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TITLE: Immunological changes induced in peach allergy patients with systemic reactions by Pru p 3 sublingual immunotherapy.

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Abbreviations used:

APCs: Antigen-presenting cells

CFSE: 5,6-carboxyfluorescein diacetate N-succinimidyl ester

DBPCFC: double-blind, placebo-controlled food challenge

DP: Dermatophagoides pteronyssinus

FA: Food allergy

IT: Immunotherapy

moDCs: Monocyte-derived dendritic cells

MTD: maximum tolerated dose

nsLTP: Non-specific lipid transfer protein

PC: Plasma cell

PD-L1: Programmed cell death ligand 1

SCIT: Subcutaneous immunotherapy

SIT: Specific immunotherapy

SLIT: Sublingual immunotherapy

SPT: Skin prick test

Treg: Regulatory T cell

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Keywords: Dendritic cells, food allergy, nsLTP, specific sublingual immunotherapy, T cells, food tolerance.

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Abstract

Scope: Sublingual immunotherapy using peach extract enriched in Pru p 3 (Pru p 3-enriched-SLIT) brings a new perspective to treating patients with allergy to lipid transfer proteins. We evaluated the immunological changes induced by Pru p 3-enriched-SLIT during one year.

Methods and Results: Three groups were included: peach allergic patients who received Pru p 3-enriched-SLIT, peach allergic untreated patients and controls. Peripheral blood mononuclear cells were obtained before treatment and different time-points. Monocyte-derived dendritic cells (moDCs) maturation and lymphocyte proliferation were assessed by flow cytometry.

Data showed a significant reduction of moDCs maturation status during one year of treatment and an increase in PD-L1. Moreover, we observed a significant decrease of the Pru p 3-specific proliferation of effector cells and an increase in Treg cells with higher PD-L1 expression and IL-10 production. These were observed in patients treated only.

Conclusion: Successful Pru p 3-enriched-SLIT is linked to an important immunosuppression of allergen-specific effector T cells maybe the increase of allergen-specific Treg cells. These cellular changes are orchestrated by the activity of moDCs promoting the expression PD-L1 that will participate in the regulatory response. These

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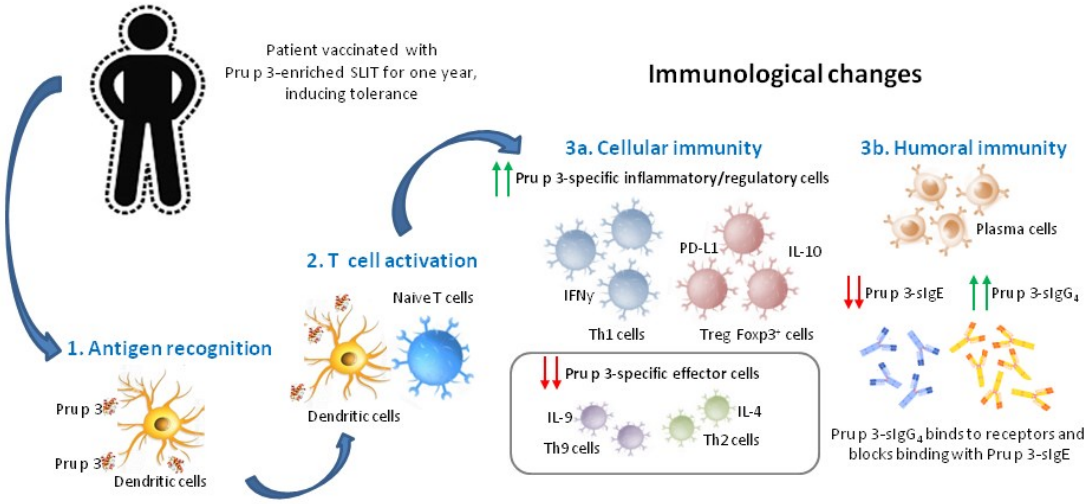
changes may serve as biomarkers during SLIT alongside other features such as IgE/IgG_4 ratio.

GRAPHICAL ABSTRACT



Peach is an important cause of plant allergy in Mediterranean areas. The major peach allergen is Pru p 3, a non-specific lipid transfer protein associated with the complex clinical entity known as LTP-syndrome. In this work, we show that successful SLIT with Pru p 3 is linked to the modulation of Pru p 3-specific effector cells, including Pru p 3-specific Treg cells. These cellular changes are orchestrated by the activity of dendritic cells, and are associated with reduced numbers of IgE secreting cells and increased numbers of IgG_4 secreting cells during successful treatment. The results of this study will help develop promising early biomarkers for assessing the efficacy of allergen specific SLIT on a personalized basis that could help monitor patients during treatment and guide decisions of whether a patient should continue this costly procedure or not.

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INTRODUCTION

Food allergy (FA) is an adverse immune response induced after the ingestion of certain foods that can lead to life-threatening reactions such as anaphylaxis [1]. Incidence has increased over the last 20 years, currently affecting around 8% of children and 2–3% of adults [2] . FA occurs as a consequence of the loss of tolerance to food proteins. At cellular level this intolerance is characterised by an excessive Th2 response associated with IgE-mediated [3],[4]. Persistent FA can be caused by plant-food allergens, with peach being the most prevalent in Mediterranean areas [5],[6]. The major peach allergen is Pru p 3, a non-specific lipid transfer protein (nsLTP) [7]. nsLTPs are panallergens highly distributed among all plant-food species, being therefore associated with sensitization to multiple, non-taxonomically related plant-derived foods, inducing a complex clinical manifestation defined as LTP-syndrome [8]. This is considered a serious health problem and is increasing in prevalence [9]. Current treatment is based on the avoidance of peach, however this does not rule out the possibility of accidental ingestion [10]. There is a clear need to develop safe and long-term effective therapies for peach allergy [11],[12].

