Thymic involvement in specific immunotherapy and respiratory allergy to *Dermatophagoides pteronyssinus*

Envolvimento tímico na imunoterapia específica e na alergia respiratória a Dermatophagoides pteronyssinus

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ABSTRACT

Introduction: T cell receptor excision circles (TREC) on $CD31^+$ T cells are related to recent thymic emigrant cells (RTEs). **Aim:** Evaluation of specific immunotherapy effects on number of TREC in peripheral T cells in patients allergic to *Dermatophagoides pteronyssinus* (Dpt). **Method:** 85 respiratory allergic patients (both genders), 41 (Group II) under maintenance treatment to Dpt SIT (21 sublingual, SLIT, and 20 subcutaneous, SCIT), were selected. The allergic patients (Group I) without specific treatment underwent an allergen challenge test (22 nasal and 22 conjunctival). Peripheral cell analysis was performed immediately before treatment and 60 or 240 minutes after allergenic extract administration. TREC count was performed in $CD4^+CD31^+$ and $CD8^+CD31^+$ cells. The results were expressed per 100,000 RTE-related cells. Samples from 10 healthy individuals (Control – Group III) were obtained using the same method. **Results:** The value of TRECs on RTEs was constant in control groups. Group I patients' TREC count in $CD31^+$-T cells showed relevant individual changes, even in the patients tested earlier (60 minutes), and statistical significant at 240 minutes. Both SCIT

and SLIT also demonstrated enormous individual changes, particularly on TRECs/$CD4^+CD31^+$ cell assay. Basal values in Group III were significantly higher than those observed in active patient groups. **Conclusions:** Thymic functional activity has early involvement in the allergic reaction and SIT. IgE-mediated allergy is able to induce RTEs in the periphery, particularly TRECs/$CD4^+CD31^+$ cells. Both SLIT and SCIT showed reduced RETs in the periphery, probably due to maturation of regulatory T cells. Our results suggest a crucial role of the functional thymic tissue in the central mechanism of this therapy.

Keywords: allergy mechanism, asthma, CD31 cells, Dermatophagoides pteronyssinus, lymphocytes, nasal challenge test, rhinitis, specific immunotherapy, TREC.

RESUMO

Introdução: Os círculos de excisão do receptor da célula T (TRECs) em linfócitos T-$CD31^+$ correlacionam-se com células recentemente emigradas do timo (RTEs). **Objectivo:** Avaliar o efeito da SIT no número de TRECs presentes em linfócitos T periféricos em doentes alérgicos a *Dermatophagoides pteronyssinus* (Dpt), bem como os efeitos decorrentes da resposta alérgica específica. **Metodologia:** Foram estudados 85 doentes com alergia respiratória a Dpt: 41 doentes (Grupo II) em SIT de manutenção (21 por via sublingual; 20 por via subcutânea); 44 doentes (Grupo I), sem terapêutica específica, sendo que 22 deles foram submetidos a prova de provocação específica (nasal=22; conjuntival=22). Todos foram submetidos a estudo de celularidade basal no dia do estudo. Após administração do extracto terapêutico ou da solução alergénica procedeu-se a novo estudo aos 60 ou 240 minutos. Foi quantificado o número de TRECs presentes em células $CD4^+CD31^+$ e $CD8^+CD31^+$ e os resultados expressos por 100 000 células. Amostras de 10 indivíduos saudáveis (Grupo III) foram analisadas nos mesmos tempos. **Resultados:** O valor de TRECs em RTEs foi constante no grupo controlo. Nos doentes do Grupo I a quantificação de TRECs em células $CD4^+CD31^+$ apresentaram diferenças intra-individuais relativamente ao valor basal, estatisticamente significativo aos 240 minutos. Nos doentes do Grupo II observou-se, também, marcada variabilidade intra-individual, independentemente da via de administração. Os valores basais obtidos no Grupo III foram significativamente superiores aos obtidos na determinação basal dos restantes grupos. **Conclusões:** A actividade funcional tímica está precocemente envolvida na resposta alérgica e na SIT. A exposição a alergénios determina, precocemente, a presença de RTEs na periferia, particularmente do fenótipo $CD4^+CD31^+$/TRECs. Nos doentes submetidos a SIT, a redução de RTEs circulantes poderá resultar da necessidade de maturação e diferenciação linfocitária. Estes resultados sugerem um envolvimento do tecido funcional tímico nos mecanismos centrais da alergia e da SIT.

Palavras-chave: Asma, CD31, *Dermatophagoides pteronyssinus*, imunoterapia específica, linfócitos, mecanismos alérgicos, rinite, teste provocação nasal, TREC.

INTRODUCTION

There is consensus as to the systemic character of IgE-mediated allergy and a raft of supporting evidence in the form of studies into experimental and human models[1]. The underlying immune mechanism implies the intervention of multiple immunoinflammatory cells and biological mediators, with the pathological effects of a specific response to an allergen necessarily involving central immune organs[2,3].

Specific subcutaneous immunotherapy (SCIT) or specific sublingual (SLIT) immunotherapy are currently the only forms of possible treatment which can change the natural course of a disease[5-8]. Despite being thoroughly studied, the mechanisms which lead to immunological tolerance still remain to be completely elucidated[9,10]. A study in which treatment allergen extract was administered directly into the lymphatic gland showed efficacy in a reduced treatment time and corroborated the role of the central immune structures in inducing the central immune mechanism[11].

