

ORIGINAL PAPER

Expansion of circulating Foxp3⁺ CD25^{bright} CD4⁺ T cells during specific venom immunotherapy

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Clinical and Experimental Allergy

Summary

Background Venom immunotherapy (VIT) induces long-lasting immune tolerance to hymenoptera venom antigens, but the underlying mechanisms are not yet clarified.

Regulatory T cells are thought to play an important role in allergic diseases and tolerance induction during specific immunotherapy.

Aim Characterize longitudinally the impact of VIT on the pool of circulating regulatory T cells.

Methods Fourteen hymenoptera venom-allergic patients with severe reactions (grades III–IV) were studied before, 6 and 12 months after starting ultra-rush VIT. Freshly isolated peripheral blood mononuclear cells were surface stained with a panel of markers of T cell differentiation and intracellularly for CTLA-4 and Foxp3 and analysed by flow cytometry. *foxp3* mRNA was quantified by real-time PCR. VIT responses were assessed by measuring specific IgG4 and IgE levels. Eleven individuals with no history of insect venom allergy were studied as controls.

Results VIT induces a significant progressive increase in both the proportion and the absolute numbers of regulatory T cells defined as CD25^{bright} and/or Foxp3⁺ CD4⁺ T cells. These changes are not related to alterations in the expression of activation markers or imbalances in the naïve/memory T cell compartments. *foxp3* mRNA levels also increased significantly during VIT. Of note, the increase in circulating regulatory T cell counts significantly correlates with the venom-specific IgG4/IgE ratio shift.

Conclusion VIT is associated with a progressive expansion of circulating regulatory T cells, supporting a role for these cells in tolerance induction.

Keywords CD25^{bright}, Foxp3, regulatory T cells, specific immunotherapy, venom allergy

Submitted 13 July 2007; revised 24 September 2007; accepted 5 October 2007

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Introduction

Allergic reactions to insect stings can be life threatening. Venom-specific immunotherapy (VIT) is currently considered the only approach by which long-term tolerance can be achieved, and one of the most effective specific immunotherapies [1]. Thus, it represents a particularly advantageous model to dissect the mechanisms underlying tolerance induction [2]. Successful VIT is usually associated with an increase in specific IgG antibodies, particularly of the IgG4 class, which are thought to block allergen and IgE-facilitated antigen presentation [3]. Moreover, a reduction in the numbers and activation state of basophils and mast cells have been reported [4, 5], as well as a shift from a T helper type 2 (Th2) to a Th1

cytokine profile [6, 7] and a decrease in T cell responsiveness against the allergen [3]. The role of suppressive cytokines in these processes has been investigated. VIT-treated patients have been shown to exhibit increased levels of IL-10 in the supernatants of lymphocyte cultures stimulated with venom extracts [3, 6]. Moreover, an expansion of IL-10-producing CD4⁺ T cells has been reported [8]. Besides regulatory T cells (Treg) that mediate their action via IL-10, usually referred to as type-1 regulatory T cells (Tr1), other Treg populations are thought to be implicated in allergy [9]. Circulating CD4⁺ T cells expressing high levels of CD25, the α chain of the IL-2 receptor, and the forkhead-winged helix transcription factor Foxp3 have been clearly shown to represent a well-defined and potent regulatory population [10–13].

These cells were initially described to be originated in the thymus [14–16], but increasing evidence indicates that they can also be generated in the periphery during immune responses [17, 18]. The mechanisms of action of this population are not fully characterized but they are thought to rely on contact-dependent processes rather than soluble factors [10, 12, 19]. Foxp3 was shown to be essential for the development and function of this subset [11, 20, 21]. To our knowledge, there are no data on the role of Foxp3⁺ Treg in the long-term development of tolerance during VIT. In this study, we evaluate longitudinally the effects of ultra-rush VIT on the pool of circulating Treg, defined as CD4⁺ T cells expressing high levels of CD25 and/or Foxp3.

Methods

Study population

Fourteen patients [11 males and 3 females; mean age 47 years (range 16–65)] with a history of severe systemic reactions to hymenoptera stings of grades III–IV [22] eligible for VIT according to the criteria of the European Academy of Allergy and Clinical Immunology [23] were studied. This cohort comprised 10 bee- and 4 wasp venom-allergic patients. Specific sensitization was confirmed by positive skin tests (prick and intradermal) and detection of allergen-specific IgE in the serum. Blood samples were taken at baseline (before VIT), and at 6 and 12 months after starting VIT. VIT (Stallergènes, France) was carried out according to an ultra-rush protocol [24]. Eleven individuals [mean age 51 years (range 34–72)] with no history of allergic reactions to hymenoptera stings were also studied as controls. All subjects gave informed consent to blood sampling and processing, and the study was approved by the Ethical Board of the Faculty of Medicine of Lisbon.