Allergen specific immunotherapy (SIT) represents a form of treatment in which controlled quantities of an allergen are given to a patient in order to induce tolerance. Sublingual immunotherapy (SLIT) studies have shown that the use of an enriched Pru p 3 extract (Pru p 3-enriched-SLIT) produces clinically beneficial effects for mild, moderate and even severe reactions [13-15]. In addition, SLIT with peach has been shown to induce clinical and humoral changes relevant to severe reaction induction. These changes include reduced sIgE and increased $sIgG_4$ levels [15]. However, cellular changes during the early response to SLIT are poorly understood. Studies with subcutaneous immunotherapy (SCIT) to *Dermatophagoides pteronyssinus* have shown immunological changes associated with

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clinical improvement, including decreased levels of effector cells and inflammatory plasma cells, and increased regulatory cells [16],[17]. Similar results have been found for SLIT and SCIT, although some immunological changes differ, such as the modulation of the allergic T cell response [18]. Regarding peach allergy, there is a lack of information concerning early biomarkers associated with Pru p 3-enriched-SLIT. It has been shown that Pru p 3 can induce dendritic cell (DC) maturation in peach allergic patients and induce specific T-lymphocyte proliferation and Th2-associated proinflammatory cytokine release [19]. Further, plasmacytoid and myeloid DCs exhibit phenotypic changes during SLIT that enable to regulate the response to allergens through an altered Th2 response [20].

The programmed cell death ligand 1 (PD-L1) has been shown to promote functional Treg cells by enhancing Foxp3 expression [21],[22]. Interestingly, modulation of PD-L1 is also associated with SLIT efficacy [23]. However, there is no data about the role of PD-L1 in the cellular immune response to SLIT in FA.

To dissect the early immunological events during Pru p 3-enriched-SLIT, their relationship with the clinical response, and to identify early potential biomarkers, we analysed phenotypic changes in DCs, effector and inflammatory/regulatory T cells, B and NK cells at various time points during treatment. We also evaluated changes to the humoral response by quantifying Pru p 3-specific-IgE and IgG_4 antibodies and their productive cells.

MATERIALS AND METHODS

Twenty-six patients with a clinical history of systemic allergic reactions to peach and positive skin prick test (SPT) and sIgE to Pru p 3 were included. Patients were classified as having suffered a systemic reaction if they presented at least generalized urticaria with or without angioedema. Reaction severity was classified as mild, moderate or severe according to Brown's

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anaphylaxis classification. Fifteen patients were treated with a Pru p 3-enriched-SLIT (50 μg/mL) (ALK-Abelló, Madrid, Spain) using a conventional schedule [15]; 11 individuals did not receive treatment and were referred to as non-treated. Ten non-allergic, peach tolerant individuals were also included, who did not receive treatment. Inclusion criteria consisted of: i) a clear clinical history of peach allergy and its severity, classified according to Brown *et al*. [24] as: mild (skin and subcutaneous tissues only), moderate (features suggesting respiratory, cardiovascular or gastrointestinal involvement) and severe (hypoxia, hypotension or neurological compromise), more details on clinical symptoms in Table 1; ii) a positive skin prick test (SPT), specific IgE (sIgE) to LTP (Pru p 3) and a positive double-blind placebo-controlled food challenge (DBPCFC) to peach. This was performed soon after the pollen season ended (September), and treatment was started within one month of the DBPCFC.

All subjects were prospectively evaluated at several points time: before starting treatment (T0), and 1 (T1), 6 (T6) and 12 months (T12) after the treated patients started receiving SLIT. The study was conducted according to the declaration of Helsinki and approved by the Ethics Committee of the Malaga Regional University Hospital. All the subjects signed informed consent forms before carrying out the study.

Skin tests

SPT were performed according to European guidelines [25] using commercialized extracts from grass pollen, olive, plane tree pollen, mugwort pollen, cypress pollen and nsLTP (Pru p 3-enriched) (all from ALK-Abelló). The response was considered positive if the wheal diameter was 3 mm greater than that induced by a negative saline control.

Double-blind placebo-controlled food challenge

DBPCFC was performed at T0 and T12, outside of the pollen season, as described previously [15] and following European guidelines [26].

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Sample collection and storing

Peripheral blood was obtained from all subjects at different time intervals by venopunction. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque density gradient (Rafer S.L, Zaragoza, Spain) and stored in liquid nitrogen in RPMI 1640 medium supplemented with 2mM of L-glutamine (BioWhittaker, Pittsburgh, PA), gentamicin (5mg/mL) and streptomycin (50ng/mL) (both from Normon, Madrid, Spain), 40% of foetal bovine serum (BioWhittaker) and DMSO (10%) (Sigma, St. Louis, MO). Samples were biobanked according to standard procedures of the Málaga Regional University Hospital-IBIMA Biobank, Andalusian Public Health System Biobank, for future studies. All the sequential samples taken from a patient were assayed at the same time.

Pru p 3-specific IgE and IgG_4 determinations

Serum specific-Ig-antibodies levels to Pru p 3 were determined by ImmunoCAP (Thermofisher, Uppsala, Sweden) following the manufacturer's recommendations. IgE/IgG_4 ratio were determined after conversion of KU/L (IgE) and mgA/L (IgG_4) to µg/L as described [27],[28].

ELISpot assay

The number of IgE and IgG_4-secreting cells after Pru p 3 stimulation was measured by ELISpot assay [29],[30]. ELISpot Multiscreen HTS plates (96 wells) (Millipore, Darmstadt, Germany) were coated overnight at 4°C with Pru p 3 (5µg/mL). Plates were blocked for 1 hour at 37ºC with a blocking solution (Sigma-Aldrich, St. Louis, MO); a total of $1x10^5$ cells were seeded and incubated for 48 hours. After washing, 50µl of alkaline phosphatase labelled-αIgE and -αIgG_4 (both from Southern Biotech) at 1:1000 and 1:2000 respectively,

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were added to each well and incubated overnight at 4°C. After washing, 100µl of ready to use solution of previously filtered NBT-BCIP (Sigma-Aldrich) was added. The reaction was allowed to proceed until spots were clearly evident on the positive-control sample. The reagent and plate backing material were removed and reactions were quenched with distilled water. The number of Ig secreting cells was determined using an ELISpot Bioreader(R) 6000 (BioSys, Karben, Germany).

Generation of monocyte-derived DCs (moDCs)

Monocytes were purified from PBMCs by positive selection using CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The $CD14^+$ fraction was differentiated to become DCs using 200ng/mL of recombinant human granulocyte-macrophage colony-stimulating factor and 100ng/mL of recombinant human IL-4 (both from R&D Systems Inc, Minneapolis, MN) for 5 days at 5% CO_2 and 37Cº [31]. The $CD14^-$ fraction was frozen in 10% DMSO for performing the lymphocyte proliferation test.

