Original article

Dendritic cells modification during sublingual immunotherapy in children with allergic symptoms to house dust mites

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Background: **The importance of dendritic cells (DCs) in the initiation of the Th2-mediated inflammatory response to allergens is well known and more recently it has been proposed that DCs have a pivotal role in maintaining tolerance to allergens. The aim of this study was to investigate whether the success of sublingual immunotherapy (SLIT) in allergic asthma is mediated by the induction of changes of DCs functions.**

Methods: **Ten children with allergic asthma sensitive to house dust mite were studied before and after 12 months of SLIT. Immature DCs were derived from peripheral blood monocytes cultured for 6 days in presence of interleukin (IL)-4 and GM-CSF and stimulated with lipopolysaccharide for the last 24 hours to induce maturation.**

Results: **After 12 months of SLIT, mature DCs derived from SLIT-treated patients showed a statistically significant defect of CD86 up-regulation, an increase of IL-10, and a reduction of IL-12 production.**

Conclusion: **SLIT induces changes in DCs functions that might be responsible for an impairment of T cell activation or drive T cells towards a regulatory activity, thus restoring immune tolerance to allergens.**

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Key words: **allergy;**
CD86;
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Introduction

The increasing prevalence of allergy and its impact on individual quality of life and social costs underline the need of an improvement of the treatment options in order to modify the natural course of allergic diseases. In this context, specific immunotherapy (SIT) represents the only approach currently available to redirect inappropriate immune response in atopic patients and it has been shown to be safe and effective in the treatment of clinically significant respiratory IgE-mediated diseases.[1]

Despite this clinical evidence, its mode of action has not yet been fully elucidated. In the last years, some studies indicated that the mechanism of immunotherapy might be based on the increase of number and activity of regulatory T cells. Regulatory T cells appear to control the development of autoimmune diseases and transplant rejection and may also play a critical role in controlling asthma and allergy. In particular, type 1 regulatory T cells (Tr1) are a subset of $CD4^+$ T cells that produce high levels of IL-10, low levels of IL-2 and no IL-4.[2] It has been reported that they consistently represent the dominant subset specific for common environmental allergens in healthy individuals in contrast to the allergen-specific IL-4-secreting T cells frequently found in allergic individuals.[3,4] Specific immunotherapy, by modifying Tr1 compartment, would then restore the natural immune tolerance to harmless antigens such as aeroallergens.[5-7]

Accumulating evidences suggest that the generation of T regulatory cells in periphery is orchestrated by a particular subset of dendritic cells (DCs), whose

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phenotype and modality of function are topics of intense study. Early reports showed that repeated stimulation of naïve CD4 T cells with allogenic immature DCs induce Tr1 cells.[8,9] Subsequently, it has been demonstrated that a specialized subset of DCs, termed tolerogenic DCs, can prime T cells to differentiate in Tr1 cells, thus promoting tolerance rather than immunity[10] and several protocols have been developed to generate Tr1 cells *in vitro* by using tolerogenic DCs.[11]

For years the gold standard of SIT was represented by the subcutaneous immunotherapy (SCIT), but the risk of severe systemic side effects and the need for frequent injections has limited the application of SCIT, especially in children. Nowadays, other kinds of immunotherapy such as sublingual-swallow immunotherapy (SLIT) may represent a valuable alternative to SCIT. SLIT has been shown to be safe and effective in several double-blind placebo-controlled studies in children affected by asthma and rhinitis.[12-14] In 2001, the allergic rhinitis and its impact on asthma (ARIA) extended the indications of SLIT also to children and a recent meta-analysis concluded that SLIT significantly reduces both symptoms and the requirement of medications.[15]

The immunological mechanism that underlies SLIT has only started to be investigated. It is conceivable that the sublingual administration route might induce immunological tolerance towards allergens involving cells and mediators specific of oral and intestinal mucosa. In particular, although it partially shares the same mechanisms of SCIT, it is also able to act on Langerhans cells present in oral mucosa. It seems to induce the secretion of allergen-specific IgG,[16] to increase IL-10 and TGF-β expression and to enhance Tr1 functions.[17,18]

The purpose of this study was to address the question whether SLIT promotes tolerogenic functions of DCs and whether it is possible to identify DCs specific biomarkers that may predict the success of SLIT in allergic patients.