Flow cytometric analysis

Freshly isolated peripheral blood mononuclear cells (PBMC) were stained as described previously [25]. Briefly, cells suspended in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA; Sigma-Aldrich, St Louis, MO, USA) and 0.1% sodium azide (Sigma-Aldrich) were stained with monoclonal antibodies (mAbs) for 20 min in the dark at room temperature. The following anti-human mAbs were used: anti-CD25, anti-CD3, anti-CD38, anti-CD4, anti-CD45RA, anti-CD45RO, anti-CD62L, anti-CD8, anti-CCR7 and anti-HLA-DR. All mAbs were from BD-Biosciences (San Jose, CA, USA). For intracellular cytotoxic T-lymphocyte antigen 4 (CTLA-4) staining, cells were fixed with 2% formaldehyde and permeabilized with PBS containing 1% BSA and 0.5% saponin (Sigma-Aldrich) before staining for CTLA-4

(BD-Biosciences). Foxp3 (PCH101 clone, eBiosciences, San Diego, CA, USA) intracellular staining was performed according to the manufacturer's instructions. Matched isotype controls were included. Samples were acquired on a FACSCalibur flow cytometer (BD-Biosciences). Analysis was performed using FlowJo software (Tree Star, Ashland, OR, USA). Results are presented as a proportion of total lymphocytes. Total cell numbers were computed by multiplying the corresponding percentages by the peripheral blood lymphocyte count obtained at the clinical laboratory.

foxp3 mRNA quantification

RNA was extracted from freshly isolated 10⁶ PBMC using an AbiPrism 6100 Nucleic Acid Extractor (Applied Biosystems, Foster City, CA, USA) and quantified using a NanoDrop ND-10 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Four hundred nanograms of total RNA was reverse transcribed using the Superscript II Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA). Duplicate samples were quantified by real-time PCR in 150 ng of cDNA and 50 µL PCR mixture volume containing 25 µL of Platinum quantitative PCR SuperMix-UDG, 1 µL ROX Reference Dye 50x, 5 mM of MgCl₂ (all from Invitrogen), with 300 nM of each primer (Forward: 5'-cacattcccagagttcctc-3', Reverse 5'-gcgtgtgaaccagtgtagatc-3'), 200 nM of TaqMan probe (5'-FAM-tcatccgctgggcca-MGB-3') and 150 ng of DNA using AbiPrism 7000 SDS (Applied Biosystems). A plasmid containing foxp3 sequence was serially diluted (10⁶–10 copies/reaction) and used to generate a standard curve. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control (FAM/MGB probe, non-primer limited – Applied Biosystems; standard curve generated with serial dilutions of cDNA, synthesized from total RNA extracted from pooled PBMC from three different healthy donors). Results were normalized to GAPDH internal controls and expressed as arbitrary units.

Allergen-specific immunoglobulin E and immunoglobulin G4 levels

Serum allergen-specific IgE and IgG4 concentrations were determined by UniCAP (Phadia, Sweden) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 4.00 (GraphPad Software Inc., SD). Data were tested for Gaussian distribution, repeated-measures ANOVA was used for the analysis of variance, two group comparisons were performed using a paired or an unpaired *t*-test and Pearson's coefficient was used to determine the significance of the correlation between two variables.

Results are expressed as mean $\pm$ SEM, and *P*-values <0.05 were considered to be significant.

Results

Venom-specific immunotherapy is associated with a progressive increase in the proportion and absolute numbers of CD25^{bright} and/or Foxp3⁺ regulatory T cells

A longitudinal study of 14 patients with severe systemic reactions to hymenoptera venom, grades III–IV according to Mueller's classification [22], was performed before and after starting ultra-rush VIT.

Freshly isolated PBMC were surface stained and analysed by flow cytometry to assess the frequency of CD4⁺CD25^{bright} T cells within total lymphocytes. CD4⁺CD25^{bright} T cells were defined as those CD4⁺ T cells expressing CD25 at a higher intensity of fluorescence than the CD4⁺ lymphocytes, as illustrated in Fig. 1a. The adequacy of our analysis was further confirmed by the high levels of intracellular expression of CTLA-4, a recognized Treg marker, within CD4⁺CD25^{bright} T cells (data not shown).