DC maturation studies

To assess changes in the DC maturation state after Pru p 3 stimulation during SLIT, 10^5 DCs /mL obtained at different times were incubated in 96-well plates (Thermo-Fisher AS, Roskilde, Denmark) with 25 µg/ml Pru p 3 [19], 1mg/mL lipopolysaccharide (Sigma) as positive control, or complete medium as negative control for 48 hours at 37ºC in 5% CO_2. DCs were harvested and their maturation status assessed by analysing CCR7, CD40, CD80, CD83, CD86 co-stimulatory molecules and PD-L1 expression (BD Pharmigen and Biolegend, San Diego, CA) in a FACSCanto II Cytometer (BD Biosciences, Milpitas, CA). Data were analysed using FlowJo® software (Tree Star, Inc., Ashland, OR). Results are

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expressed as maturation index (MI) calculated as the ratio between stimulated DC and non-stimulated DC and considered positive when greater than 2 [19].

Analysis of cell subpopulation frequency

The frequency of different lymphocyte subpopulations during one year was analyzed by flow cytometry. All monoclonal antibodies (MoAbs) used for lymphocytes obtained from PBMCs at the different time points were from BD Pharmigen, except CRTH2, CD19, CD138 and IL-10 from Biolegend fixed with BD Cytofix/CytoPerm Fixation/Permeabilization solution kit (BD Bioscience). Cell subpopulation phenotypes were defined as: $CD3^+CD4^+CXCR3^+IFN\gamma^+$ (Th1); $CD3^+CD4^+CRTH2^+CCR4^+$ (Th2); $CD3^+CD4^+CRTH2^-IL\text{-}9^+$ (Th9); NK cells including the subtypes, $CD16^+CD56^+IFN\gamma_{low}Perforin^{high}$ (cytotoxic NK^{Dim}) and $CD16^+CD56^+IFN\gamma^{high}Perforin_{low}$ (inflammatory NK^{Bright}); $CD19^+CD20^-CD138^+CXCR3^+IgE^+$ as IgE producing Plasma cells (IgE^+PC) and two subtypes of regulatory cells, $CD4^+CD127_{low}CD25^{high}FOXP3^+$ (Treg) and $CD4^+CD25^+FOXP3^+IL\text{-}10^+$ as IL-10 producing Treg cells ($IL\text{-}10^+$Treg). For cell staining of the latter two populations, human Foxp3 buffer was used (BD™ PharMingen). Results are expressed as percentages.

Pru p 3-specific proliferative response of different cell subpopulations

The specific proliferation of different lymphocyte subpopulations was evaluated at different time points using as antigen presenting cells (APCs) autologous DCs pre-stimulated with Pru p 3 (25 μg/mL) for 48h [19]. Proliferation was determined using a 5,6-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE) dilution assay. A total of $1.5x10^6$/mL pre-labelled PBMCs were cultured with Pru p 3-pre-stimulated-DCs (10:1 ratio) at a final volume of 250 μL of complete medium, in 96-well plates for 6 days at 37ºC and 5% CO_2. Ten μg/mL phytohemaglutinin (Sigma) was used as positive proliferative control and culture with

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unstimulated DCs as negative proliferative control. The proliferative responses were assessed by flow cytometry, analyzing $CFSE_{low}$ expression in the different cell subsets. Results were expressed as proliferation index (PI), calculated for each subset as (%$CFSE_{low}$ stimulated lymphocytes+DC- (%$CFSE_{low}$ unstimulated lymphocytes+DC)/ %$CFSE_{low}$ lymphocytes and considered positive when was greater than 2 [19]. In addition, we measured the mean florescence intensity (MFI) and the percentage of PD-L1 in Pru p 3-specific-proliferative regulatory cells at the different time points by flow cytometry.

Statistical analysis

All variables were analysed using Kruskal-Wallis test when comparing the three groups of subjects (Tolerant, Non-treated and Treated) being significant differences between the groups when a p-value lower than 0.05 was obtained. In the case that significant differences were detected, this was followed by the Mann-Whitney U test between pairs of samples, resulting in three post-hoc tests. Bonferroni correction was used to reduce the threshold for significance to 0.0167.

For comparison of related samples, (T1, T6 and T12) to the starting point (T0), the Friedman test was used, using a p-value threshold of 0.05. In the case that significant differences were detected, this was followed by the Wilcoxon signed-rank test between the different pairs of time points (T0vsT1, T0vsT6 and T0vsT12). Again, Bonferroni correction was used to reduce the threshold for significance to 0.0167**.** All reported *p*-values represent 2-tailed tests.

RESULTS

We evaluated a total of 26 peach allergic patients with systemic symptoms, either mild/moderate symptoms, of whom 15 were treated with Pru p 3-enriched-SLIT and 11 non-treated. Clinical and demographic data for the groups are shown in Table 1, and are in line

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with those from a larger study from our group [15]. We also included a group of peach-tolerant subjects (n=10). The majority of patients were female but no statistically significant differences were found between the two groups (66.7% for treated and 63.6% for non-treated). There was no significant difference in age (mean age 29.6 years for treated and 29.5 years for non-treated) (Table 1).

The time interval between the last reported reaction in clinical history and the study ranges from 8.1 to 9.17 years. All patients presented mild/moderate symptoms after peach intake, and peach allergy was confirmed with challenge at T0. There were no significant differences in wheal size after SPT to nsLTP, Pru p 3-sIgE levels or the maximum tolerated dose (MTD) of peach needed to induce a reaction via challenge between the groups of peach allergic patients at T0.

At T12, there was a significant decrease in SPT wheal size compared to T0 in the treated group, with no changes for non-treated patients (Figure 1A). A significant increase in MTD was observed for the treated group, in fact all treated patients could tolerate the maximum dosage of 150 g at T12, increasing from a median of 50 g (IR:30-60) at T0, in a DBPCFC. We did not find any difference in the amount of peach tolerated for non-treated patients, with a median value of 43 g (IR:15-60) at T0 and 49 g (IR:30-60) at T12 (Figure 1B).

Pru p 3-enriched-SLIT affects Pru p 3-sIgE and -sIgG$_4$ levels in sera and secreting cell numbers

At T0, Pru p 3-sIgE and sIgG$_4$ levels in peach allergic treated and non-treated patients were higher than tolerant controls although with no significant differences.

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Determinations of Pru p 3-sIgE levels over the study period showed no significant differences for any group, although a small, non-significant decrease was noted for treated patients. Conversely, a non-significant increase of Pru p 3-sIgG_4 levels was observed in treated patients only (Figure 2A). There was a significant decrease in Pru p 3-sIgE/sIgG_4 ratio at T6 and T12 compared to T0 in treated patients only (Figure 2A).

