

ORIGINAL ARTICLE Clinical Mechanisms in Allergic Disease

Allergen-specific CD4⁺ T cell responses in peripheral blood do not predict the early onset of clinical efficacy during grass pollen sublingual immunotherapy

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Summary

Background Surrogate biomarkers of efficacy are needed in support of allergen-specific immunotherapy.

Objective The aim of this study was to relate changes in peripheral CD4⁺ T cell responses to clinical efficacy during sublingual immunotherapy (SLIT).

Methods Allergen-specific CD4⁺ T cell responses were assessed in peripheral blood mononuclear cells (PBMCs) from 89 grass pollen-allergic individuals enrolled in a double-blind placebo-controlled SLIT study conducted in an allergen exposure chamber (ClinicalTrials.gov NCT00619827). Surface phenotype, proliferative responses, cytokine production and gene expression were analysed in coded samples at baseline, and after 2 and 4 months of SLIT, in PBMCs after *in vitro* allergen stimulation or among MHC class II/peptide (pMHCII)-tetramer-positive CD4⁺ T cells.

Results SLIT induced a 29.3% improvement of the average rhinoconjunctivitis total symptom score in the active group, when compared to the placebo group. In parallel, only minor changes in proportions of CD4⁺ T cells expressing Th1 (CCR5⁺, CXCR3⁺), Th2 (CRTh2⁺, CCR4⁺) and Treg (CD25⁺, CD127⁻, Foxp3⁺) markers were detected. A down-regulation of IL-4 and IL-10 gene expression and IL-10 secretion ($P < 0.001$) were observed, as well as a decrease in the frequency of potential “pro-allergic” CD27⁻ Th2 cells from patients receiving active tablets ($P < 0.001$), but without any correlation with clinical benefit. pMHCII-tetramer analyses failed to document any major impact in both numbers and polarization of circulating Phl p 1- and Phl p 5-specific CD4⁺ T cells, confirming that early clinical improvement during SLIT is not associated with dramatic alterations in T lymphocyte responses.

Conclusion & Clinical Relevance Changes in patterns of peripheral CD4⁺ T cells are not markers for the early onset of efficacy during SLIT.

Keywords allergen, biomarker, CD4⁺ T lymphocyte, grass pollen, MHC class II tetramers, sublingual immunotherapy

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Introduction

There is a major interest in monitoring immunological changes induced by allergen-specific immunotherapy to identify surrogate markers of clinical efficacy. Herein, we focus on sublingual immunotherapy (SLIT), well established as an efficacious treatment for type I respiratory allergies [1, 2]. SLIT has been proposed to reori-

ent allergen-specific CD4⁺ T cells from a Th2 to a Th1 profile (immunodeviation) [3–7]. In addition, it induces regulatory T cells exhibiting a suppressive activity, via IL-10 or TGF- β secretion as well as cell–cell contact [3–15]. Although most of these changes have been observed in peripheral blood, recent studies have suggested an alteration in patterns of CD4⁺ T cell responses within the nasal mucosa [13]. In contrast,

other studies failed to document changes in allergen-specific T cell responses during SLIT longitudinal studies [16, 17]. Importantly, in all circumstances, the link between modulation of circulating allergen-specific CD4⁺ T cells and clinical improvement remains to be established.

One inherent difficulty to document the impact of immunotherapy on CD4⁺ T cells is to rely upon well-standardized assays [18]. In this regard, allergen-specific CD4⁺ T cell responses are commonly analysed by monitoring proliferation, activation and cytokine secretion in peripheral blood mononuclear cells (PBMCs) following *in vitro* allergen stimulation. More recently, the availability of MHC class II-peptide (pMHCII)-tetramers made it possible to investigate at a single-cell level the polarization of adaptive immune responses against selected epitopes within a given allergen [19–26]. We also focused on recently described 'pro-allergic' Th2 cells [27], corresponding to a subset of CD45RO⁺CD27⁻ terminally differentiated CD4⁺ T cells expressing CRTh2, the prostaglandin D2 receptor used as a marker for Th2 cells [28]. These CD27⁻ cells contain most allergen-specific CD4⁺ T cells with a capacity to produce high levels of Th2 cytokines [27]. Herein, combining those approaches, we took advantage of a double-blind placebo-controlled study conducted in an allergen challenge chamber to thoroughly investigate early effects of SLIT on allergen-specific CD4⁺ T cell responses. In this study, clinical efficacy was documented at an individual patient level in a cohort of grass pollen-allergic patients as early as after 1 month of SLIT and over the 4-month study period [29], thereby offering an opportunity to investigate a potential correlation with changes in T cell polarization. We report that changes in phenotype and cytokine secretion in grass pollen-specific CD4⁺ T cells induced by SLIT in such a time frame are limited and unrelated to clinical improvement.

Methods

Design of the VO56.07A SLIT study conducted in an allergen challenge chamber

Details of this study have been published elsewhere [29]. Briefly, this randomized, double-blind placebo-controlled study was performed in an allergen exposure chamber, at the Allergy Center in Vienna West (Austria) after approval by a local Ethical committee, Ethikkommission der Österreichischen Arbeitsgemeinschaft für klinische Pharmakologie und therapie (Protocol 05/07 (EudraCT-2007-001651-20) n° VO56.07A-2607) (ClinicalTrials.gov NCT00619827). After screening and providing written informed consent, 89 subjects suffering from moderate-to-severe grass pollen-related allergic rhinoconjunctivitis were randomized 1 : 1 to receive

sublingually once a day for 4 months, either a placebo or a 300-IR SLIT tablet containing a five-grass pollen extract (Stallergenes SA, Antony, France) [30]. Patients were challenged with grass pollens before treatment (baseline challenge), as well as after 1 week and 1, 2 and 4 months of SLIT, with nasal and eye symptoms quantified to establish an average rhinoconjunctivitis total symptom score (ARTSS) [29].