An increase in both the proportion (Fig. 1b) and the absolute counts (Fig. 1c) of CD4⁺CD25^{bright} T cells in the peripheral blood was observed during VIT. The majority of these cells exhibited a memory phenotype, and the proportion of CD45RA⁺ cells did not change significantly during the follow up ($92.72 \pm 1.5\%$, $91.56 \pm 1.3\%$ and $90.35 \pm 2.1\%$ at baseline, 6 and 12 months of VIT, respectively).

Of note, the frequency of CD4⁺CD25^{bright} T cells was significantly reduced in patients at baseline as compared with age-matched non-allergic individuals studied in parallel ($0.46 \pm 0.06\%$ vs. $0.80 \pm 0.13\%$, respectively;

$P = 0.0228$, unpaired *t*-test). Furthermore, their frequency increased to levels similar to those observed in non-allergic individuals after 6 months of immunotherapy.

As Foxp3 has been shown to be essential for Treg development and function [21, 26, 27], we compared the foxp3 transcriptional activity in PBMC at baseline and 1 year after starting VIT. Foxp3 mRNA was quantified by real-time PCR and was found to increase significantly during VIT (Fig. 2a). Moreover, we were also able to assess Foxp3 protein expression by flow cytometry in a subgroup of patients, as illustrated in Fig. 2b. Both the proportion (Fig. 2c) and the absolute number (Fig. 2d) of CD4⁺Foxp3⁺ T cells increased progressively under VIT, reaching statistical significance for the absolute numbers.

As reported previously [13], the Foxp3 expression was found to be mainly restricted to CD4⁺ T cells expressing CD25 at a high intensity (Fig. 3a). Moreover, the frequency of CD4⁺Foxp3⁺ T cells was found to correlate strongly with CD4⁺CD25^{bright} T cells (Fig. 3b). The analysis of CD4⁺ T cells co-expressing both markers, CD25^{bright}Foxp3⁺, revealed a marked increase in both the proportion (Fig. 3c) and the absolute number (Fig. 3d) of this population during VIT. Of note, these increases were much more pronounced than the ones observed when the analysis was restricted to the expression of only one of the markers.

The expansion of circulating CD25^{bright} and/or Foxp3⁺ CD4⁺ T cells during venom-specific immunotherapy is not associated with an increase in activation markers or naïve/memory T cell imbalances

T cell activation is known to induce transient up-regulation of CD25, and recent data suggested that it also