We determined the number of sIg-antibody secreting cells after stimulation with Pru p 3 by ELISpot assay. Differences were found in treated patients only, with a significant decrease of Pru p 3-sIgE secreting cells at T12 of Pru p 3-enriched-SLIT, achieving levels similar to those of tolerant controls. For Pru p 3-sIgG_4 secreting cells, we found a significant increase from T1 to T12 (Figure 2B). No differences in the number of Pru p 3-sIgE or -sIgG_4 secreting cells were found between time points for either non-treated or tolerant groups. Pru p 3-sIgE/sIgG_4 ratio secreting cells significantly decreased in treated patients only, from T1 onwards, compared to T0 (Figure 2B).

Pru p 3-enriched-SLIT induces changes in DC maturation

We evaluated whether one year of Pru p 3-enriched-SLIT induced changes in DC subpopulation levels after stimulation with Pru p 3. We first analysed the expression of all markers at T0 in all groups and found that there was a significantly higher frequency of cells expressing activation markers (CCR7, CD40 and CD83), maturation markers (CD80 and CD86) and PD-L1 in peach allergic treated and non-treated patients compared to tolerant subjects (Figure 3A).

Changes in the expression were observed for all markers, for treated patients only. These changes were maintained throughout the study, and by T12 these levels were similar to those of the tolerant control group for most markers. Regarding PD-L1, we observed a significant increase at T12 compared to T0 in treated patients only (Figure 3A). Markers CCR7, CD40,

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CD83,and CD86 showed changes after only one month of treatment. No changes were found for any DC markers in non-treated patients or tolerant controls.

PD-L1/CD80 and PD-L1/CD86 ratios in DCs were similar at T0 in all groups, however there was a significant increase at T12 for both PD-L1/CD80 and PD-L1/CD86 ratios compared to T0, in treated patients only (Figure 3B).

Pru p 3-enriched-SLIT induces changes in the frequency of different cell subsets

The frequency of different cell subpopulations of effector cells (Th2, Th9 and $IgE^{+}PC$) and inflammatory/regulatory cells (Th1, Treg and IL-10^{+}Treg cells) as well as NK cells (inflammatory NK^{Bright} and cytotoxic NK^{Dim}), were evaluated. At T0 there was a significantly higher frequency of all effector cells except for NK cells (Figure 4), however these changes were not significant after multiple testing corrections. Analysis of cell subpopulation frequency at different time points showed a progressive reduction of effector cells in treated patients, being significant from T1 onwards for Th2 and Th9 cells, and from T6 for $IgE^{+}PC$ cells (Figure 4A). Conversely, for Th1 cells we observed a significant increase from T1 that was maintained until T12 (Figure 4B) and for regulatory cells we observed a significant increase at T12. For IL-10^{+}Treg cells, we observe an increase during one year of treatment, although this was not significant. No changes were observed in non-treated patients and tolerant controls for any of the subpopulations.

Finally, changes in the frequencies of NK cells were analyzed observing an increase of NK^{Bright} and a decrease of NK^{Dim} with changes at T12 in treated patients only, although not statistically significant (Figure 4C).

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Pru p 3-enriched-SLIT induces changes in the Pru p 3-specific proliferation of different cell subpopulations

In order to investigate whether the phenotypic changes in cell subpopulation described above were specific to Pru p 3, we studied the proliferative response of the different cell subpopulations to this nsLTP using flow cytometry with CFSE dilution in co-cultures with Pru p 3-pre-stimulated-DCs. We analysed the Pru p 3-specific-proliferative response in the different groups at T0 and observed significantly higher proliferation for effector cells in peach allergic patients (treated and non-treated) compared to tolerant controls (Figure 5).

We analysed the proliferative response at later time points and observed a significant decrease in the Pru p 3-specific proliferative response of Th2, Th9 and $IgE^{+}PC$ in treated patients only; this was found to be significant at T6 and T12 compared to T0 (Figure 5A-5C). The opposite trend was found for Th1, Treg, IL-10^{+}Treg cells with a significant increase at T12 and for NK^{Bright} cells at T6 (Figure 5B-5C). No changes were observed for the non-treated and tolerant groups.

Pru p 3-enriched-SLIT modulates PD-L1 expression in Treg cells

PD-L1 is expressed on various cell types including Treg cells. We measured the MFI and the percentage of PD-L1 in Pru p 3-specific-proliferative Treg and IL-10^{+}Treg cells. The MFI data at T0 showed similar values for peach allergic patients (treated and non-treated) and tolerant controls, with a significant increase over time in treated patients only for Treg and IL-10^{+}Treg cells (Figure 6B). The percentage of PD-L1 in proliferative Treg or IL-10^{+}Treg cells was significantly lower for peach allergic patients compared to non-allergic controls at T0. Only in treated patients was there a significant increase in the percentage of PD-L1 Treg cells from T1 (Figure 6B).

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DISCUSSION

SIT is widely accepted as being the only available therapy that modifies the natural course of allergic diseases. It has been shown to be safe and effective for food allergens [13],[15],[32],[33]. The main aim of SIT is to achieve tolerance through the modulation of cellular subtypes, such as long-lived B or IgE^{+}PCs, resulting in lower sIgE levels [16, 34]. We analysed the immune response to Pru p 3 in peripheral blood samples from patients receiving Pru p 3-enriched-SLIT for one year. We found that patients receiving SLIT showed significant clinical improvement, demonstrated by a decrease in SPT wheal area and an increase in the amount of peach tolerated. This improvement was related to the modulation of cellular function in terms of the Th1/Treg response to Pru p 3.

DCs are crucial for the modulation of the immune response towards a tolerant response and may represent a potential therapeutic target for SIT [35],[36]. Therefore, we analyzed changes in Pru p 3 induced DC maturation due to Pru p 3-enriched-SLIT. Previously, our group confirmed that Pru p 3 induced DC maturation in peach allergic patients only [19]. All DC markers showed lower expression in treated patients; in fact, levels of the treated patients resembled those of the tolerant controls at T12. The reduction of these markers is likely to impair DC migration to lymph nodes and reduce their capacity to present the allergen to T cells, as has been shown previously [37]. Taken together, our data suggest that changes induced by SLIT to the different DC markers can contribute to the regulation of T cell functions, as observed for other food allergens [38], [39].