Surface phenotyping, proliferative responses and analysis of cytokine secretion and gene expression

Venous blood samples were collected from each patient into citrate tubes before (visit 3 or V3) and after 2 (visit 6 or V6) and 4 (visit 7 or V7) months of treatment, prior to *in vivo* allergen challenge. PBMCs were isolated by centrifugation on Ficoll-Paque Plus (PAA, les Mureaux, France). All analyses, except surface phenotyping and proliferative responses, were conducted on frozen cells. All samples were coded to the operators with respect to their origin (i.e. patient from active or placebo group, clinical responder vs. non-responder) until completion of biological analyses. Baseline and post-treatment samples from a given patient were thawed and analysed within the same experiment. The mean $\pm$ SD viability of PBMCs after thawing was $84 \pm 6\%$.

Surface phenotyping and proliferative responses were evaluated as described elsewhere [22]. Potential highly 'pro-allergic' Th2 (i.e. CD27⁻ Th2) cells were detected among a subset of terminally differentiated memory Th2 (i.e. CD45RO⁺/CD27⁻/CRTh2⁺) cells in frozen PBMCs.

Cytokine secretion and gene expression were assessed using PBMCs, frozen at visits V3, V6 and V7 after restimulation with grass pollen or recall allergens, as described elsewhere [22].

pMHCII-tetramer analysis of CD4⁺ T cells specific for Phl p 1 and Phl p 5

pMHCII-tetramer analyses were performed as previously described [22, 27, 31] in patients with HLA-DRB1*0101 (DR1), HLA-DRB1*0301 (DR3), HLA-DRB1*0401 (DR4) or HLA-DRB1*1501 (DR15) haplotypes. To expand CD4⁺ T cells specific for Phl p 1 and Phl p 5, cells from DR1 and DR3 patients were stimulated for 14–16 days with Phl p 1_{153–172} or Phl p 1_{171–185} peptides (used at 10 μ g/mL) in presence of 20 UI/mL recombinant IL-2 (Roche, Meylan, France). Cells from DR4 patients were incubated with 20 μ g/mL Phl p 1_{100–114} peptides in the form of li-key conjugate peptide mixes [26, 32] and IL-2. Cells from patients with a DR15 haplotype were stimulated with 10 μ g/mL of a pool of Phl p 5a_{33–52}, Phl p 5a_{233–252} and Phl p 5b_{129–148} peptides in presence of IL-2. All pMHCII-tetramers used in this study were

produced by the Benaroya Research Institute (Seattle, WA, USA) [33]. pMHCII-tetramers loaded with irrelevant peptides were used as controls. Surface phenotyping assays were performed as described elsewhere [22]. Cytokine secretion was assessed in Phl p 1-specific CD4⁺ T cells using a cytokine secretion assay (CSA) (Miltenyi Biotech, Bergish, Germany) [21], and in Phl p 5-specific CD4⁺ T cells using intracellular cytokine staining [27]. Cells were stimulated for 4–5 h with appropriate peptide mixes (10–20 µg/mL) for CSAs and with 50 ng/mL PMA and 1 µg/mL ionomycin (Sigma-Aldrich, St Louis, MO, USA) in presence of 10 µg/mL monensin (BD Pharmingen, San Jose, CA, USA) for intracellular cytokine staining. Cells were analysed using either an LSRII flow cytometer (Becton Dickinson, San Jose, CA, USA) or an FC500 cytometer (Beckman Coulter), with a minimum of 100 (phenotype) or 500 (cytokine measurement) pMHCII-tetramer-reactive cells acquired.

Statistical analysis

We used a Wilcoxon matched-pairs signed rank test to study variations of biological parameters between Visit 3 and Visit 7 within groups, and a Mann–Whitney test to compare results obtained at Visit 7 between active and placebo patients. Spearman tests were used for correlation analyses. Data are represented using Tukey box plots summarizing median, upper and lower quartiles. Outliers (i.e. data not included between the whiskers corresponding to values inferior or superior by 1.5-fold to lower and upper quartiles respectively) were plotted as dots.

Results

Summary of clinical efficacy results

Efficacy results of the clinical study were previously reported in details elsewhere [29]. Briefly, in the active group, the ARTSS mean improvement from baseline reached 30.9% and 41.8% after 1 month and 4 months of active treatment, respectively, and 12.9% and 18.5% after 1 and 4 months of placebo exposure respectively. Changes of ARTSS from baseline were significantly higher in the active group when compared to the placebo group ($P < 0.001$), thus establishing unambiguously the clinical efficacy of this SLIT protocol [29].

Changes in the frequency of peripheral blood CD4⁺ T cells expressing particular phenotypes

PBMCs isolated from each patient before (V3), and after 2 (V6) and 4 (V7) months of SLIT, were surface stained with antibodies specific for various markers associated with either Th1, Th2 or Treg cells. Percentages of Th2

(i.e. CRTh2⁺ or CCR4⁺) cells among CD4⁺ T cells significantly decreased in both active and placebo groups after treatment ($P < 0.001$, Fig. 1a). Proportions of CXCR3⁺ and CCR5⁺ Th1 cells significantly decreased after 4 months of SLIT, in both placebo and active groups ($P < 0.05$ and $P < 0.001$ for CXCR3 and CCR5 respectively). The frequency of potential CD4⁺ Treg (i.e. CD25⁺ CD127[−] Foxp3⁺) cells increased substantially after 4 months of immunotherapy, irrespective of the treatment group ($P < 0.001$). CTLA-4⁺ CD4⁺ T cells were marginally increased from baseline after 2 months of treatment in both active and placebo groups (data not shown), whereas proportions of GITR⁺ CD4⁺ T cells decreased during treatment in both placebo and active groups ($P < 0.01$ and $P < 0.001$ respectively) (Fig. 1a). Although not shown, no variations were observed regarding CD45RO⁺/CD62L⁺, CD45RO⁺/CD62L[−], CD30⁺ and CD103⁺ CD4⁺ T cell subsets.