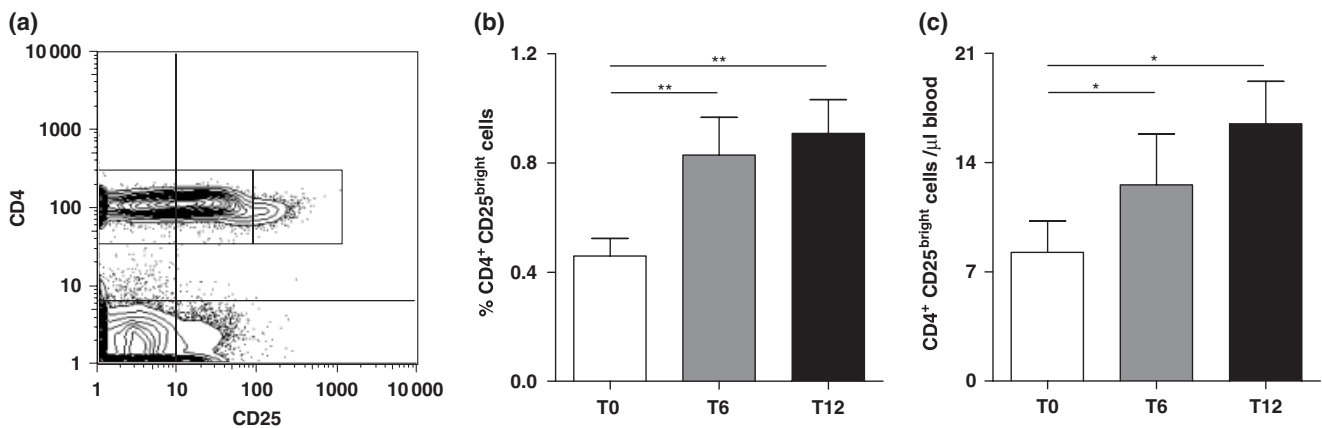


Fig. 1. Progressive increase of circulating CD4⁺CD25^{bright} regulatory T cells during ultra-rush venom immunotherapy (VIT). Freshly isolated peripheral blood mononuclear cells were stained for CD4 and CD25, and analysed by flow cytometry, before (T0) and after 6 (T6) and 12 (T12) months of starting VIT. (a) Representative contour plot of CD4⁺CD25^{bright} analysis: after gating on lymphocytes according to forward and side scatter, the CD4⁺CD25^{bright} cells were defined as the CD4⁺ cells expressing CD25 at a higher intensity than the CD4⁺ lymphocytes. (b) Proportion of CD4⁺CD25^{bright} T cells within total lymphocytes along VIT ($n = 14$). (c) Absolute numbers of circulating CD4⁺CD25^{bright} T cells during VIT ($n = 9$). Bars represent mean $\pm$ SEM. Differences between time-points were compared using a paired *t*-test, and *P*-values are shown as follows: * $P < 0.05$; ** $P < 0.01$.

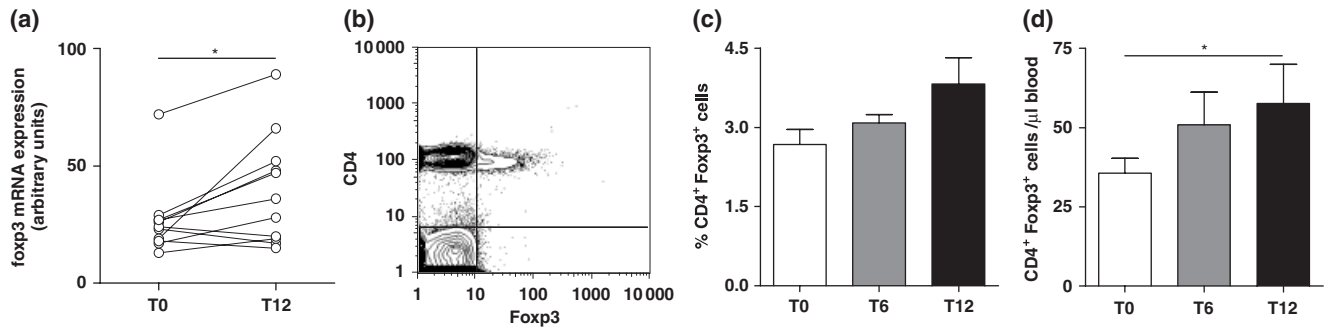


Fig. 2. Progressive increase of Foxp3 expression during ultra-rush venom immunotherapy (VIT). (a) *foxp3* mRNA expression in peripheral blood mononuclear cells (PBMC) was determined by real-time PCR before (T0) and after 12 months (T12) of starting VIT. Results were normalized to GAPDH internal controls and expressed as arbitrary units. Each circle represents one individual. (b) In parallel, freshly isolated PBMC were stained for CD4 and intracellular Foxp3 and analysed by flow cytometry before (T0) and after 6 (T6) and 12 (T12) months of VIT. Representative contour plot of the flow cytometric analysis performed on lymphocytes gated according to forward and side scatter. (c) Proportion of CD4⁺Foxp3⁺ T cells within total lymphocytes along VIT ($n = 5$). (d) Absolute numbers of circulating CD4⁺Foxp3⁺ T cells during VIT ($n = 4$). Bars represent mean $\pm$ SEM. Differences between time-points were compared using a paired *t*-test, and *P*-values are shown as follows: * $P < 0.05$.