At the T cell level, evaluation indicated a shift from a Th2 to Th1/Treg response in treated patients. Moreover, we confirmed that these changes were allergen-specific by measuring cell proliferation after Pru p 3 presentations by autologous DCs. We found a decrease of the proliferative response of effector T cells, and an increase of inflammatory/regulatory cells early in the treatment. These results corroborate those obtained in other studies with inhaled

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allergens [16], [40]. The immediate response to therapy was associated with the induction of Treg cells, with increased IL-10 production in treated patients compared to non-treated and tolerant groups. In agreement with other studies, we found an increase of Th1 and Treg cells at beginning of SIT [34],[41]. Other studies have asserted the importance of IL-10 and Treg cells in allergen-SIT [42]. Here we found that all treated patients showed increased IL-10 levels after 6 months, indicating that SLIT may induce tolerance via IL-10 production [43].

Regarding PD-L1, it is a costimulatory molecule expressed by various immune cells that is thought to play a key role in immune evasion of tumour cells [44]. Recently, it has been associated with the production of IL-10 and IFNγ and the induction of functional $Foxp3^{+}$Treg cells [45]. In our study, although no differences were initially found between peach allergic patients and tolerant controls, PD-L1 expression in DCs eventually increased during treatment by T12, in line with other studies [23]. Moreover, we have observed an increase in PD-L1/CD80 and PD-L1/CD86 ratios for DCs, which has been shown previously as necessary for the induction and maintenance of Treg cells [46],[47]. These ratios were significantly increased at T6 and T12 in treated patients. These indicate that Pru p 3-enriched-SLIT is able to regulate PD-L1 expression in DCs.

Furthermore, other studies have suggested that higher PD-L1 levels could deliver signals to T cells to promote regulatory responses, producing IL-10 [45]. In addition, PD-L1 is also expressed on activated Treg cells [48]. The present study revealed that PD-L1 expression on Treg subsets cells was up-regulated by Pru p 3-enriched-SLIT. It is known that Tregs expressing PD-L1 are associated with IL-10 production, which can trigger inhibitory signals for effector T cells, mediating the tolerance response [22],[49].

Finally, we analysed the sIgE/$sIgG_4$ ratio, which is considered a biomarker for SIT [50]. We found a decrease of Pru p 3-sIgE/$sIgG_4$ serum ratio over time in treated patients only. This was accompanied by a decreased ratio of Pru p 3-sIgE/-$sIgG_4$ secreting cells during early

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treatment stages [34]. As reported by other studies, increased $sIgG_4$ levels are associated with the effect of SLIT via blocking the effector activity of IgE on mast cell/basophil activation [28].

In conclusion, our results show that successful SLIT with Pru p 3 is linked to the modulation of allergen specific effector cells, probably due to the increase of IL-10 producing allergen-specific Treg cells. These cellular changes are orchestrated by the activity of DCs that underwent changes during Pru p 3-enriched-SLIT, leading to decreased migration and effector activation. Moreover, increased expression of PD-L1 appears to have a role in the regulatory response. These changes could be responsible for reducing the immune response to the allergen. This occurred in parallel with reduction of IgE secreting cells and an increase of IgG_4 secreting cells during treatment. It would be interesting to see whether these changes are maintained for a longer period of time, particularly after treatment has finished. It would also be interesting to repeat the study on a larger population, and for SIT with other allergens. We suggest that the role of PD-L1 in the cellular response in combination with changes in the secretion of sIgE and $sIgG_4$ could putatively be used to help develop a potential Pru p 3-enriched-SLIT signature. The results shown here may thus help develop promising early biomarkers for allergen specific SLIT that could help monitor patients during treatment and guide decisions of whether or not a patient should continue, potentially saving a time and money for the patient and the health system.

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Author contributions

TMJ and MC designed the study and wrote the manuscript. PF, GF and PJR performed and analyzed the experiments including statistical analyses. GF, BG, CP and AP recruited the study participant and provided patients' samples and clinical dates. D-PA provided reagents. PF, PJR, RMJ and BD edited the manuscript. TMJ and MC jointly supervised the study. All authors approved the final version of the article.

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FIGURES LEGENDS

Figure 1. Clinical changes due to Pru p 3-enriched-SLIT. Comparative analysis for treated (n=15) and non-treated patients (n=11) at T0 and T12. A) Changes in wheal diameter after SPT with peach at T0 and T12. B) Changes in peach threshold after DBPCFC at T0 and T12. The statistical test used was the Wilcoxon test for related samples.

