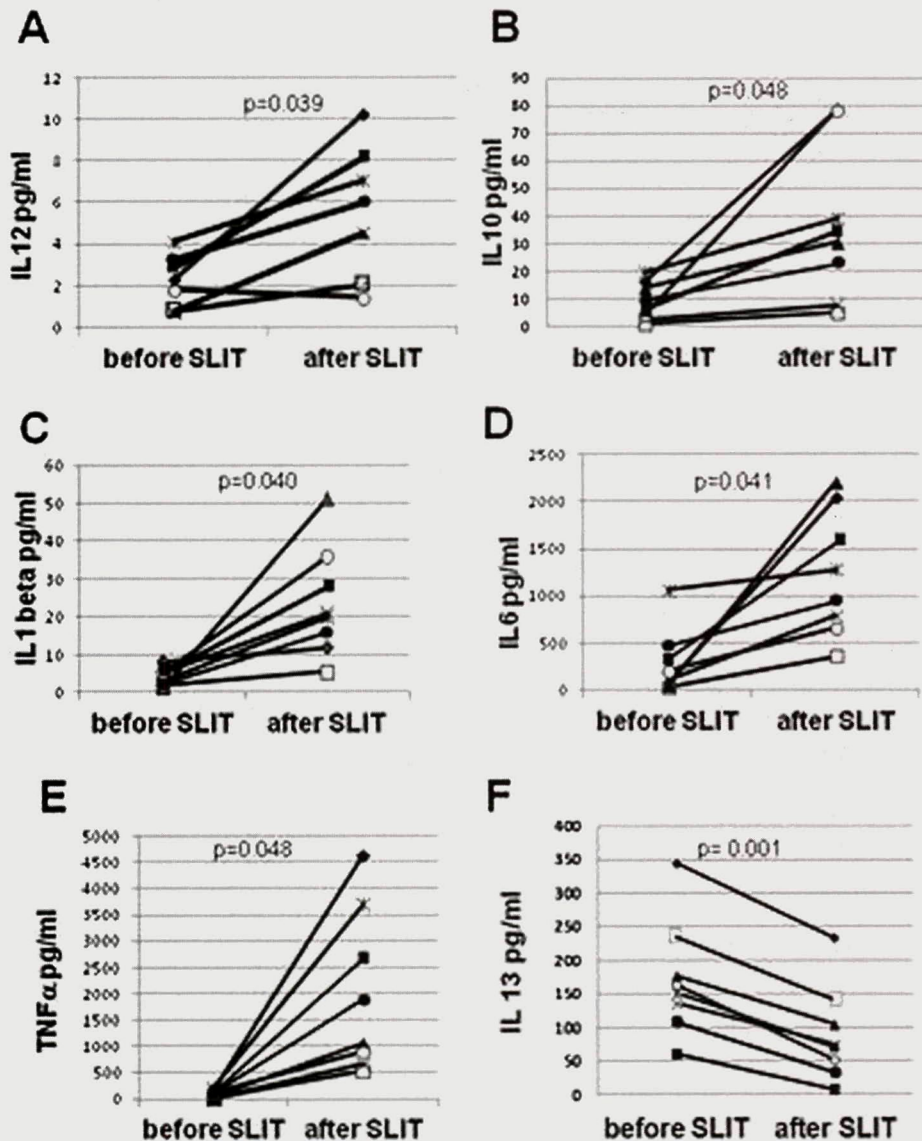


Figure 2. Cytokine production from culture supernatants stimulated with nBet v1 of birch-allergic patients, before and after sublingual immunotherapy (SLIT).

Similarly, Casas *et al.* showed that MoDCs from birch-allergic patients, when stimulated with nBet v1, have impaired maturation that results in significantly lower levels of IL-12 compared with normal subjects.¹¹ The decreased production of proinflammatory and Th1 cytokines compared with nonallergic subjects suggests that allergic patients have an allergen-specific impairment of natural immunity and the Th1 immune response¹² and the interaction between T cells and MoDCs is able to reveal this imbalance. These results confirm our hypothesis that allergic patients compared with controls have a decreased Th1 and proinflammatory response to the allergens. We did not observe an increased production of Th2 cytokines in lymphocytes and MoDCs cultures of our allergic patients, as reported in experiments with fully differentiated MoDCs.⁸⁻¹⁰ In our experimental setting, DCs were not stimulated with maturing factors (*e.g.*, lipopolysaccharide, IL-1, or Th1 cytokines). The major defect of

allergic response seems to be the altered ratio between Th1/Th2 cytokines, in favor of Th2 cytokines, either because of decreased Th1 cytokines or because of increased Th2 cytokines. After SLIT we observed increased proinflammatory and Th1 cytokines of our allergic patients. This observation suggests that immunotherapy may modify the link between the innate and adaptive immune system.¹⁷ Actually, according to a recent study, allergen immunotherapy increases DCs innate immune function.¹⁸ These observations suggest that SLIT may enhance allergen-specific natural immunity and the Th1 response.¹⁹ Most recent studies implicate the T-regulatory cell response as central to the development of a tolerogenic state in response to allergen immunotherapy, with both IL-10 and transforming growth factor β playing crucial roles in the development of this cell subset.

We found that IL-10 levels were lower in allergic patients than in controls and significantly increased

after a single course of SLIT. This observation suggests a possible role of regulatory T cells in our experimental system. IL-10 producing regulatory T cells (CD4⁺/CD25⁺ cells) have been shown to be significantly increased after SLIT and to promote a specific suppression of nBet v1 T-cell response.²⁰ The low levels of IL-10 may negatively influence DCs maturation, causing a lack of tolerance for allergens.²¹

We did not observe a significant difference in Th2 cytokines in atopic subjects compared with healthy controls. This is not surprising because DCs must be fully matured^{9,22} to show Th2 polarization in allergic patients. After SLIT, we observed a significant decrease in IL-13 without any changes in IL-4 and IL-5 and a trend toward specific IgE reduction. These results may be related to down-regulation of Th2 lymphocytes after the course of SLIT.²³

One limitation of this study, similar to most others using cell cocultures, is that it is unknown which cells produce the cytokines measured in the supernatants. However, the aim of this study was to discern whether there was a significant difference in the immune response of the DCs/T-cells system between allergic patients and healthy control subjects. Additional studies are needed to evaluate the precise source of the cytokines produced in this system.

In conclusion, our results suggest that one course of SLIT may modify the allergen presentation by DCs that in turn correct the phenotype of allergen-specific T cells, switching from the Th2-type response, typical of allergic inflammation, to a Th1-type as well T-cell regulatory responses.

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