To complement those analyses conducted on global T cell populations irrespective of their specificity, potential 'pro-allergic' CD4⁺ T cells producing high levels of Th2 cytokines in response to allergens, further referred as CD27[−] Th2 cells, were detected within PBMCs with a combination of five antibodies [27]. Percentages of CD27[−] Th2 cells among CD4⁺ T cells remained stable in the placebo group, whereas they slightly decreased after 4 months of SLIT in the active group (from 100% to $82.8 \pm 38\%$, $P < 0.001$) (Fig. 1b, left panel). At an individual level, a decrease in percentages of CD27[−] Th2 cells was observed in most patients within the active group, whereas a larger heterogeneity was seen for placebo patients (Fig. 1b, right panels). Collectively, although several changes in frequencies of peripheral CD4⁺ T cells expressing specific polarization markers were detected in this study, only a small decrease in CD27[−] Th2 cells, thought to contain most allergen-specific CD4⁺ T cells, was observed in the active but not the placebo group, with a significant difference between active and placebo patients after treatment ($P < 0.01$).

Changes in proliferative response, gene expression and cytokine production in allergen-stimulated PBMCs

The proliferative capacity of allergen-stimulated PBMCs was further assessed by measuring [³H]-Thymidine incorporation before, and after 2 and 4 months of SLIT, following *in vitro* stimulation with either a grass pollen extract, purified Dac g 5 or recall antigens. A slight but significant decrease in cell proliferation in response to the grass pollen extract- or Dac g 5- stimulation was observed in placebo-treated patients after 4 months of SLIT ($P < 0.01$ and $P < 0.001$ respectively) (Fig. 2, upper panels). However, the relevance of such variations is questionable as a decrease in proliferative responses to recall antigens was also seen over time,