An IgE-mediated reaction determines a very early systemic effect, one which develops simultaneously to the immunoinflammatory mechanism at the site of exposure to the allergen. *In vivo* studies show that in parallel to the inflammatory activity at the site where the allergenic challenge occurs, there is also involvement of the adjacent regional lymphoganglion structures simultaneously with recirculation of circulating cells which infiltrate related central immune systems structures, particularly bone marrow and functional thymic tissue[12]. Focalisation of inflammatory activity in anatomical areas reporting to the central immune system presupposes both the systemic effect of IgE-mediated allergy and the swift and central involvement of the immune system. Equally so, we can observe that specific immunotherapy (SCIT or SLIT) determines early intervention of these structures with possible implications in the immunomodulatory mechanism[13,14]. There is a very early onset of the effect of administering specific allergens, with the systemic effect swifter than the local inflammatory effect. There are no significant differences in the lenght of the response in the central immune organs in terms of the type of extract and route of administration. That said, there is a distinct local inductor mechanism between SLIT and SCIT, although with an identical systemic effect, depending on the phenotypic heterogeneity of the dendritic cells (DCs) in the sublingual mucosa and the subcutaneous tissue.

If the J.A. Denburg *et al*. studies leave no question remaining as to the role of bone marrow in allergic inflammation, the role and persistence of thymic activity throughout life is harder to show directly. Our group was able to show this activity in IgE-mediated allergic response and that which occurs with the administration of specific immunotherapy, independently of being administered via SLIT or SCIT)[12,13] and in the latter, the type of extract (aqueous, depot or polymerized).

It has been traditionally assumed[15] that thymus involutes with age. Several recent studies have shown that thymic function persists throughout life in healthy individuals. In humans the pool of circulating T cells begins developing in the foetal period after the passage and maturation of thymocytes through the thymic microenvironment and the later migration of mature thymocytes to the periphery, lymphatic ganglions and spleen[12].

The thymus is the only lymphoid organ responsible for production of *naïve* T cells with self-tolerance and also for T lymphocytes naturally regulators for self--specific antigens[17]. Despite the different magnitude, the absolute number of *naïve* T cells in children and the elderly is relatively stable, and T-CD4$^+$ immunity is unequivocally maintained in adults although these cells may proliferate in a post-thymic state, maintaining the *naïve* functional phenotype[18]. Two subtypes of these cells are frequently considered: one quiescent, highly representative of cells recently emigrated to the thy-

mus (recent thymic emigrants – RTEs) and a second subtype understood to be *naïve* T-CD4$^+$ which proliferate in the periphery[19].

The surface molecule CD31 (platelet endothelial cell adhesion molecule-1 – PECAM-1) could be of great use in distinguishing *naïve* T-CD4$^+$ thymic cells/CD31$^+$ cells in the peripheral blood of healthy humans[18].

In addition to the CD4$^+$CD31$^+$ phenotype which sustains the RTEs, the T receptor cells excision circles (TRECs) are another interesting marker[18-20]. TRECs are stable DNA episomes formed during the rearrangement of the T receptor in α/β cells located in the cellular cytoplasm[19]. In the constitution of the receptor there is a TREC joint signal generated during the rearrangement process in around 2/3 of Th α/β cells[20]. As TRECs do not replicate during mytosis, this allows evaluation of the RTE cell phenotype[18].

In the healthy individual the variability in TREC count in peripheral cells is greatly reduced over time, supporting the idea that thymic function is maintained in a relatively constant way[20]. CD4$^+$ and CD8$^+$, T-CD45RA$^+$ and T-CD45RO$^+$ cells are detected in RTEs or in circulating mononuclear cells[18]. Thus, parallel analysis of CD31-TRECs is by definition a marker of development tightly related to thymic function, and the concentration in the periphery could be recognised to stimulate immune production and reconstitution.

This study aimed to evaluate the production of RTE cells and the number of lymphocytic TRECs, which might support the role of functional thymic tissue in allergic aggression and in inducing the immune tolerance underlying specific immunotherapy which is fully documented in the literature.

MATERIAL AND METHODS

Patients and healthy population

Patients with respiratory allergy to *Dermatophagoides pteronyssinus* (Dpt), with the diagnosis of moderate persistent bronchial asthma[21], associated moderate--severe persistent rhinitis[22], followed at Allergology and Clinical Immunology appointments at Hospitais da Universidade de Coimbra were selected. All these adult patients, men and women, gave their informed written consent, and the study was approved by the hospital's ethics committee. The study ran between January--March 2009.

No patient presented another pathology beyond allergic disease, namely inflammatory or infectious disease or mental imbalance. No drugs were being taken apart from those required for allergic disease, except oral contraceptive pills by some women. Pregnant women were excluded from the study.

The control group was selected from a population of healthy individuals of potential organ donors with allergic disease excluded.

The following patients groups were studied:

– Group I: 44 allergic patients with no former specific immunotherapy treatment who underwent specific allergen challenge test to Dpt (nasal or conjunctival).
– Group II: 41 allergic patients under maintenance treatment with specific immunotherapy (SCIT or SLIT) for less than one year and in whom it was possible to show clinical efficacy demonstrated by the complete remission of symptoms, lack of preventative anti-allergic medication and/or symptom medication in crises, and the favourable evolution of laboratory parameters, namely reduced cutaneous reactivity to the allergen in skin prick tests and reduced serum concentration of specific IgE compared to the start of treatment. All treatment extracts were from Bial/Aristegui (Bilbao, Spain): SLIT (aqueous 0.97µg/ml of Der p 1 and Der p2, in a 5-drop dose) and SCIT (polymerized, 1.95µg/ml of Der p 1 and Der p2, subcutaneous injection of 0.5cc), administered under strict hospital supervision. The day of administration was that programmed in the maintenance treat-

ment scheme (monthly for SCIT and 3 times a week for SLIT).

– Group III: 10 healthy individuals.

Allergic patients were asked to suspend their systemic antihistamine and/or treatment for 3 days and topical corticosteroids and systemic anti-leukotrienes in the 8 days prior to the study.