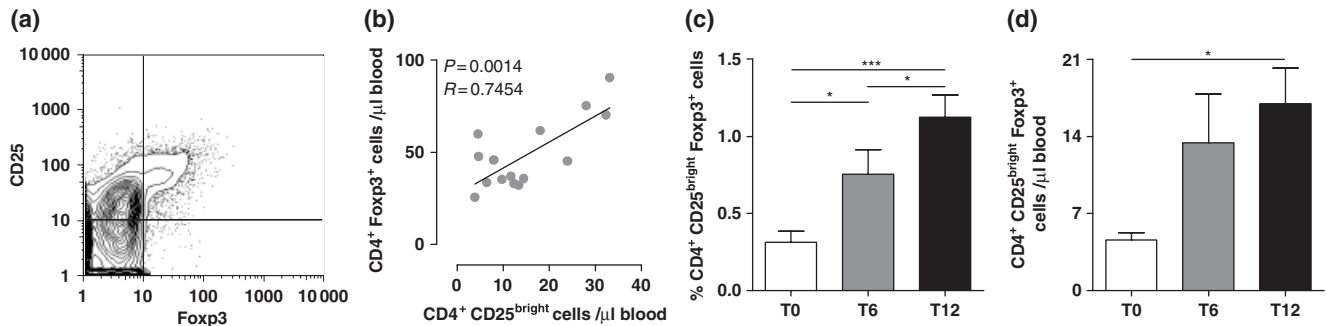


Fig. 3. Progressive increase of CD4⁺CD25^{bright} Foxp3⁺ T cells during ultra-rush VIT. Freshly isolated peripheral blood mononuclear cells were stained for CD4, CD25 and intracellular Foxp3 and analysed by flow cytometry before (T0), and after 6 (T6) and 12 (T12) months of starting VIT. (a) Representative contour plot of CD25 and Foxp3 staining gated on CD4⁺ lymphocytes. (b) Correlation between CD4⁺CD25^{bright} and CD4⁺ Foxp3⁺ T cells. Each circle represents one individual. Pearson's coefficient was used, and *P*- and *R*-values are shown. (c) Proportion of CD4⁺CD25^{bright} Foxp3⁺ T cells within total lymphocytes along VIT ($n = 5$). (d) Absolute numbers of circulating CD4⁺CD25^{bright} Foxp3⁺ T cells during VIT ($n = 4$). Bars represent mean $\pm$ SEM. Differences between time-points were compared using a paired *t*-test, and *P*-values are shown as follows: * $P < 0.05$; *** $P < 0.001$.

induces Foxp3 expression in human T cells [13, 18, 28]. In order to exclude activation as a driving force for the observed expansions, HLA-DR expression was assessed on T cells and was found to remain stable throughout the period of follow-up ($4.31 \pm 1.3\%$, $4.44 \pm 1.4\%$ and $4.04 \pm 1.1\%$ for CD4⁺ T cells and $8.72 \pm 3.5\%$, $10.80 \pm 3.2\%$ and $8.89 \pm 2.7\%$ for CD8⁺ T cells at baseline, 6 and 12 months of VIT, respectively, not statistically different).

Moreover, the observed expansions were not due to imbalances in the relative representation of naïve or memory CD4⁺ T cell subsets, because their proportions did not change significantly during the study. The frequency of naïve cells within the CD4⁺ T cell subset, as defined by CD45RA⁺CD62L⁺ expression, was $42.40 \pm 4.9\%$, $40.63 \pm 6.4\%$ and $44.30 \pm 4.5\%$ and the proportion of memory cells (CD45RO⁺) was $53.88 \pm 4.3\%$, $49.66 \pm 6.4\%$ and $51.79 \pm 4.4\%$ at baseline, 6 and 12 months of VIT, respectively.

The increase in regulatory T cell count is in correlation with the specific immunoglobulin G4/immunoglobulin E ratio shift

VIT has been shown to induce an increase in allergen-specific IgG4 levels and a parallel decrease in specific IgE [3, 29], leading to an enhanced IgG4/IgE ratio. In order to assess a putative role of Treg in tolerance induction, we correlated Treg frequency and IgG4 and IgE levels. Although no significant correlation was observed between the changes of the Treg frequency in the peripheral blood as defined by CD25^{bright} and/or Foxp3⁺ expression and IgG4 or IgE levels, we demonstrated a positive correlation between the changes in CD4⁺CD25^{bright} T cell counts during therapy and the changes observed in the allergen-specific IgG4/IgE ratio (Fig. 4a). The shift in this ratio during VIT was even more strongly correlated with the changes in CD4⁺CD25^{bright} Foxp3⁺ T cell counts (Fig. 4b).