Methods

Patients

We recruited 10 children (6 males and 4 females), aged 6.3 years on average (range: 4.5-9.2 years), affected by intermittent asthma and/or rhinitis, due to house dust mites (HDM) allergy. All children had positive skin prick test and/or specific IgE for *Dermatophagoides Pteronyssinus* (Dt.Pt.) and/or *Dermatophagoides Farinae* (Dt.F.). At the beginning and at the end of the study we assessed clinical score evaluating nasal symptoms (sneezing, itching nose, watery running and nasal blockage) and asthma daily and nocturnal symptoms (wheeze/breathless and dry cough during the night) using 0-4 scales (0=none, 1=mild intermittent, 2=mild persistent, 3=moderate persistent, 4=severe persistent).[19,20] Patients' characteristics are detailed in Table 1. At the time of the enrollment all patients started SLIT (SARM, Rome, Italy), consisting the sublingual administration of allergen extract (HDM maximum dose 6000 BU/monthly).

The Ethical Committee of the Hospital approved the study and informed consent was obtained from every study participant after the nature of the study was explained.

Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from the patients, before HDM-SLIT (T0) and after 12 months (T12), by Ficoll density gradient centrifugation.

Generation of DCs

Immature DCs (iDCs) were generated from peripheral blood monocyte precursors. Monocytes were isolated from peripheral blood mononuclear cells (PBMCs) by positive selection, using anti-CD14-conjugated magnetic Microbeads (MACS, Miltenyi Biotec, Belgish Gladbach, Germany) routinely resulting in >95% purity of the $CD14^+$ population, as assessed by flow cytometry. DCs were derived from $CD14^+$ cells cultured for 6 days in RPMI-1640 (Sigma, Milano, Italy) containing 10% FCS (Gibco, Carlsbad, CA, USA) and supplemented with GM-CSF (50 ng/mL, Leucomax, Sandoz AG, Nürnberg, Germany) and IL-4 (10 ng/mL, Sigma-Aldrich, St Louis, MO, USA), at the concentration of 4×10^4 cells/ml.

Myeloid DCs, defined as $CD14^-$, $CD1a^+$, $CD11c^+$

Table 1. Clinical and allergological characteristics of the cohort

Patients (n=10)*	Baseline (T0)	Follow-up (T12)
Clinical score†	6 (0-8)	3.5 (0-8)
Total IgE (UI/dl)	506.3 (71-1000)	402.5 (68-1000)
IgE to Dt.Pt. (UI/dl)	68.0 (3-200)	70.6 (10-145)
Dt.F. (UI/dl)	105.0 (4-205)	35.1 (9-65)‡
Skin prick test (mm)		
Dt.Pt.	6 (3-12)	5 (3-9)
Dt.F.	8 (3-9)	6.5 (3-9)
FEV1 (% predicted value)	88 (80-90)	90 (82-94)
Presence of nasal eosinophils	Baseline (T0)	Follow-up (T6)
Eosinophilia (% blood count)	8 (1-17)	6.5 (0-15)

Values for each parameter are expressed as mean and (range). *: Patients were considered allergic if they were positive for allergen-specific IgE and/or skin prick tests to house dust mites and had a history of bronchospasm and/or rhinitis after allergen exposure; †: Clinical scores were evaluated according to Allergic Rhinitis and its Impact on Asthma[19] and the Global Initiative for Asthma[20]; ‡: T0 *vs.* T12, P= 0.038. Dt.Pt.: *Dermatophagoides Pteronyssinus*; Dt.F.: *Dermatophagoides Farinae*.

cells, were then analyzed or further stimulated with lipopolysaccharide (LPS) from *E. Coli* (1 μg/mL, Sigma Chemicals, St Louis, MO, USA) for 24 hours to induce maturation.

Analysis of DCs phenotype by flow cytometry

DCs were stained for 20 minutes on ice with monoclonal antibodies conjugated to FITC or PE, specific for CD14, CD1a, CD11c, HLA-DR, CD80, CD86, CD83, as well as with isotype controls (all from Pharmingen, San Diego, CA, USA). For each sample, a minimum of 1×10^4 events were acquired with a flow cytometer (FACSCalibur, Beckton Dickinson, San Jose, CA, USA) interfaced with Cellquest software (Beckton Dickinson).