Specific challenge tests: nasal and conjunctival

A Dpt (23µg/ml of Der p 1, Bial/Aristegui, Bilbao, Spain) allergen extract was used, diluted to 1/10. This concentration was selected as it was the one guaranteed to induce a minimum skin wheal (prick) 3 mm in diameter and which in former studies had ensured positive response in the nasal and conjunctival challenge test[12]. The procedures were held in the morning and after a 30 minute adaptation to room temperature.

The nasal challenge test was performed with the unilateral application of 2 consecutive sprays (160µl volume) of allergenic aerosolised solution via the nose to the middle nasal turbinate in expiration.

The conjunctival challenge test consisted of the unilateral application of a drop (50µl) of allergenic solution in the external lower quadrant of the ocular conjunctiva.

Nasal and conjunctival symptoms were assessed for 5 minutes after the challenge test using standardised clinical scores which assured a positive response[23,24].

Treatment allergenic extract

All patients were treated with Bial/Aristegui, Bilbao-Spain extracts. In the subcutaneous extracts an extract modified with glutaraldehyde was used, at a 0.50cc volume, and 5 drops of an aqueous extract of Dpt administered sublingually using common techniques[5,8]. In all patients the maintenance dose was administered respecting absolutely the individual scheme in progress.

LABORATORY PROCEDURES

Blood samples

After venipuncture, 30ml of peripheral blood was collected by heparin-lined tube and by PAXgene Blood RNA Tubes (Qiagen®).

All patients and controls had their blood samples collected twice: at T0 prior to the challenge test or administration of specific subcutaneous or sublingual immunotherapy, and again at 60 minutes (T60) or 240 minutes (T240) after diagnostic or treatment procedure.

The healthy Group III subjects had three samples collected on the same day at the same time intervals as the active study group.

Separation of the T lymphocyte populations

The CD4+ and CD8+ populations were isolated from circulating polymorphonuclears by positive immunoselection using the specific magnetic Dynal beads required for the proceedings in question (Dynal, Oslo, Norway).

Cell study to determine the surface cell receptors was performed using direct immunofluorescence in FACSCalibur (Beckton-Dickinson, USA) flow cytometer using fluorochromes bound to the specific monoclonal antibodies for CD3, CD4, CD8, CD31, CD45RA and CD45RO.

TREC PCR COUNT IN REAL TIME

A cell count of TRECs in $CD4^+/CD31^-$, $CD4^+/CD31^+$, $CD8^+/CD31^-$ and $CD8^+/CD31^+$ cells by PCR in real time (PCR-rt) with 5´-nuclease (TaqMan) in an ABI 7900 (Perkin-Elmer, Norwalk, CT, USA) system was made. The cells were classified by use of combination of monoclonal antibodies: CD3-PE (Beckman Coulter), CD4-APC (Beckman Coulter), CD8-PercP Cy5.5 (Becton Dickinson) CD31-FITC (BD Pharmingen, San Diego, USA). DNA was extracted by cellular lysis with 5µl of proteinase K solution (100 µg/ml) for at least 1 hour of incubation at 56.°C followed by incubation at 95.°C

for 15 minutes. PCR-rt in 5µl of cell lysate (±50000 cells) with F-5´-CACATCCCTTTCAACCATGCT and R-5´-GCCAGCTGCAGGGTTTAGG primers and probe 5´-[6FAM]ACACCTCTGGTTTTTGTA AAGGT-GC CCACT[TAM] (Sigma-Aldrich, USA) was performed. The reaction took place at 0.125µl of Taq polymerase, 3.5 µl MgCl2 25 mM, 0.5 µl dNTPs 10 mM, 1 µl of each primer 12.5 uM, 1 µl probe 5 uM, 0.25 µl of reference BD636 (Megabases) for a total of 25µl of water. The conditions require heating at 95.°C for 5 minutes, after at the same temperature for 30 seconds and at 6.°C for a minute, for 40 cycles. A standardised curve was traced using 5ml of standard with 10^3, 10^4 and 10^5 plasmid molecules, and the number of TRECs in the samples was obtained using SDS2.0 Perkin-Elmer, Norwalk, CT, USA) software.

The number of copies of TRECs in each sample was calculated by interpolation of the standard curve and the results given in 100,000 cells. The results were made in duplicate for each sample to minimalise error.

Statistical study

The concentration was expressed in mean and standard deviation for 100,000 cells. The Wilcoxon signed rank test (paired samples) was used to compare the determination at distinct times. The Kruskal-Wallis (one-way analysis of variance) and the Mann Whitney U tests were used to check differences between distinct groups. A significance level of $p<0.05$ was set.

RESULTS

Table I shows the demographic and clinical characteristics of the sample of patients and healthy controls.

All patients who underwent specific allergic challenge testing had clinical scores compatible with positive tests. No adverse local or systemic effects were seen in patients who underwent specific immunotherapy. There were no relevant differences in clinical characteristics of patients studied in the different groups and subgroups.

No significant differences were seen in the total number of $CD4^+$ and $CD8^+$ cells in the patients who underwent specific nasal and conjunctival allergy challenge testing at the two time points in question. The same was true in the group of patients who underwent subcutaneous or sublingual immunotherapy treatment.