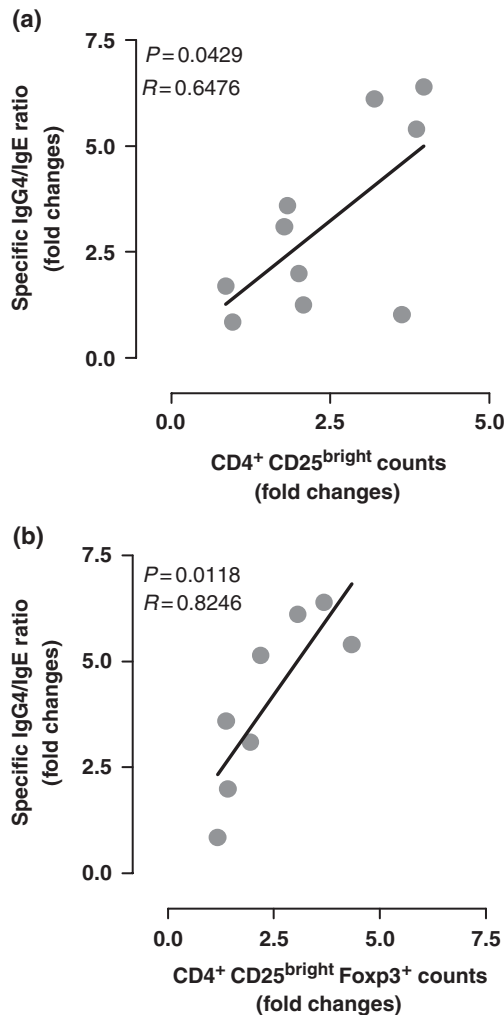


Fig. 4. The changes in the circulating regulatory T cell subset correlate positively with the changes in the venom-specific IgG4/IgE ratio during ultra-rush venom immunotherapy (VIT). Venom-specific IgG4 and IgE serum levels were determined by UniCAP, and the venom-specific IgG4/IgE ratio was calculated before starting VIT and after 1 year of immunotherapy. Graphs illustrate the correlation between the changes in this ratio and the increases documented in the number of circulating CD4⁺CD25^{bright} T cells (a) and CD4⁺CD25^{bright} Foxp3⁺ T cells (b) during VIT. Each circle represents one individual. Pearson's coefficient was used to determine significance between variables, and P - and R -values are shown.

Although these correlations do not establish a causative link between the alterations in regulatory T-cell numbers and IgG4/IgE levels, they are consistent with a possible role for this population in tolerance induction.

Discussion

In this study, we show that VIT is associated with a progressive increase in the proportion and absolute numbers of regulatory T cells defined as CD25^{bright} and/or Foxp3⁺ CD4⁺ T cells. These effects were observed in individuals with severe allergic reactions and correlated

positively with the changes observed in the ratio between specific IgG4 and IgE, supporting a role for Treg in the induction of tolerance in this clinical setting. Importantly, no changes of other T cell subsets or activation markers were documented during the follow up, suggesting that the increase in the Treg pool is not activation related or the result of imbalances in naïve/memory compartments. It should also be noted that, in comparison with non-allergic individuals, the patients studied showed significantly reduced frequencies of CD4⁺CD25^{bright} and CD4⁺CD25^{bright} Foxp3⁺ T cells at baseline, which normalized after 6 months of VIT.

Treg are critical players in peripheral immune tolerance [9–11, 30]. Specific immunotherapy was shown to induce IL-10-producing Treg able to suppress allergen-specific T cell responses and to regulate allergen-specific IgG4 and IgE synthesis [3, 6]. Recently, in a study by Mamessier et al. [8] IL-10-producing T cells and CD4⁺CD25^{high} T cells were shown to increase simultaneously during VIT. In their study, a less stringent criterion to define CD4⁺CD25^{bright} T cells was used and Foxp3 expression was not evaluated. Furthermore, this study was limited to the initial 45 days of treatment and a significantly less marked expansion of CD4⁺CD25^{high} T cells was observed in patients with severe disease as compared with those with only mild symptoms [8]. These data raised doubts about the impact of VIT on the Treg pool of the most suitable candidates to start VIT namely the ones with more severe allergic reactions. Our data expand upon these findings, as it shows a progressive and sustained expansion of CD4⁺CD25^{bright} Foxp3⁺ T cells in individuals with severe reactions during a period of at least 1 year of VIT. Mamessier et al. [8] have shown that a cellular fraction highly enriched in CD4⁺CD25^{high} T cells was able to suppress venom-specific proliferation. Accordingly, in the few patients in which we were able to reveal *in vitro* proliferative responses against the allergen at baseline using commercially available extracts, a decrease in their magnitude was observed during the follow-up (data not shown). These data are compatible with a suppression of the proliferative responses by the expanded population. Interestingly, Smith et al. [31] did not find an increase in the intrinsic suppressive capacity of CD25⁺Treg during cat allergen peptide immunotherapy, despite the significant decrease of the *in vitro* proliferative responses to the allergen. However, their study did not include a quantification of the circulating regulatory T cells. Thus, an increase in the relative representation of this population similar to that described here could contribute to the reduced allergen-specific proliferative responses during immunotherapy.

To our knowledge, this is the first study reporting a diminished basal frequency of Treg in the peripheral blood of patients with severe allergic reactions to insect stings compared with individuals without a history of allergic

diseases. Previous studies have shown that the capacity of CD4⁺CD25⁺ T cells from allergic individuals to suppress allergen-specific T cell responses was reduced or even absent [9, 32], and moreover a recent study by Hartl *et al.* [33] in paediatric asthma reported a significant increase in CD25^{high} CD4⁺ T cells after corticotherapy both in the peripheral blood and in the bronchoalveolar lavage fluid. Taken together, these data further highlight the importance of Treg in maintaining immunologic tolerance, supporting the need to explore therapeutic approaches aimed at boosting this population as a strategy to control allergy [1, 9, 34].