Cytokines production assay

IL-12p70 and IL-10 concentrations in iDCs and mature DCs (mDCs) culture supernatants were determined by enzyme-linked immunosorbent assay (Human IL-12 and IL-10 ELISA, Endogen, Woburn, MA, USA). The test was performed in duplicate according to the instructions of the manufacturer.

Statistical analysis

The SPSS for Windows 17.0 was used for statistical analysis. Experimental data were tested for distribution according to Kolmogorov-Smirnov, and all the surface cell markers were normally distributed. The differences between groups were therefore analyzed with Student's *t* test and a value of $P<0.05$ was considered statistically significant.

Results

Clinical outcome

During the observational period, none of the children experienced severe exacerbations of asthmatic symptoms necessitating either hospital admission or treatment with a short course of oral steroids, without viral or bacterial infections. As described in Table 1, after 12 months of treatment we observed an improvement of both nasal and asthma symptoms, as indicated by the reduction of the score. Concerning laboratory parameters, during SLIT treatment, we observed a significant modification only for specific IgE to Dt. F. ($P=0.038$).

DCs phenotypes

We analyzed expressions of HLA-DR, CD80, CD86 and CD83 on DCs derived from SLIT-treated allergic asthmatic children ($n=10$) at T0 and T12 (Fig. 1). After 12 months of follow-up, no statistically significant changes were found in the expressions of HLA-DR, CD80 and CD83 on both iDC and mDC. A slight reduction of HLA-DR and CD80 expressions was detected (Table 2 and Fig. 2A).

Interestingly, the expression of CD86 was reduced in both iDCs and mDCs at T12 compared to T0, and the decrease was statistically significant for mDC ($P=0.00006$) (Table 2 and Fig. 2A).

IL-10 and IL-12p70 production by DCs

Upon maturation with LPS, mDCs from allergic patients produced detectable amount of cytokines as determined in culture supernatants. IL-10 production was increased after 12 months of SLIT (373±521 *vs*. 276±281 pg/ml), while IL-12 production was decreased (141±257 *vs*. 1598±1614 pg/ml) (Fig. 2B).

In the supernatants of iDCs cultures of both IL-10 and IL-12p70 were undetectable.

Discussion

To elucidate the immunological changes induced by SLIT, we studied the function of DCs in asthmatic patients receiving SLIT for 12 months. We observed a statistically significant reduction over time of the expression of CD86, an important costimulatory molecule for T cells, on mDCs. Differently, CD80 expression, as well as HLA-DR expression, was substantially unchanged. CD86 appears indeed to be more important than CD80 for the induction of the allergic immune response.[21,22] In murine models of allergic airway inflammation, blockade of CD86, but not of CD80, abolishes airway eosinophilia and airway hyperresponsiveness.[23,24] To note, the defective up-regulation of CD86 on mDC at T12 was consistent with the increase of IL-10 that can be detected at the same time point. Indeed, IL-10 can cause a down-regulation of costimulatory molecules expression[25] leading to an impairment of T cell-stimulatory ability of DCs, as well as to an induction of T anergy.[26,27] Therefore, the reduction of CD86 expression that we observed during SLIT might have an important role in the induction of

Table 2. Expression of dendritic cells surface markers in sublingual immunotherapy (SLIT) treated allergic patients before (T0) and after 12 months (T12) of follow-up.

	T0		T12	
	iDC	mDC	iDC	mDC
HLA-DR	28.8±21.8	107.3±120.1	21.4±14.6	96.2±77.8
CD80	11.7±9.7	56.6±37.7	11.4±4.1	37.6±17.9
CD86	23.2±20.3	202.7±68.9	16.9±12.6	99.3±61.5*
CD83	5.6±3.2	27.9±19.9	7.0±2.3	34.7±25.9

All values represent average mean fluorescence intensity (MFI) ± SD. *: mDC T0 *vs*. T12: $P=0.00006$. iDC: immature dendritic cells; mDC: mature dendritic cells.