Figure 1

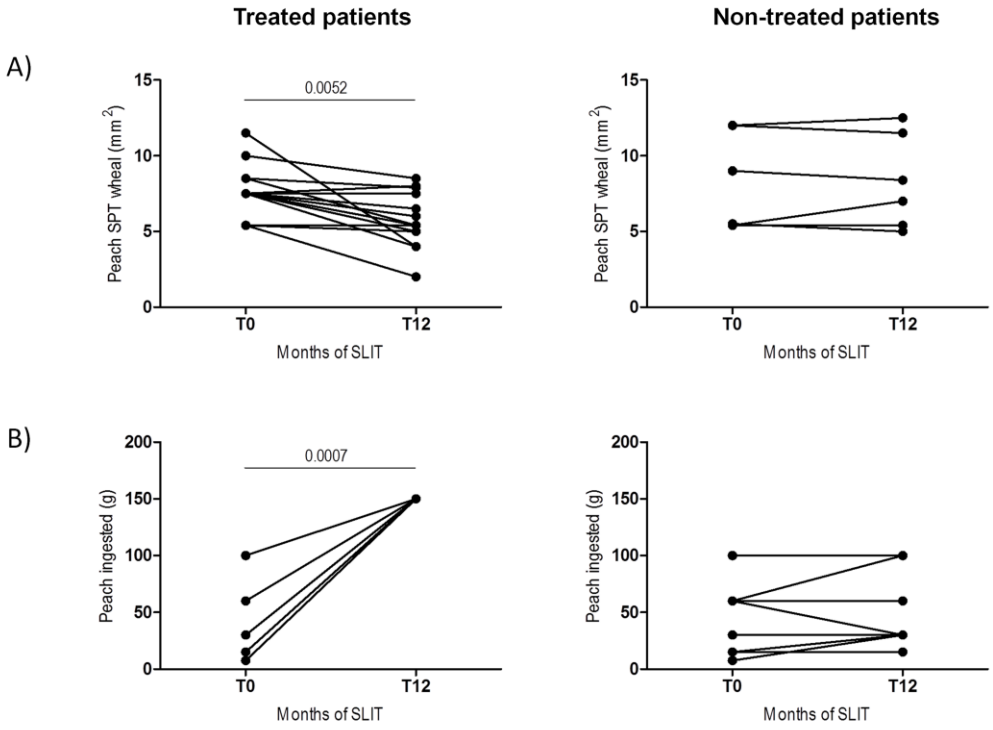

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Figure 2. Pru p 3-specific immunoglobulin determination. A) Serum Pru p 3-sIgE (Ku/L) and $sIgG_4$ (mgA/L) levels, and Pru p 3-sIgE/$sIgG_4$ (µg/L) ratio; B) Number of Pru p 3-sIg and sIgE/$sIgG_4$ ratio secreting cells throughout T12 of Pru p 3-enriched-SLIT in treated (line in bold) (n=15), non-treated patients (n=11) and tolerant controls (n=10). Results represented as median and error using interquartile range. Significant differences of T1, T6 and T12 compared to T0 (related samples, Wilcoxon test) are shown as p-values (significant when < 0.0167).

Figure 2

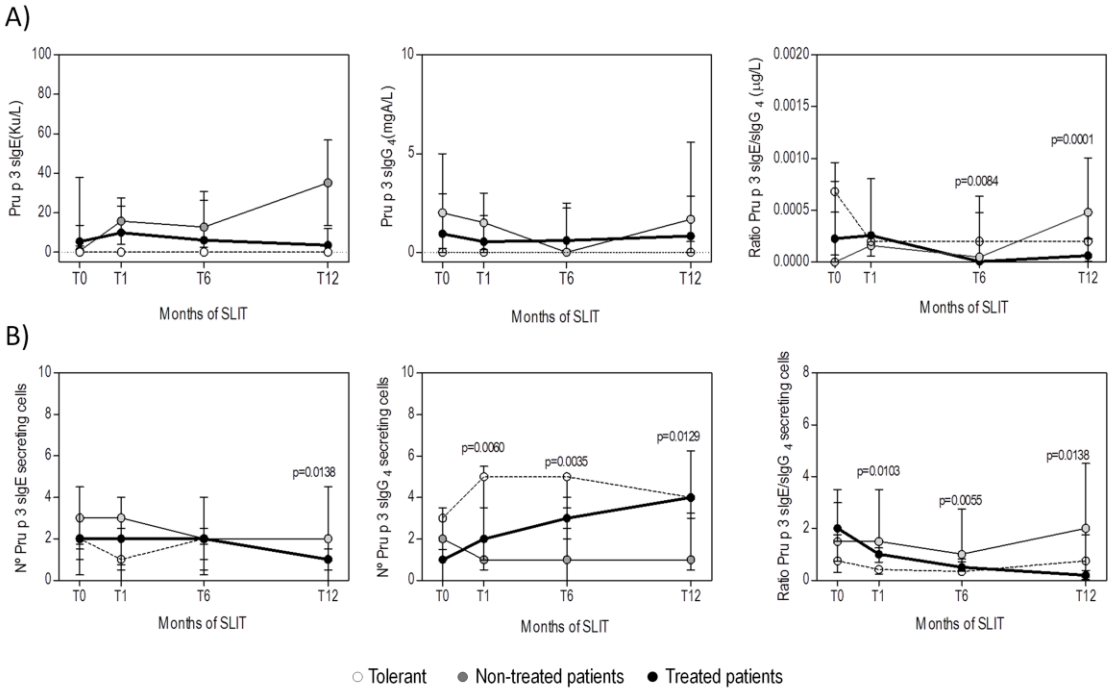

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Figure 3. Pru p 3-enriched-SLIT changes DC marker expression. A) Bars represent mean and SD of maturation indexes of all DC markers after stimulation with Pru p 3 (25µg/ml); B) Results represented as median and error using interquartile range of PD-L1/CD80 and PD-L1/CD86 ratios during Pru p 3-enriched-SLIT. All study groups, treated patients (n=15), non-treated patients (n=11) and tolerant controls (n=10) were compared using Mann-Whitney test for pairwise comparisons between unrelated groups, representing significant differences as * ($p < 0.0167$) and Wilcoxon test for related samples, significant differences are represented p values and considered as significant when ($p < 0.0167$, Bonferroni correction).