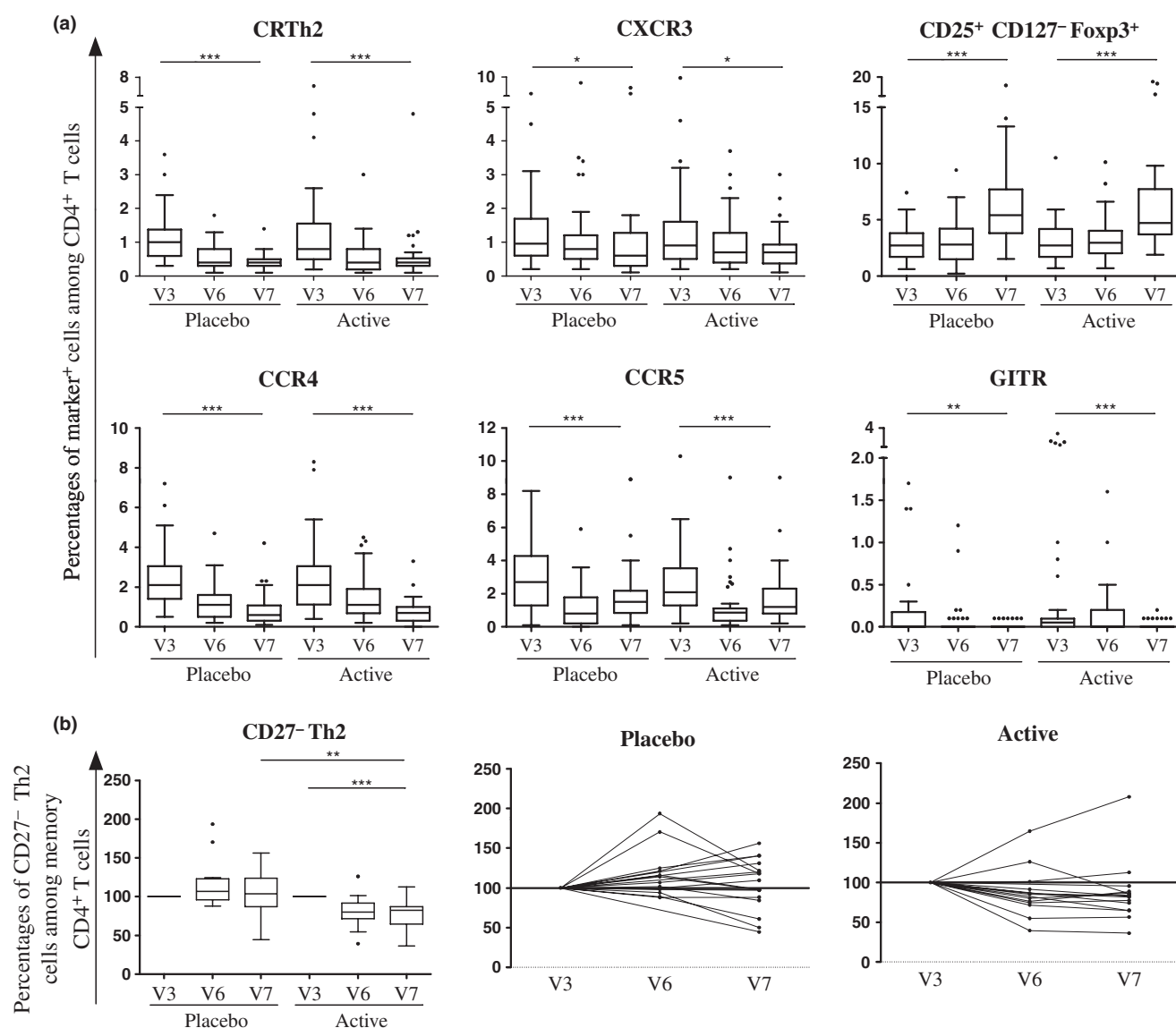


Fig. 1. Surface phenotype of peripheral blood CD4⁺ T cells during grass pollen sublingual immunotherapy (SLIT). Fresh peripheral blood mononuclear cells (PBMCs) collected before (V3), after 2 (V6) and 4 months (V7) of SLIT in grass pollen-allergic patients receiving active or placebo tablets were stained with antibodies directed against markers of T cell polarization. (a) Percentages of CD3⁺ CD4⁺ T cells expressing each of the markers tested are represented using Tukey box plots. Outliers (i.e. values inferior or superior by 1.5-fold to lower and upper quartiles respectively) were plotted as dots. (b) Assessment of 'pro-allergic' Th2 (i.e. CD27⁻ Th2) cells among memory (i.e. CD45RO⁺ CD4⁺) T cells at the cohort (left panel) and individual level (right panels). Percentages of CD27⁻ Th2 cells were normalized to 100 for each patient at baseline (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

even if responses to PHA stimulation remained stable (Fig. 2 lower panels).

We subsequently analysed at various time points during SLIT the expression of genes encoding transcription factors or cytokines in PBMCs stimulated for 1 and 5 days with either a five-grass pollen extract or recall antigens. Regarding transcription factors, the expression of T-bet, but not GATA3 or Foxp3, was up-regulated in PBMCs obtained after 2 months of SLIT from patients in both placebo and active groups, but it did not differ from baseline levels after 4 months (Fig. 3a, upper

panels). TGF- β gene expression was significantly up-regulated at V7 in PBMCs from the placebo group ($P < 0.001$) (Fig. 3a, lower left panel). Although not shown, similar trends were observed after recall antigen stimulation for both T-bet and TGF- β gene expression. IFN- γ and IL-17 gene expression were not significantly modified during SLIT (Fig. 3a). A decrease in IL-10 mRNA was detected in both placebo and active groups, but this down-regulation was stronger in the active group ($P < 0.01$ and $P < 0.001$ respectively) (Fig. 3a). Such variations were not seen for recall antigen-induced

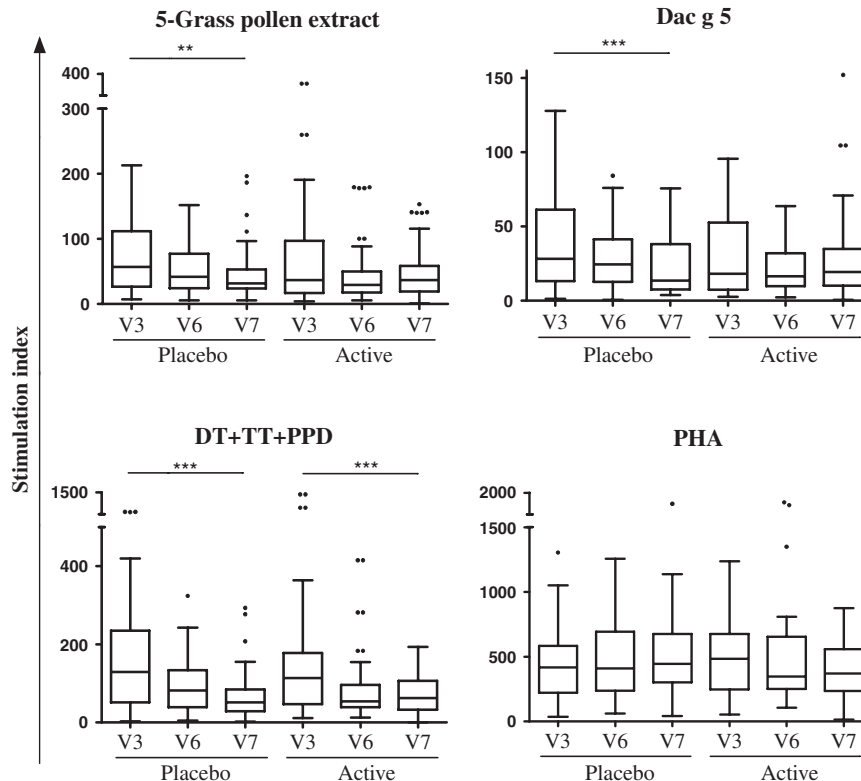


Fig. 2. Proliferative responses of allergen-stimulated peripheral blood mononuclear cells (PBMCs) during grass pollen sublingual immunotherapy (SLIT). At each time point before (V3) and after 2 (V6) and 4 (V7) months of SLIT, PBMCs were stimulated for 6 days with either 1000 IR/mL five-grass pollen extract, 10 µg/mL Dac g 5 or 10 µg/mL DT+TT+PPD recall antigens, or for 3 days with 1 µg/mL PHA, as a positive control. Proliferation was assessed in quadruplicates using [³H]-Thymidine incorporation as described in Methods. Data are expressed as a stimulation index and represented using Tukey box plots. Outliers (i.e. values inferior or superior by 1.5-fold to lower and upper quartiles respectively) were plotted as dots. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

IL-10 mRNA levels (data not shown). IL-4 mRNA levels significantly decreased from baseline after 4 months of treatment ($P < 0.001$) in the active group only, with a significant difference between active and placebo patients after treatment ($P < 0.05$) (Fig. 3a).