No relevant individual difference was seen in the TREC count in 100,000 $CD4^+CD31^+$ or $CD8^+CD31^+$ cells in the

Table I. Characterisation of the sample

	Group I				Group II				Group III
	Nasal		Conjunctival		SCIT		SLIT		
	T0-T60	T0-T240	T0-T60	T0-T240	T0-T60	T0-T240	T0-T60	T0-T240	
n	12	10	11	11	10	10	10	11	10
F/M	6/6	6/4	8/3	7/4	6/4	64	91	8/3	6/4
Age(y)	32,5±8,7	25,3±11,7	28,36±5,0	27,9±6,8	31,9±7,5	35,8±11,7	30,4±9,6	28,3±10,3	29,5±6,4
Evol (a)	13,4±4,5	12,7±5,2	10,7±7,1	11,9±4,9	12,1±4,3	13,1±6,1	9,9±5,5	10,6±6,7	
sIgE-1	31,24±12,5	32,5±13,8	37,2±28,1	41,2±17,8	12,4±9,2	13,2±12,7	18,4±15,5	17,4±14,2	
sIgE-2					17,8±15,8	22,7±19,5	21,6±14,6	22,9±16,5	

n= number, F: Female; M: Male; y: years; Evol: Evolution of disease in years; sIgE-1: Specific IgE to Dpt before treatment; sIgE-2: Specific IgE to Dpt at time of study. IgE results given in KU/L.

healthy individual control group. In these individuals the counts obtained 1 hour and 4 hours after the first collection did not show relevant oscillations, supporting the idea of a constant metabolism.

Technical difficulties and the quality of the DNA samples made it impossible to obtain results in some patients, particularly in the group under treatment with SCIT studied 1 hour after administration.

In the allergic patients who underwent specific challenge (Group I), there were obvious significant variations in the number of TRECs in peripheral lymphocytes. The nasal allergenic challenge seems to induce a more expres-

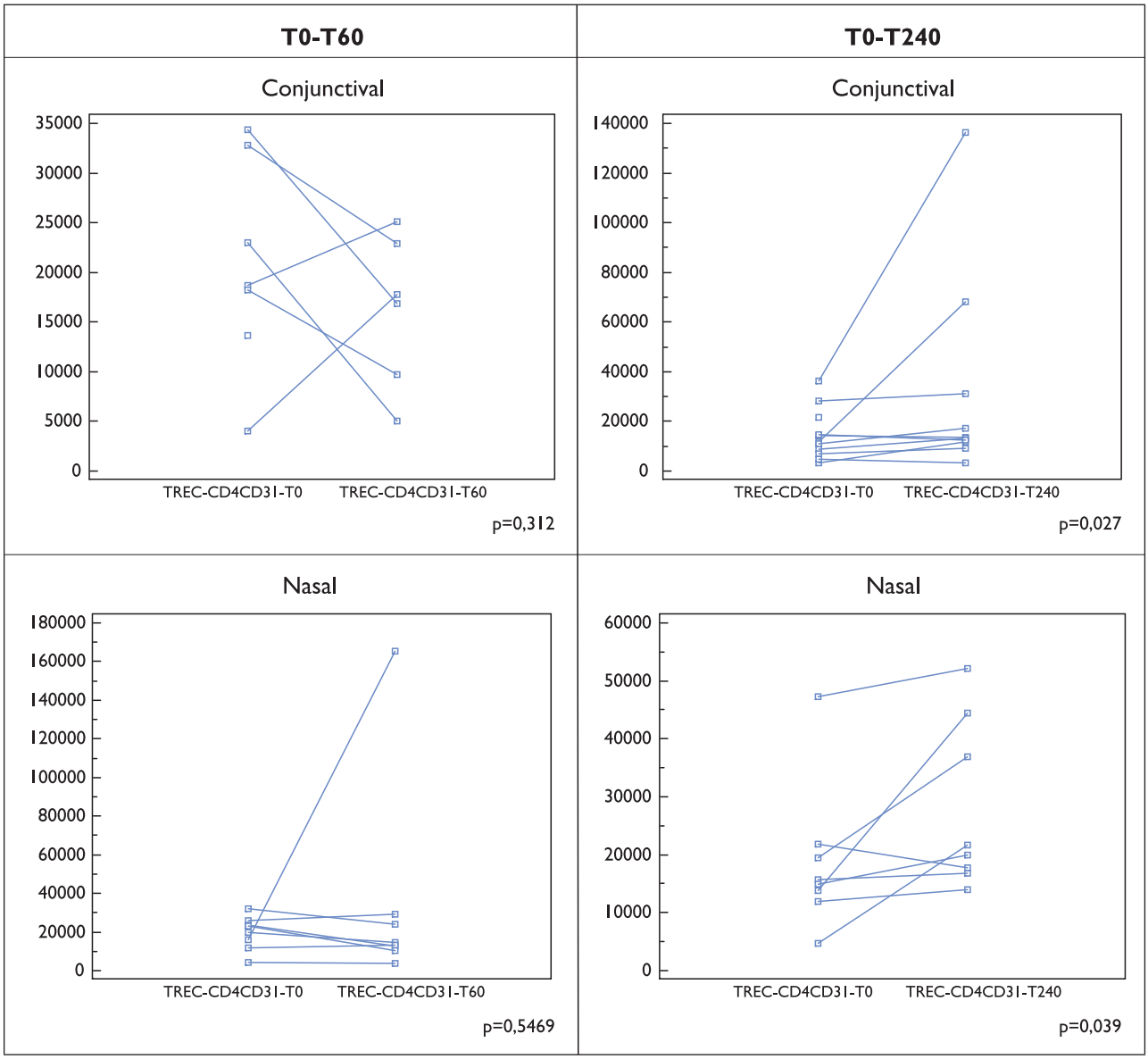

Figure 1. Number of TRECs per 100,000 CD4 + CD31 + cells in patients (Group I) undergoing specific allergy challenge test (nasal and conjunctival). Results given for each patient, basal and at 60 or 240 after first blood sample taken.

sive response than conjunctival administration of the allergen (TRECs/$CD4^+CD31^+$), with a more marked elevation, particularly in the group in which the second sample was at 4 hours (fig. 1).

The number of TRECs in $CD4^+CD31^+$ cells seemed to decrease under the effects of specific immunotherapy, particularly in the group studied after 240 minutes of administration of treatment extract, despite some patients showing divergent effects (fig. 2).