The origin and maintenance of circulating Foxp3⁺ Treg are not fully understood [34]. Peripheral Foxp3⁺ cells may arise from the expansion of thymic-derived Treg and/or may be generated concomitantly with effector or memory T cells during immune responses [15–17, 34]. Both pathways may contribute to the expansion that we document under VIT. Another important question to be addressed in future studies regards the specificity of the expanded Treg: are they allergen-specific or does this expansion result from some bystander amplification process? The second hypothesis would seem more likely, given the magnitude of the expansion and previous evidence in favour of cross-protection against further sensitizations and allergies in individuals undergoing specific immunotherapy [35–37].

In conclusion, our results support a role for CD25^{bright} and/or Foxp3⁺ CD4⁺ T cells in tolerance induction during VIT. Studies are ongoing to evaluate the kinetics of this population throughout the entire course of VIT and more importantly whether this expansion is maintained after discontinuing therapy.

Acknowledgements

We thank Russell B. Foxall for technical advice and discussion.

Financial Support: APB and RSS received scholarships from GlaxoSmithKline and Fundação para a Ciência e Tecnologia, Portugal, respectively.

Disclosures: The authors have no competing financial interests.

References

- Jutel M, Akdis M, Blaser K, Akdis CA. Are regulatory T cells the target of venom immunotherapy? *Curr Opin Allergy Clin Immunol* 2005; 5:365–9.
- Golden DB. Insect sting allergy and venom immunotherapy: a model and a mystery. *J Allergy Clin Immunol* 2005; 115:439–47; quiz 448.
- Akdis CA, Blesken T, Akdis M, Wuthrich B, Blaser K. Role of interleukin 10 in specific immunotherapy. *J Clin Invest* 1998; 102:98–106.
- Jutel M, Muller UR, Fricker M, Rihs S, Pichler WJ, Dahinden C. Influence of bee venom immunotherapy on degranulation and leukotriene generation in human blood basophils. *Clin Exp Allergy* 1996; 26:1112–8.
- Pierkes M, Bellinghausen I, Hultsch T, Metz G, Knop J, Saloga J. Decreased release of histamine and sulfidoleukotrienes by human peripheral blood leukocytes after wasp venom immunotherapy is partially due to induction of IL-10 and IFN- γ production of T cells. *J Allergy Clin Immunol* 1999; 103:326–32.
- Bellinghausen I, Metz G, Enk AH, Christmann S, Knop J, Saloga J. Insect venom immunotherapy induces interleukin-10 production and a Th2-to-Th1 shift, and changes surface marker expression in venom-allergic subjects. *Eur J Immunol* 1997; 27:1131–9.
- Jutel M, Pichler WJ, Skrbic D, Urwyler A, Dahinden C, Muller UR. Bee venom immunotherapy results in decrease of IL-4 and IL-5 and increase of IFN- γ secretion in specific allergen-stimulated T cell cultures. *J Immunol* 1995; 154:4187–94.
- Mameessier E, Birnbaum J, Dupuy P, Vervloet D, Magnan A. Ultra-rush venom immunotherapy induces differential T cell activation and regulatory patterns according to the severity of allergy. *Clin Exp Allergy* 2006; 36:704–3.
- Ling EM, Smith T, Nguyen XD *et al.* Relation of CD4+CD25+ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. *Lancet* 2004; 363:608–15.
- Asano M, Toda M, Sakaguchi N, Sakaguchi S. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *J Exp Med* 1996; 184:387–96.
- Bacchetta R, Passerini L, Gambineri E *et al.* Defective regulatory and effector T cell functions in patients with FOXP3 mutations. *J Clin Invest* 2006; 116:1713–22.
- Jonuleit H, Schmitt E, Stassen M, Tuettenberg A, Knop J, Enk AH. Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. *J Exp Med* 2001; 193:1285–94.
- Roncador G, Brown PJ, Maestre L *et al.* Analysis of FOXP3 protein expression in human CD4+CD25+ regulatory T cells at the single-cell level. *Eur J Immunol* 2005; 35:1681–91.
- Cosmi L, Liotta F, Lazzeri E *et al.* Human CD8+CD25+ thymocytes share phenotypic and functional features with CD4+CD25+ regulatory thymocytes. *Blood* 2003; 102:4107–14.
- Cupedo T, Nagasawa M, Weijer K, Blom B, Spits H. Development and activation of regulatory T cells in the human fetus. *Eur J Immunol* 2005; 35:383–90.
- Liston A, Rudensky AY. Thymic development and peripheral homeostasis of regulatory T cells. *Curr Opin Immunol* 2007; 19:176–85.
- Vukmanovic-Stejić M, Zhang Y, Cook JE *et al.* Human CD4+CD25hi Foxp3+ regulatory T cells are derived by rapid turnover of memory populations in vivo. *J Clin Invest* 2006; 116:2423–33.
- Walker MR, Kasprowicz DJ, Gersuk VH *et al.* Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25[–] T cells. *J Clin Invest* 2003; 112:1437–43.
- Levings MK, Sangregorio R, Roncarolo MG. Human cd25(+)cd4(+) t regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. *J Exp Med* 2001; 193:1295–302.