To confirm and extend these results, cytokines were further quantified in supernatants of PBMCs, following stimulation with a five-grass pollen extract. Levels of IFN- γ , IL-5, IL-13 and IL-17 secreted by PBMCs were stable in both active and placebo groups during the 4 months of SLIT relative to baseline (Fig. 3b). The secretion of TGF- β increased in placebo, but not in active patients between V3 and V7, whereas levels of TNF- α decreased in both groups. We observed a sustained decrease of IL-10 secretion during SLIT, reaching significance only in the active group (Fig. 3b).

Monitoring of Phl p 1- and Phl p 5-specific CD4⁺ T cells using pMHCII-tetramers

To further investigate changes in allergen-specific CD4⁺ T cells, analyses with pMHCII-tetramers were conducted in PBMCs from 32 subjects exhibiting either DR1, DR3,

DR4 or DR15 haplotypes, following *in vitro* peptide-driven amplification for 14–16 days in presence of IL-2, as described in Materials and Methods. We detected Phl p 1- or Phl p 5-specific CD4⁺ T cells in PBMCs from 29 patients, irrespective of the time point considered (Fig. 4a). Higher percentages of pMHCII-tetramer-positive cells were detected in CD4⁺ PBMCs from patients exhibiting DR4 or DR15 haplotypes, likely due to the higher avidity of corresponding tetramers. Beyond individual variations, no significant trends were observed during SLIT in patients treated with placebo or active tablets, regarding both numbers of circulating pMHCII-tetramer-positive CD4⁺ T cells as well as mean fluorescence intensities, the latter reflecting tetramer-binding avidities (Fig. 4a, left and right panels respectively). Phenotypic and functional properties of Phl p 1- and Phl p 5-specific CD4⁺ T cells were further characterized at a single-cell level in samples from patients displaying sufficient (i.e. > 0.5%) pMHCII-tetramer-reactive cells among CD4⁺ T cells. Surface phenotypes could be assessed in six patients from the active group and functional analyses were conducted using either CSA ($n = 4$ active patients) or intracellular cytokine staining ($n = 5$,