There were differences seen in the number of TRECs in the $CD8^+CD31^+$ population, induced by both specific challenge and the effect of treatment (figs. 3 and 4). Com-

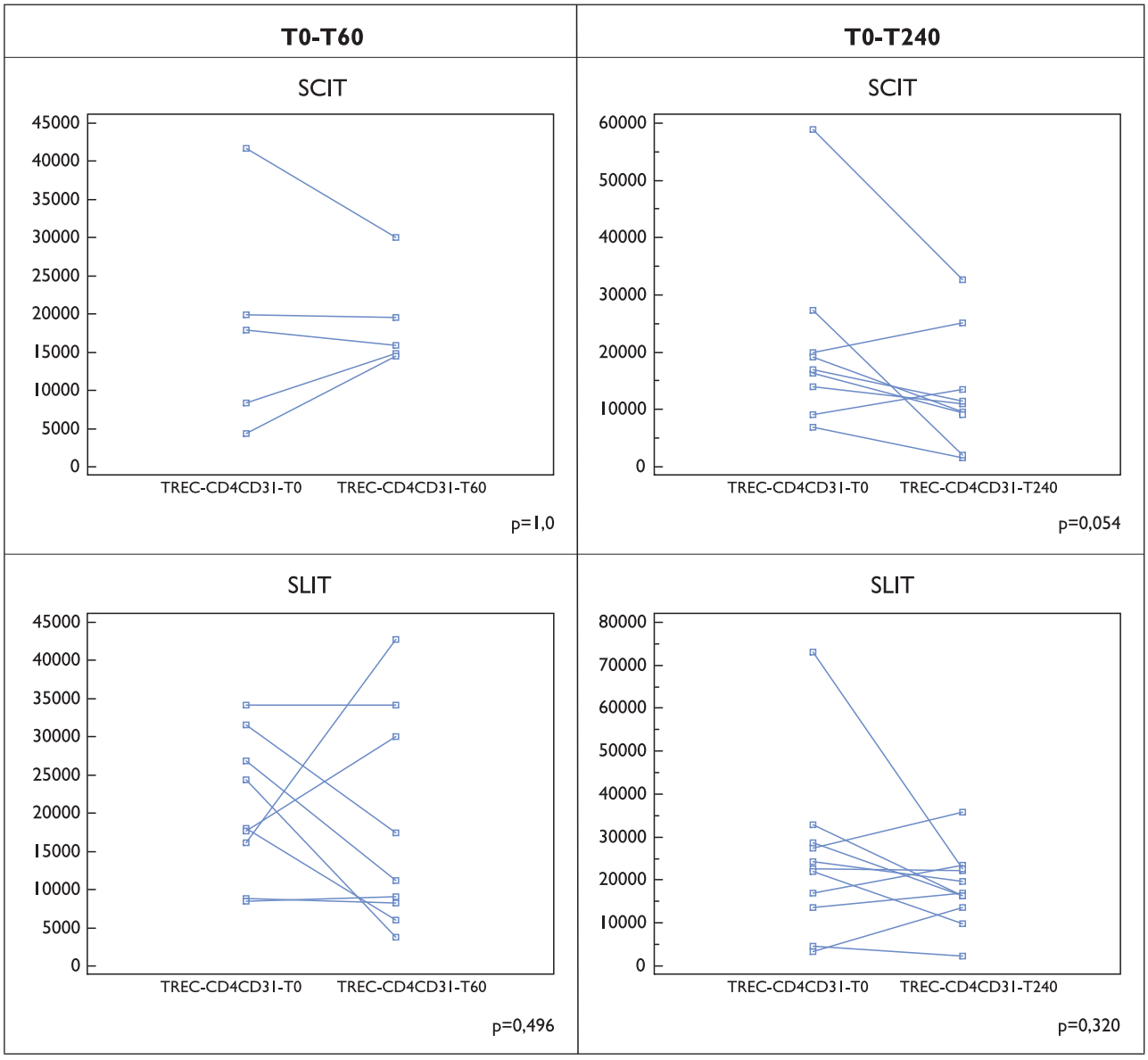

Figure 2. Number of TRECs per 100,000 CD4 + CD31 + cells in patients (Group II) who underwent specific immunotherapy (SCIT and SLIT). Results given for each patient, basal and at 60 or 240 after first blood sample taken.

pared to the $CD4^+CD31^+$ cell results there was a greater inconsistency in the results, with very divergent numbers seen in the patients of the different subgroups.

While evaluating individual behaviour of the dynamic response to the allergen or to the treatment, we also analysed the mean basal values of the TRECs/100,000 $CD4^+CD31^+$ cells obtained in the 3 groups in the study. The mean value in the maintenance stage SIT patients who had excellent response to treatment was higher but still with no statistical significance (p=0.32) compared to the allergic patients not undergoing specific treatment (fig. 5).