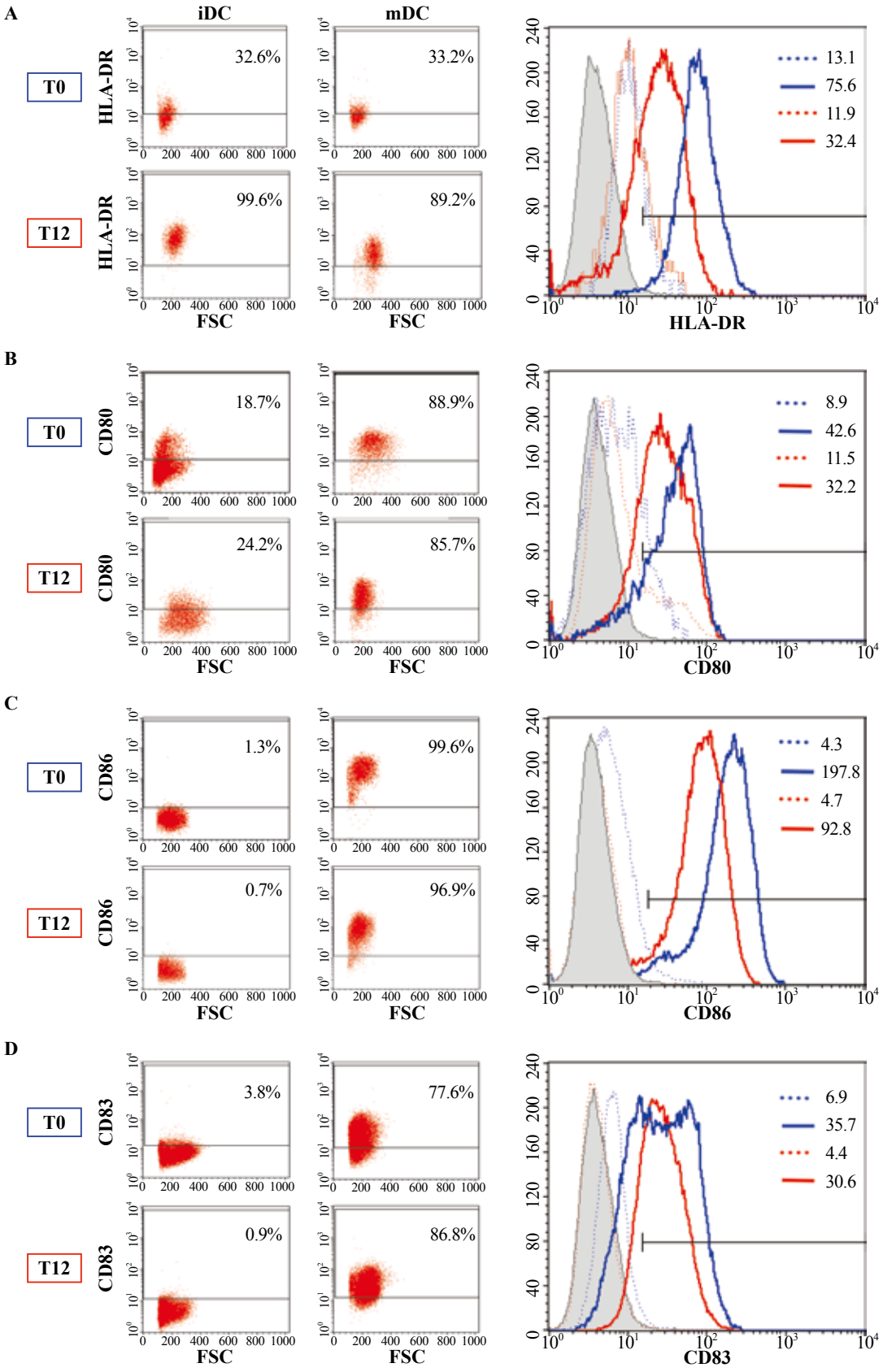

Fig. 1. Phenotype of immature DCs (iDCs, dotted line) and LPS-matured DCs (mDCs, solid line) before (T0, blue line) and after (T12, red line) 12 months of sublingual immunotherapy (SLIT). Expression levels of HLA-DR (**A**), CD80 (**B**), CD86 (**C**) and CD83 (**D**) were evaluated by flow cytometry, isotype control was also included (filled grey histogram). Data from one representative patient of 10 are shown as dot plots (left panels) and as histograms (right panels). In the left panels, numbers represent the percentages of positive cells. In the right panels, numbers represent the MFI values.