- 20 Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003; 299:1057–61.
- 21 Williams LM, Rudensky AY. Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. *Nat Immunol* 2007; 8:277–84.
- 22 Mueller HL. Diagnosis and treatment of insect sensitivity. *J Asthma Res* 1966; 3:331–3.
- 23 Bonifazi F, Jutel M, Bilo BM, Birnbaum J, Muller U. Prevention and treatment of hymenoptera venom allergy: guidelines for clinical practice. *Allergy* 2005; 60:1459–70.
- 24 Birnbaum J, Charpin D, Vervloet D. Rapid hymenoptera venom immunotherapy: comparative safety of three protocols. *Clin Exp Allergy* 1993; 23:226–30.
- 25 Albuquerque AS, Cortesao CS, Foxall RB, Soares RS, Victorino RM, Sousa AE. Rate of increase in circulating IL-7 and loss of IL-7R α expression differ in HIV-1 and HIV-2 infections: two lymphopenic diseases with similar hyperimmune activation but distinct outcomes. *J Immunol* 2007; 178:3252–9.
- 26 Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 2003; 4:330–6.
- 27 Yagı H, Nomura T, Nakamura K *et al.* Crucial role of FOXP3 in the development and function of human CD25+CD4+ regulatory T cells. *Int Immunol* 2004; 16:1643–56.
- 28 Wang J, Ioan-Facsinay A, van der Voort EI, Huizinga TW, Toes RE. Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. *Eur J Immunol* 2007; 37:129–38.
- 29 van Neerven RJ, Wikborg T, Lund G *et al.* Blocking antibodies induced by specific allergy vaccination prevent the activation of CD4+ T cells by inhibiting serum-IgE-facilitated allergen presentation. *J Immunol* 1999; 163:2944–52.
- 30 Sakaguchi S. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol* 2004; 22:531–62.
- 31 Smith TR, Alexander C, Kay AB, Larche M, Robinson DS. Cat allergen peptide immunotherapy reduces CD4(+) T cell responses to cat allergen but does not alter suppression by CD4(+) CD25(+) T cells: a double-blind placebo-controlled study. *Allergy* 2004; 59:1097–101.
- 32 Cavani A, Nasorri F, Ottaviani C, Sebastiani S, De Pita O, Girolomoni G. Human CD25+ regulatory T cells maintain immune tolerance to nickel in healthy, nonallergic individuals. *J Immunol* 2003; 171:5760–8.
- 33 Hartl D, Koller B, Mehlhorn AT *et al.* Quantitative and functional impairment of pulmonary CD4+CD25hi regulatory T cells in pediatric asthma. *J Allergy Clin Immunol* 2007; 119:1258–66.
- 34 Akbar AN, Vukmanovic-Stejić M, Taams LS, Macallan DC. The dynamic co-evolution of memory and regulatory CD4+ T cells in the periphery. *Nat Rev Immunol* 2007; 7:231–7.
- 35 Des Roches A, Paradis L, Knani J *et al.* Immunotherapy with a standardized Dermatophagoides pteronyssinus extract. V. Duration of the efficacy of immunotherapy after its cessation. *Allergy* 1996; 51:430–3.
- 36 Eng PA, Borer-Reinhold M, Heijnen IA, Gnehm HP. Twelve-year follow-up after discontinuation of preseasonal grass pollen immunotherapy in childhood. *Allergy* 2006; 61:198–201.
- 37 Pajno GB, Morabito L, Barberio G, Parmiani S. Clinical and immunologic effects of long-term sublingual immunotherapy in asthmatic children sensitized to mites: a double-blind, placebo-controlled study. *Allergy* 2000; 55:842–9.