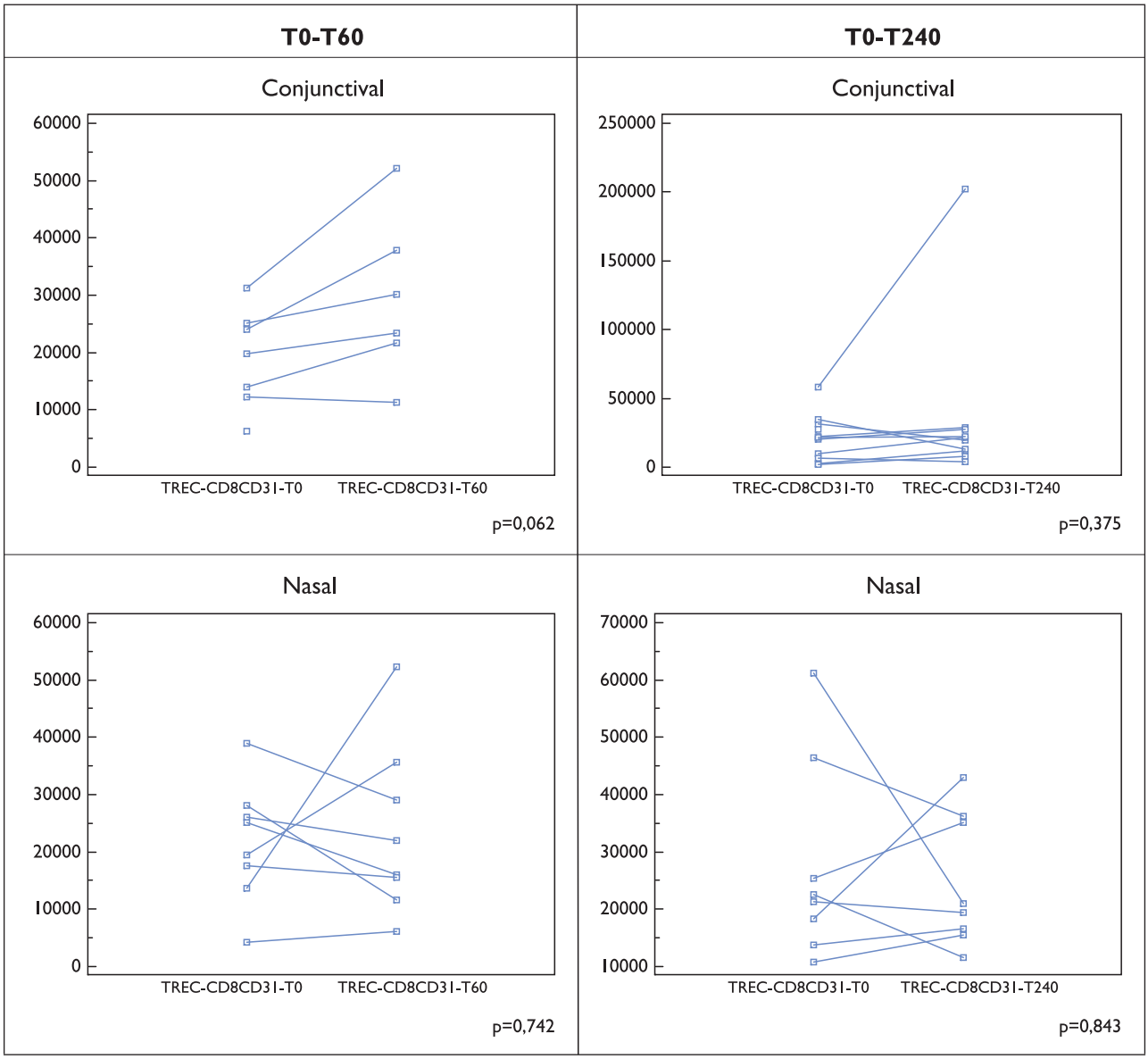

Figure 3. Number of TRECs per 100,000 CD8 + CD31 + cells in patients (Group I) undergoing specific allergy challenge test (nasal and conjunctival). Results given for each patient, basal and at 60 or 240 after first blood sample taken.

Clinically, both nasal and conjunctival challenge determined positive allergic reaction scores. Equally so, both SCIT and SLIT had an excellent clinical response. The results gleaned, particularly for the number of TRECs/100,000 $CD4^+CD31^+$ cells, showed variations at the different time points which were very different from those seen in healthy individuals.

Analysing mean values gleaned for the total patients who underwent the challenge tests showed that the allergic response in patients evaluated 1 hour and 4 hours after allergen exposure determined a progressive rise with no statistical significance, the reverse of that was seen after administration of treatment extract.