Figure 3

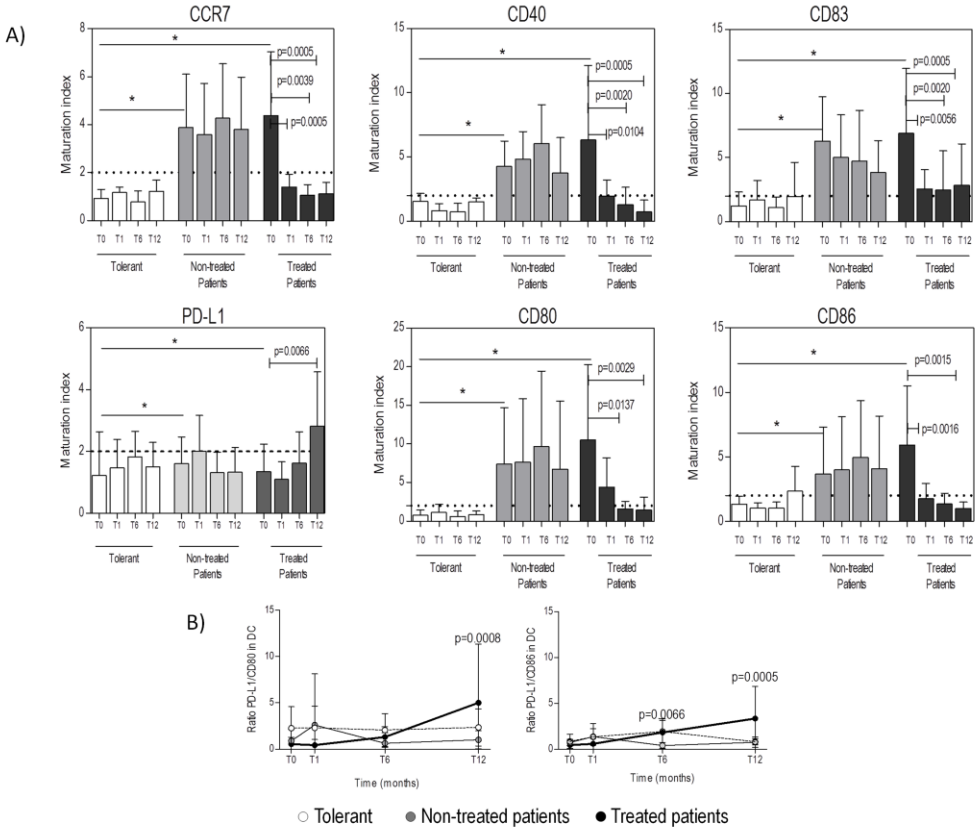

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Figure 4. Changes in T cell populations due to Pru p 3-enriched-SLIT. Percentages of A) effector cells; B) Th1 and Treg cells C) NK cells, during the treatment in peach allergic (treated, n=15 and non-treated, n=11) patients and tolerant controls (n=10). Box-plots represent mean and interquartile ranges. Mann-Whitney test was used for pairwise comparisons between unrelated groups, representing significant differences as * ($p < 0.0167$) and Wilcoxon test for related samples, significant differences are represented as p values and considered as significant when ($p < 0.0167$, Bonferroni correction).

Figure 4

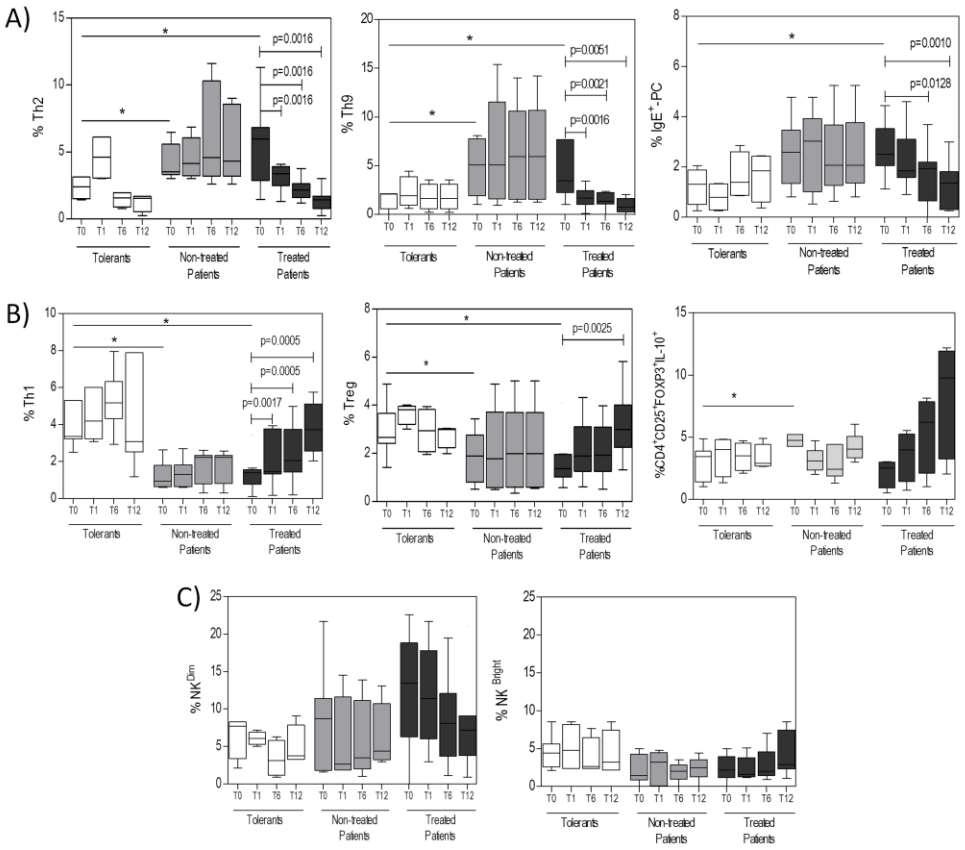

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Figure 5. Pru p 3 specific proliferation assay in lymphocyte subpopulations during Pru p 3-enriched-SLIT. Bars represent means and SD of Proliferation indexes of A) effector cells; B) Th1, Treg and Il-10^{+}Treg cells; C) NK cells, during treatment for the different groups: treated patients (n=15), non-treated patients (n=11) and tolerant controls (n=10). Mann-Whitney test was used for pairwise comparisons between unrelated groups, representing significant differences as * ($p < 0.0167$) and Wilcoxon test for related samples, significant differences are represented as p values and considered as significant when ($p < 0.0167$, Bonferroni correction).