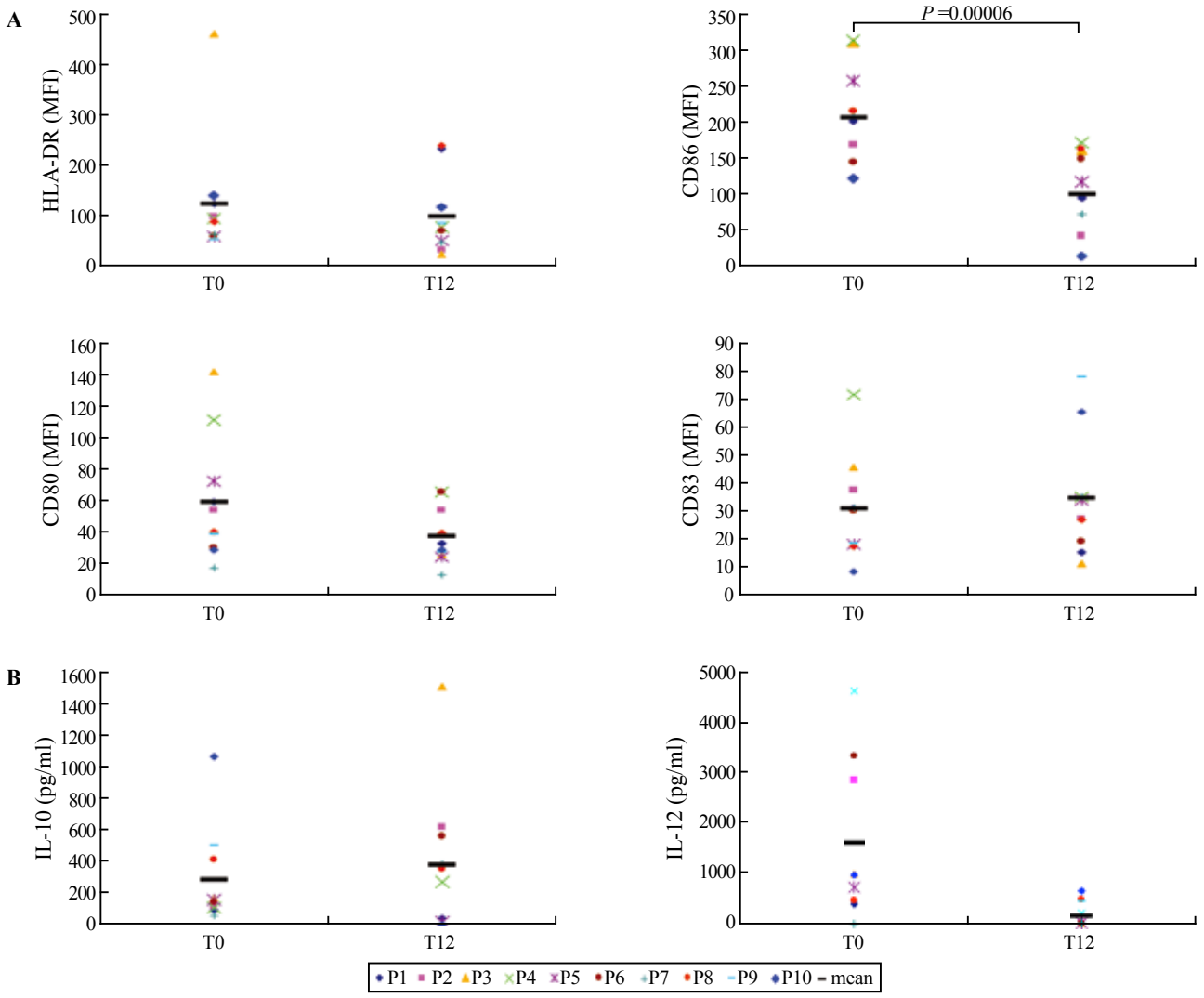

Fig. 2. LPS-matured DCs (mDCs) phenotype (**A**) and cytokine production (**B**) of allergic patients before (T0) and after (T12) 12 months of sublingual immunotherapy (SLIT). Expression levels of HLA-DR, CD80, CD86 and CD83 were evaluated by flow cytometry. IL-10 and IL-12 concentrations were determined by ELISA in the supernatants of DCs cultures after 24 hours of stimulation with LPS. Expression levels of mDCs surface markers and cytokine production are shown for each patient. Horizontal bars represent the mean values.