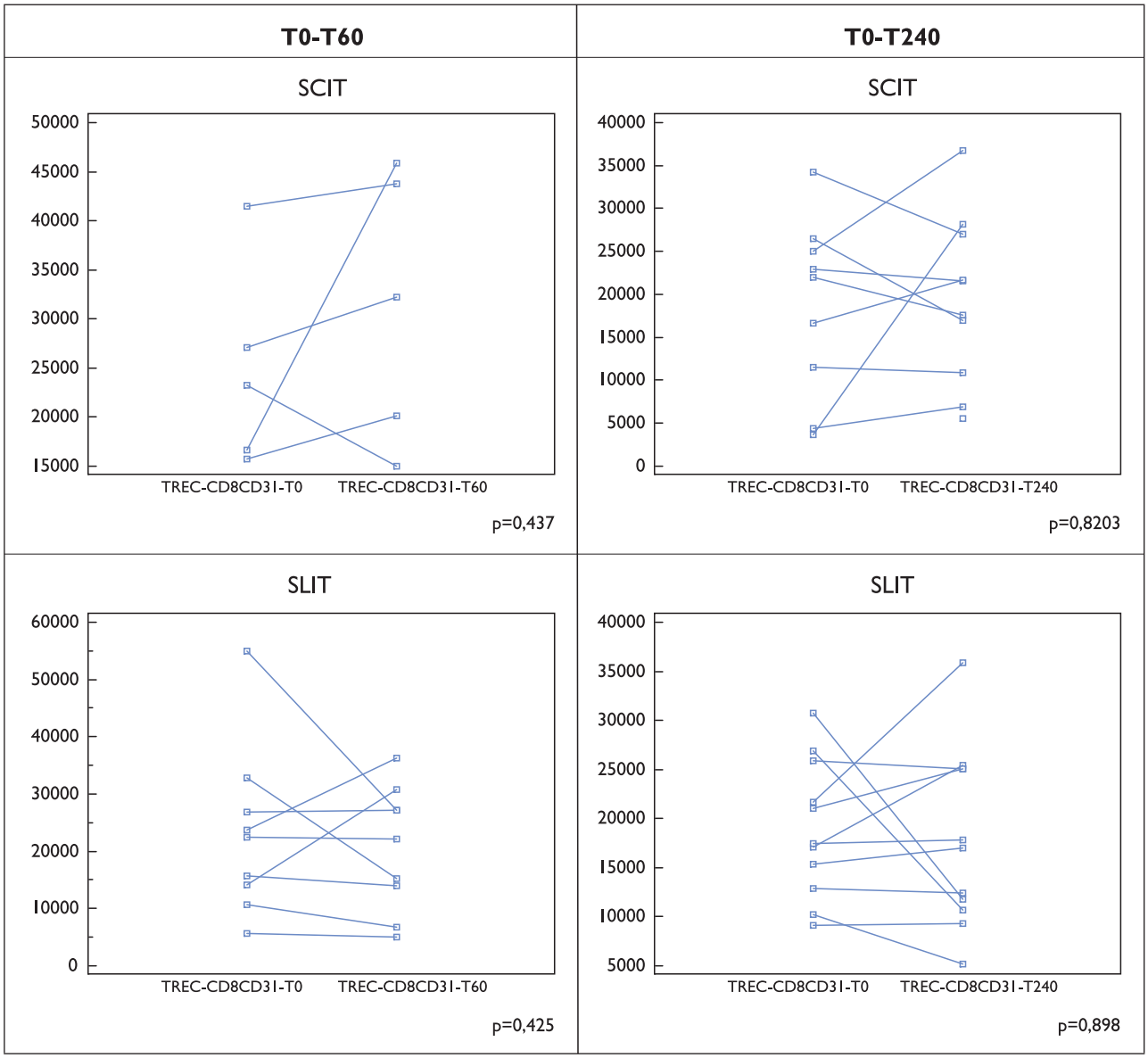

Figure 4. Number of TRECs per 100,000 CD8 + CD31 + cells in patients (Group II) who underwent specific immunotherapy (SCIT and SLIT). Results given for each patient, basal and at 60 or 240 after first blood sample taken.

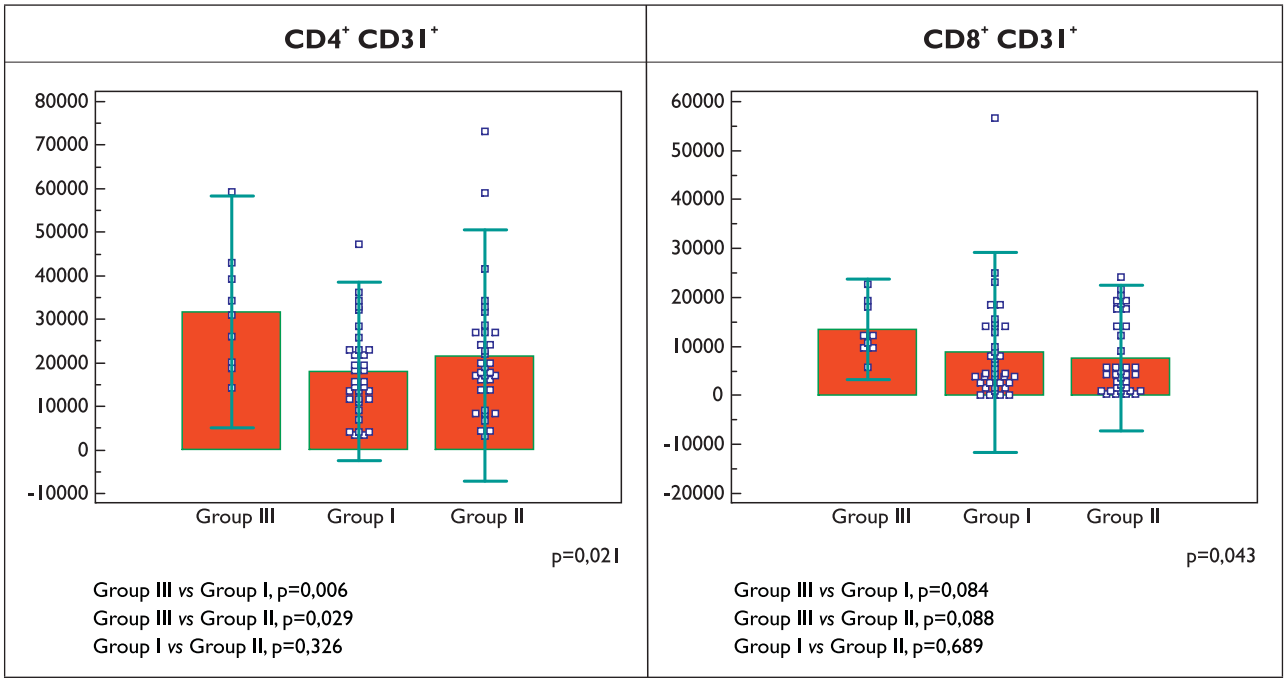

Figure 5. Mean number of TRECs per 100,000 cells obtained in the 3 groups (III-healthy control; I-allergic challenge test, II-administration of specific immunotherapy) basal (T0).

DISCUSSION

Determining thymic output via TREC count is a line of investigation which is of enormous interest in transplantation, infection, autoimmunity and immunodeficiencies[25]. We are so far unaware if this methodology has been used to study IgE-mediated respiratory allergy.

The healthy individual had a greatly reduced variability in TREC count over time, supporting the idea of the thymus's functionality and functional metabolism despite decline with age[20]. We found in the control group values for each patient in very varying spectra but ones which were stable for at least the 4 hours of the observation. Several studies highlight the reduction in the total number of TRECs in chronic inflammatory diseases compared with healthy controls, namely in atopic dermatitis, rheumatoid arthritis or erythematous lupus[20,29,30].