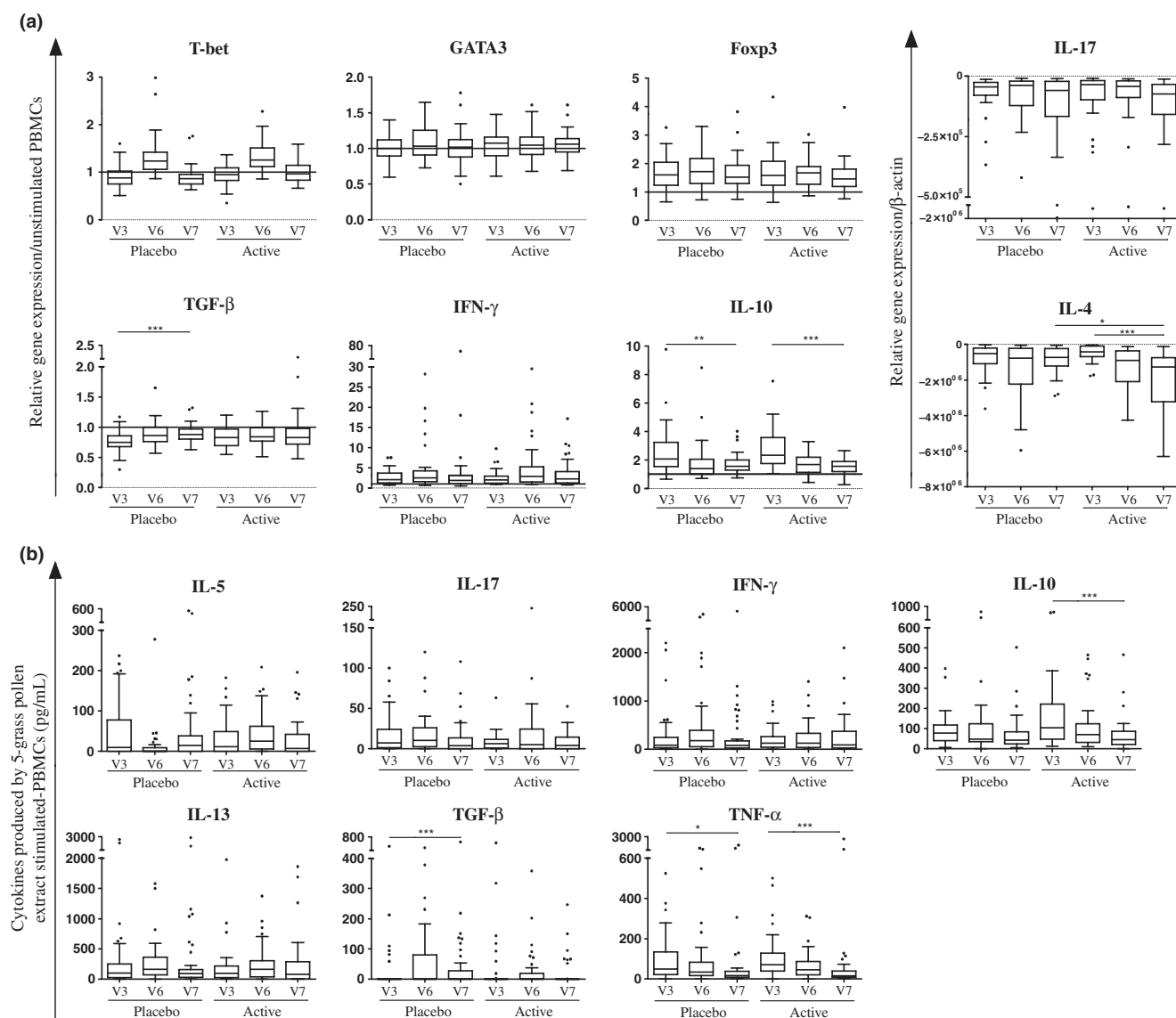


Fig. 3. Transcription factor/cytokine gene expression and cytokine production during grass pollen sublingual immunotherapy (SLIT). Thawed peripheral blood mononuclear cells (PBMCs) were stimulated with 400 IR/mL five-grass pollen extract for 1 or 5 days. Gene expression and cytokine production were assessed before (V3), after 2 (V6) and 4 months (V7) of SLIT. (a) Transcription factor and cytokine gene expression were assessed by quantitative RT-PCR. T-bet, GATA3, Foxp3, IFN- γ , IL-4, IL-10 gene expression were analysed following 1 day of stimulation, whereas TGF- β and IL-17 gene expression were quantified after 5 days of stimulation. For all genes, mRNA levels were normalized to levels of β -actin mRNA. For T-bet, GATA3, Foxp3, TGF- β , IFN- γ and IL-10, gene expression was expressed as relative amounts of mRNA in stimulated *vs.* unstimulated cells. Fold-changes are represented using Tukey box plots. (b) IL-10 and TNF- α cytokine levels were measured by CBA in cell culture supernatants after 1 day of grass pollen extract stimulation, whereas amounts of secreted IFN- γ , IL-5, IL-13, IL-17, TGF- β were measured after 5 days. Data are represented using Tukey box plots. Outliers (i.e. values inferior or superior by 1.5-fold to lower and upper quartiles respectively) were plotted as dots. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

including 4 placebo patients and 1 active patient analysed with three different peptides). No clear trend discriminating patients from placebo or active groups was observed during SLIT in the polarization of pMHCII-tetramer⁺ CD4⁺ T cells, irrespective of the epitope considered, when comparing markers associated with either Th2 (i.e. CCR4⁺, IL-4⁺ and IL-5⁺), Th1 (i.e. CXCR3⁺ and IFN- γ ⁺), Treg (i.e. CTLA-4⁺, and IL-10⁺) or Th17 (i.e.

CCR4⁺/CCR6⁺ and IL-17⁺) subsets (Fig. 4b–d). Proportions of effector and central memory-specific CD4⁺ T cells did not fluctuate during treatment (data not shown). Altogether, pMHCII-tetramer-based analyses suggested that a 4-month SLIT course does not induce any dramatic depletion of Th2 cells nor a significant variation in the polarization of peripheral allergen-specific CD4⁺ T cells.