Figure 5

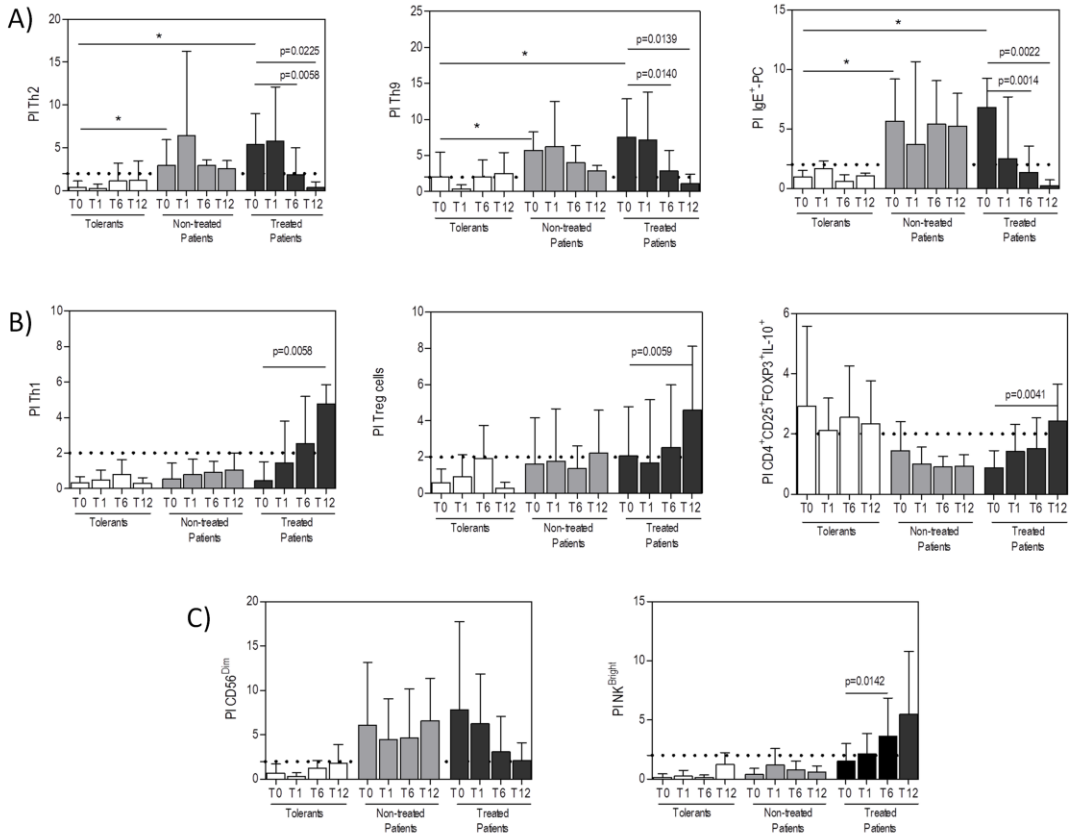

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Figure 6. PD-L1 expression for proliferative Treg subsets.

A) An illustrative example of the flow cytometry gating strategy for PD-L1 expression on proliferative in Treg (top) and IL-10+Treg cells (bottom). Left: Strategy for obtaining proliferative cells based on expression of CFSE. Right: PD-L1 expression at different times points of the treatment. Solid line represents Pru p 3-incubated cells and dashed line cells without stimulus (control). Peak fluorescence was normalised to the mode to allows to visualize the differences in relative fluorescence of cell populations of interest. B) Bars represent means and SD MFI or percentage of PD-L1 in proliferative Treg cells subsets during Pru p 3-enriched-SLIT. Mann-Whitney test was used for pairwise comparisons between unrelated groups, representing significant differences as * ($p < 0.0167$) and Wilcoxon test for related samples, significant differences are represented as p values and considered as significant when ($p < 0.0167$, Bonferroni correction).

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Figure 6

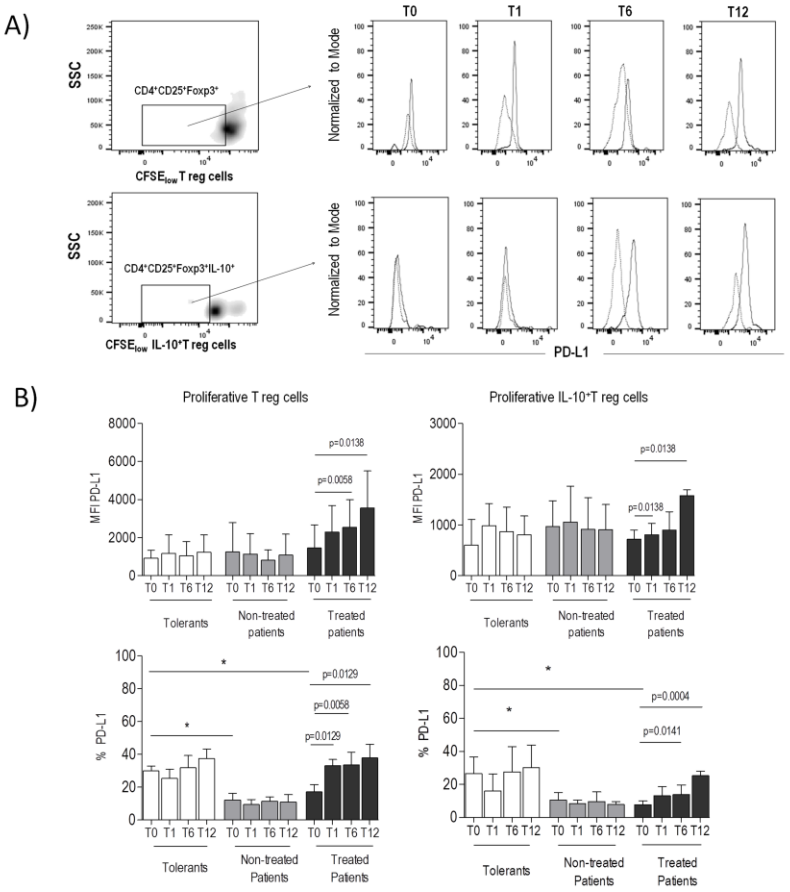

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Table 1. Demographic and clinical data of peach allergic patients

	Treated	**Non-treated**	**P-value**
N (%)	15	11	
Age (Median, IR in years)	29.6 (18-33)	29.5 (20-37.5)	0.93
Gender (N, % of females)	10 (66.7)	7 (63.6)	0.96
Onset peach symptoms (Median, IR in years)	17 (16.6-25)	20.5 (13-28)	0.52
Peach last episode (y)	9.07 (2-14)	8.09 (5.5-15)	0.76
Peach symptoms (N, %)			
• Mild	9 (60)	5 (45.4)	0.39
• Moderate	6 (40)	6 (54.5)	0.55
Pollen sensitization (N, %)	10 (66.6)	8 (72.7)	0.56
• Grass pollen	7 (70)	4 (50)	0.90
• Olive pollen	9 (90)	7 (87.5)	0.89
• Plane tree pollen	5 (50)	3 (37.5)	0.97
• Mugwort pollen	6 (60)	5 (62.5)	0.31
• Cypress pollen	2 (13.3)	2 (25)	0.55
Pollen symptoms (N, %)	5 (33.4)	4 (36.6)	0.72
• Rhinitis	4 (80)	3 (75)	0.59
• Rhinitis and asthma	1 (20)	1 (25)	0.55
Other vegetables allergies* (N,%)	13 (86.7)	9 (81.8)	0.85
Wheal size Pru p 3-SPT (mm,Median,IR)	7.64 (5.6-8)	7.75 (5-9)	0.15
sIgE peach (kUA/L, Median, IR)	14.6 (2.6-19.5)	8.79 (2.5-16.5)	0.56
MTD of peach in DBPCFC at T0 (g, Median, IR)	50 (30-60)	45.32 (15-60)	0.53

IR: Interquartilic range; SPT: Skin prick test; DBPCFC: Double blind placebo controlled food challenge; P-Value represents comparison between groups; MTD: Maximum tolerated dose of peach in DBPCFC at T0

*Other vegetables allergies include fruits, tree-nuts and vegetables.

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