Our group was able in earlier studies to show an early *in vivo* response to allergens in the central immune organs[12-14], much swifter than those supported by several studies in medullar biopsies[26-28]. Life-long persistence of T cell immunological integrity is guaranteed by a thymic functional activity, despite anatomical involution which can be seen by cintography of stained leucocytes, and this also takes place very early on in IgE-mediated allergy.

The TREC count reflects thymic function and is also influenced by peripheral T cell metabolism. Counting the TRECs in 100,000 $CD31^+$ cells of the two T populations allows a closer approach to effective thymic activity, shown by young and immature RTEs. These are *naïve* peripheral T cells with no peripheral proliferation or antigenic selection[18]. While there is no exclusive marker of these cells, the methodology we designed, the TREC count in $CD31^+$ cells, is the current closest parameter for verifying thymic activity in humans.

In this context, evaluating the individual results gleaned in the different subgroups is extremely important in that the great individual variability does not allow for an un-

equivocal evaluation of the sample's mean values. A control group of allergic patients does not seem to be relevant, as the T0 in group I patients constitutes a control.

In the different groups and subgroups studied, the analysis of the results with the mean values obtained as basal is of little importance, in that it is more important to evaluate the individual dynamic of response at the times defined in the study. In fact, despite each patient showing an appreciable variation of the RTE values reported, there were no significant differences detected in the mean values of the percentage of $CD4^+CD31^+$ or $CD8^+CD31^+$ cells in the face of the total number of $CD4^+$ or $CD8^+$ cells, respectively.

In our patients with respiratory allergy, despite the study having taken place during a period of clinical stability, the base value of TRECs in $CD4^+CD31^+$ cells was lower than that in the control group. This basal value was, however, higher in the group of patients undergoing treatment with SIT, corroborating the central mechanism of this treatment.

The variability of determination in the majority of active groups in the study was very expressive, testament to the functional activity of the central immune system. It was also evident that each patient's individual response was very variable, depending naturally on the patient's genetic expression, despite a similar clinical picture and an identical allergic sensitisation, in this case to *Dermatophagoides pteronyssinus*.

The specific nasal and ocular challenge test determined an identical output profile of RTEs in peripheral blood, right after the first 60 minutes. While in the same patient a second determination was not made, the group of patients studied after 4 hours seemed to have a statistically significant time-dependent increase (fig. 2).

Although the total circulating -$CD4^+$ and $CD8^+$ T cells were conserved in the allergic patients compared with the healthy controls, reduction in RTEs could be the result of the chronic inflammatory state[20,29].

Mediator mast cells freed locally increase the expression of adhesion molecules in post-capillary veins. This could permit a homing of circulating leucocytes, making distance cellular infiltration a possibility. There is ample evidence to support that in the initial stages of allergic reaction, there is a selective recruitment of T-CD4+ lymphocytes to the extravascular compartment of the sites where allergenic stimulation is taking place[31]. This cellular recirculation and later focalisation makes IgE-mediated allergic disease a dynamic and systemic process. Earlier results show that cell response begins at very early periods subsequent to the onset of reaction, in the immediate stage of type I hypersensitivity reaction[12]. IgE-mediated response induces immunolymphatic involvement in adjacent structures. The later amplification of the allergic reaction to locoregional lymphoid organs is determinant, in tandem with recirculation of circulating leucocytes to primary lymphoid structures, namely bone marrow and functional thymic tissue. These structures are thus responsible for inducing a systemic immune response following specific and controlled allergenic exposure.

The population most concerned in allergic reaction seems to be TREC/$CD4^+CD31^+$, contrary to that seen in atopic dermatitis[20]. In the majority of patients we saw an overall increase in circulating *naïve* T cells, which could mean a capacity for a maturation output of thymic cells able to control the magnitude of their own reaction.

In specific immunotherapy, the induction of T-regulatory cells is currently one of the most important effects on the modulatory mechanism[6]. Therapeutic intraganglional administration naturally presupposes the direct intervention of the immune system[11], although the subcutaneous and sublingual routes induce an identical effect[13,14]. We found less circulating RTEs in our patients who were under maintenance treatment than in the healthy controls, although with mean values higher than the group of allergic patients not undergoing this treatment. The early involvement of the bone marrow and functional thymic tissue was also seen, but obviously the mechanisms and targets are safely distinct from the allergic reaction.

The induction of T-reg cells is determinant in the mechanism of immunotherapy, despite constituting a heterogeneous population which includes natural $CD4^+CD25^+$ and those induced in the periphery following exposure to the

antigen (Tr1, Th3 and regulatory $CD8^+$)[32]. In analysing the results we admit this population needs time for maturation in the thymic tissue, the reason why there is a reduction in circulating RTEs (TRECs/$CD31^+$) following administration of treatment extract.

Our results support that the central immune system is effectively a target for IgE-mediated reaction and in SIT. Functional thymic tissue intervenes very early once the output of RTEs shows great variability, very different from what occurs in a healthy individual, in whom these levels are constant over time. We admit that in allergic reaction the ready release of *naïve* thymic cells could be the result of the pathogenic mechanisms themselves and/or the regulation of the reaction itself. In the SIT mechanism, the seeming reduction in these cells could signify a cellular influx in functional thymic tissue for posterior maturation of T-reg lymphocytes.

We presume that an evaluation at a later stage of an allergy challenge test or SIT would show differences, more significant in individual and group analysis. We feel that additional studies characterising the cell markers specific to these RTE populations, and capable of regulating the reconstitution of the T compartment, exploring new treatment strategies in IgE-mediated allergy and allergic disease would be of interest.

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Conflict of interest statement: This study was performed using the exclusive resources of the Allergology and Clinical Immunology Department of the Hospitais da Universidade de Coimbra and those of the Histocompatibility Centre, Coimbra. The project is under the aegis of the Pulmonology Department, Universidade Coimbra – FCT. No author declares any financial or commercial conflict of interest.

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