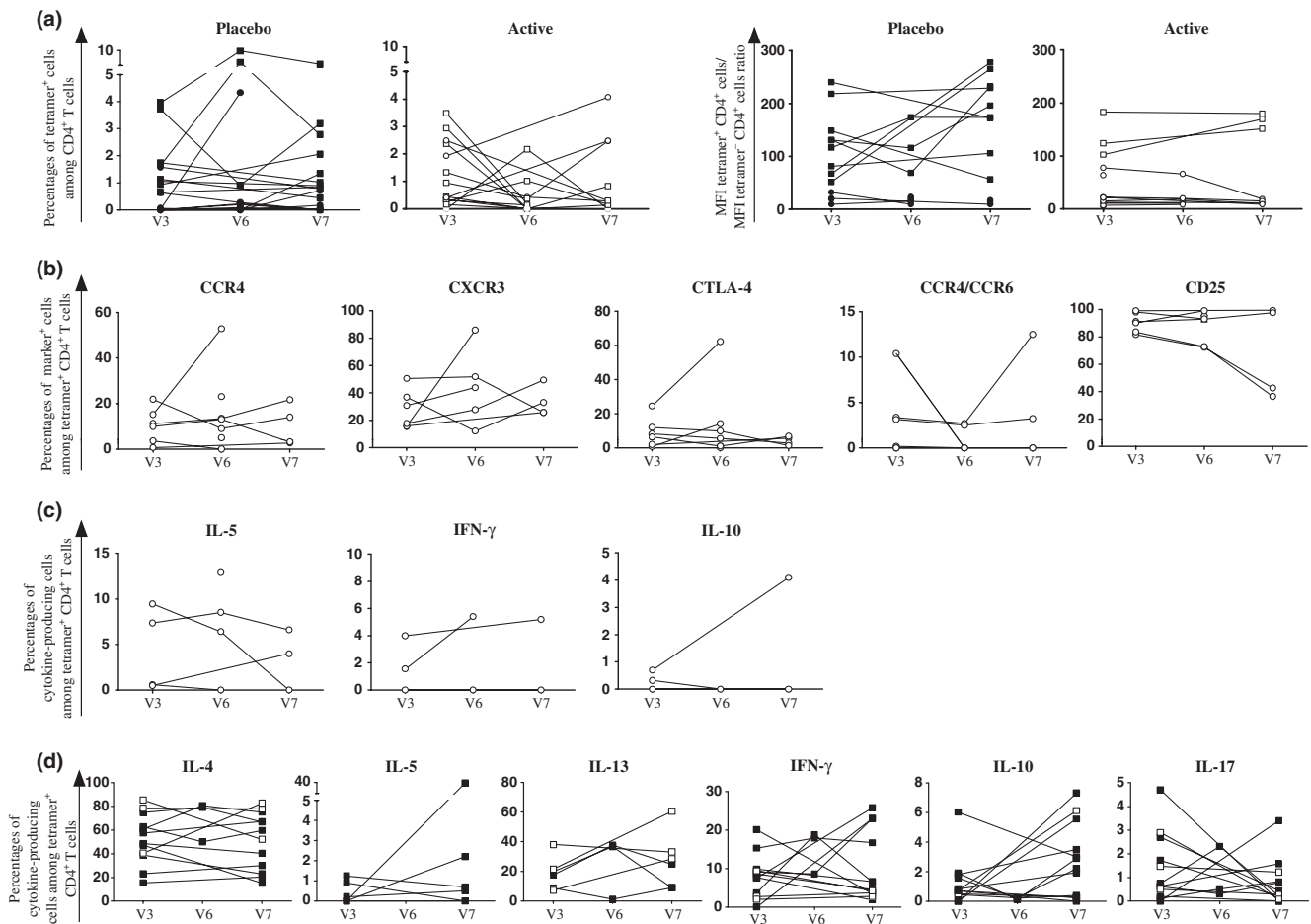


Fig. 4. Detection and characterization of peripheral Phl p 1- and Phl p 5-specific CD4⁺ T cells using pMHCII-tetramers during sublingual immunotherapy (SLIT). Before (V3), after 2 (V6) and 4 (V7) months of SLIT, thawed peripheral blood mononuclear cells (PBMCs) from 32 patients with either a DR1, DR3, DR4 or DR15 HLA background were stimulated *in vitro* for 14–16 days and subsequently stained with appropriate pMHCII-tetramers. (a) pMHCII-tetramer⁺ CD4⁺ T cell frequency and avidity. The latter was expressed as a ratio between MFIs from tetramer⁺ and tetramer⁻ CD4⁺ T cells. (b) Phenotype of pMHCII-tetramer⁺ CD4⁺ T cells. (c) Cytokine production by pMHCII-tetramer⁺ CD4⁺ T cells assessed by cytokine secretion assay (CSA) following Phl p 1 peptide stimulation. (d) Cytokine production by Phl p 5-specific pMHCII-tetramer⁺ CD4⁺ T cells measured by intracellular staining after PMA/Ionomycin stimulation • Phl p 1-specific CD4⁺ T cells (DR1, DR3, DR4, placebo patients); ○ Phl p 1-specific CD4⁺ T cells (DR1, DR3, DR4, active patients); ■ Phl p 5-specific CD4⁺ T cells (DRB5, placebo patients); □ Phl p 5-specific CD4⁺ T cells (DRB5, active patients).

Correlation between biological changes and clinical efficacy

We further analysed the potential correlation between the few biological changes detected in PBMCs related to treatment (i.e. observed in patients from the active, but not placebo group) and clinical efficacy of SLIT. Biological changes were expressed as ratios, given the asymmetric distribution of results. Specifically, we focused on those variations suggesting a potential decrease in Th2 responses, which were plotted against improvement in the ARTSS documented for each individual patient. As shown in Fig. 5 (centre and right panels), the down-regulation of IL-4 or IL-10 gene expression as well as IL-10 secretion by allergen-stimulated T cells was unrelated to clinical benefit. If anything, an inverse

relationship was found between clinical efficacy and the decrease observed in the active group of circulating CD27⁺ Th2 cells (Fig. 5, left panels).

Discussion

CD4⁺ T cells play a major role in both allergic inflammation and its resolution, through the production of distinct sets of cytokines [4, 5, 7, 34]. Th2 cells contribute directly to allergen-driven pathogenesis, by controlling B-cell differentiation and antibody class switching towards IgE production on one hand, and by recruiting and activating proinflammatory eosinophils, basophils and mast cells in target organs on the other hand [7, 34]. Multiple studies have proposed that immunotherapy in general and SLIT in particular