tolerance or in the suppression of immune response to allergens.

Moreover, we observed a contemporary decrease of IL-12 production by DCs at T12. This could be secondary to the increased secretion of IL-10 since it is known that IL-10 can downregulate the immune response and counteracts the activity of IL-12.[28] Although the amounts of IL-10 produced by mDCs were quite variable among patients, here we provide the first evidence, to our knowledge, of an increased production of IL-10 by DCs during SLIT. This finding is supported by previous studies demonstrating that SLIT increases IL-10 production in PBMCs of allergic patients.[29-32] In particular, in the study by Ciprandi et al,[29] IL-10 production was induced by *Dermatophagoides Farinae*, but also by recall antigens and phytohemagglutinin, thereby raising the possibility that some IL-10 secreting cells were not T lymphocytes.

Our findings suggest that the immunological mechanism of SLIT might reside in the induction of IL-10 production by DCs. Some previous reports on cohorts of patients treated with SLIT described that an early increase of IL-10 mRNA positively correlates with an improvement of the clinical score.[33,34] It has been recently shown that IL-10 can induce the differentiation of T cells with antigen-specific regulatory activity, directly or via induction of an IL-10 producing subset of DCs.[34-37] Therefore, it is conceivable that an increase of IL-10 production by the DCs during the first months of SLIT might drive an expansion of the T regulatory cell population, which would be responsible

for the clinical improvement usually observed after the first year of immunotherapy. This interpretation is supported by the recent work of Koya et al,[38] who demonstrated that DCs treated with IL-10 produced, in turns, considerable amounts of IL-10, and that the adoptive transfer of IL-10-treated DCs leads to an increase of $CD4^+IL\text{-}10^+T$ cells in the lung of sensitized recipient mice. Interestingly, the negative regulatory effects exhibited by the IL-10–treated DCs were dependent on endogenous production of IL-10 because DCs from IL-10–deficient mice, despite differentiation in the presence of IL-10, failed to exhibit the negative regulatory effects in any of these assays.[38]

In summary, our results are consistent with the hypothesis that the induction of tolerance by SLIT involves a modification of DCs functions, although further studies on a larger group of patients might help to elucidate if IL-10 or CD86 is a reliable biomarker of the success of immune therapy. In the light of the recent research it appears that DCs have to manage two opposite tasks: on one hand they promote pro-inflammatory reactions and actively induce T-cell responses, on the other hand they have an important function as 'silencers' within the immune system by sending out anti-inflammatory, tolerance inducing signals. The last unique capacity of DCs has opened several exciting possibilities to exploit DCs for recovering tolerance to allergens and protecting from allergic reactions. The importance of therapies specifically targeting the immune system as the only disease-modifying treatment for allergic diseases is evident. In particular there is a great need for therapies aimed to prevent or to block the initial development of the allergic inflammation. The work discussed herein might help to define the role of DCs in the pathogenesis of allergy and to implement therapies attempting to intervene in the early phases of allergic inflammation.

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Competing interest: The authors declare no conflicts of interest.

Contributors: Angelini F had primary responsibility for protocol development, performed the final data analysis and the writing of the manuscript. Pacciani V performed most of the experiments. Di Cesare S performed part of the flow cytometry experiments. Corrente S, Silenzi R, Di Pede A, Polito A, Riccardi C, and Yammine ML performed patient screening, enrollment and outcome assessment. Rossi P supervised laboratory activities. Moschese V participated in the development of the protocol and patient screening. Chini L had primary responsibility for protocol development, supervised the design and execution of the study and contributed to the writing of the manuscript.

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