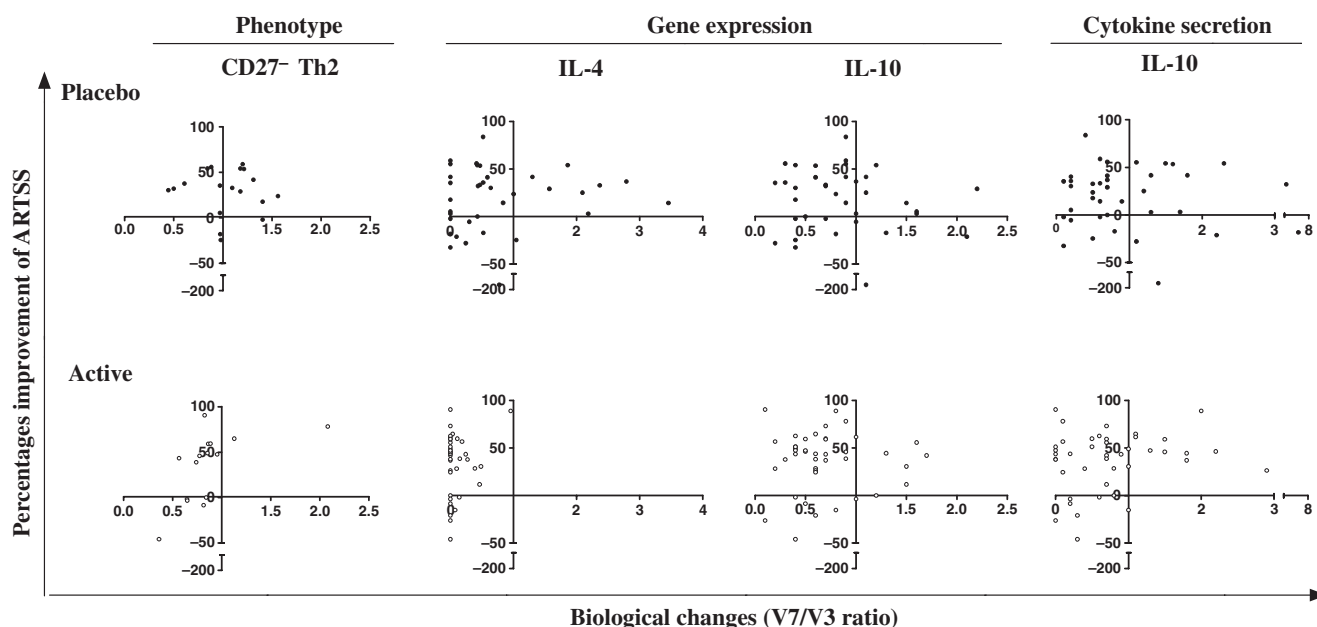


Fig. 5. Correlation between clinical efficacy of grass pollen sublingual immunotherapy (SLIT) and changes in Th2 markers. Changes in CD27⁺ Th2 cell percentages, IL-4 and IL-10 gene expression or IL-10 secretion after grass pollen extract stimulation of peripheral blood mononuclear cells (PBMCs) are plotted for each individual patient from the placebo (upper panels) or active (lower panels) groups, as a ratio between results obtained for each parameter at V7 vs. V3. In ordinate, clinical responses are expressed for each individual patient as a percentage of improvement in the average rhinoconjunctivitis total symptom score (ARTSS) after 4 months of SLIT relative to baseline levels (i.e. before treatment). From left to right diagrams, *P* values for correlations were 0.60, 0.54, 0.61, 0.73 and 0.02, 0.84, 0.95, 0.44 for placebo and active patients respectively (Spearman test).

induce a decrease in Th2 responses, together with the induction of allergen-specific CD4⁺ T cells producing IFN- γ , IL-10 and TGF- β (i.e. Th1/Treg switch) [3–15]. Noteworthy, most studies investigating the impact of SLIT on the polarization of peripheral blood CD4⁺ T lymphocytes were open studies conducted in small cohorts of patients, and as such could not establish any firm link with clinical efficacy at an individual level. Thus, with the aim to strengthen the scientific rationale behind SLIT, further investigations of changes in allergen-specific CD4⁺ T cell responses remain necessary to determine whether they contribute to clinical benefit, and beyond, to identify potential surrogate markers of efficacy.

To this aim, we took advantage of a double-blind placebo-controlled SLIT study conducted in 89 grass pollen-allergic patients to assess the impact of treatment on peripheral blood CD4⁺ T cells. In this trial, individual clinical responders were distinguished from non-responders, as multiple grass pollen challenges were performed in an allergen exposure chamber before and during SLIT [29]. As reported elsewhere, the grass pollen tablet was highly efficacious in reducing symptoms as early as after 1 month of treatment, when compared with the placebo group, with a clinical benefit sustained during the three subsequent months of treatment [29]. In such a setting, using biological samples coded to the operators, phenotype, proliferation, cyto-

kine production and gene expression were measured in PBMCs from allergic patients, before, as well as after 2 and 4 months of immunotherapy.

Several biological changes observed during SLIT with active tablets were also detected in control groups, and thus are not considered relevant to explain clinical efficacy. For example, we observed a small decrease in grass pollen extract- and Dac g 5-induced proliferative responses in the placebo group during treatment. Furthermore, this trend was also seen with recall antigen stimulation. Similarly, the proportions of CD4⁺ T cells expressing markers associated with Th1 (CCR5) or Th2 (CRTh2, CCR4) cell polarization were decreased, whereas regulatory T cells were rather up-regulated during SLIT, in both active and placebo groups. The expression of a number of genes encoding transcription factors or cytokines was altered during treatment in PBMCs from patients receiving placebo tablets. The latter is likely due to interassay variability, although we cannot exclude an impact on immune parameters of allergen exposure in the challenge chamber. These observations demonstrate the importance of using well-controlled experimental conditions, as well as proper standardized T cell assays to draw conclusions on comparative immunomonitoring data generated during longitudinal studies [18].

Some of our results are in agreement with previous reports. For example, we observed an induction of T

cells with a potential Treg phenotype after SLIT, but in our study, similar changes were shown in the placebo group. Interestingly, a study in house dust mite-allergic patients reported that Treg cells increased in both active and placebo groups after 6 months of SLIT [10], but this up-regulation was sustained after a year only in the active group, raising the possibility that a 4-month treatment course is too short to elicit significant changes in CD4⁺ T cell polarization detectable in peripheral blood. The latter does not preclude the occurrence of potential local alterations in T cell polarization, i.e. at mucosal surfaces, as was recently detected during subcutaneous immunotherapy (SCIT) [35] and SLIT protocols [13]. Although an increase in the secretion of IL-10 or TGF- β , or both of these regulatory cytokines has been reported previously in PBMCs following sublingual immunotherapy [5, 8–12, 14, 15], we did not observe those alterations, in accordance with others studies [17, 18]. We did not detect any significant induction of Th1 (i.e. CXCR3⁺ and IFN- γ ⁺) cells in agreement with another study evaluating a 1-year birch pollen SLIT protocol [10].

Interestingly, some significant changes were induced in patients receiving the active tablet, but not the placebo. For example, we observed that IL-4 gene expression as well as IL-10 secretion was selectively decreased in the active group. Although such variations suggest a potential down-regulation of allergen-specific Th2 cells following SLIT, no correlation with clinical benefit at an individual patient level was noticed. One potential limitation of those analyses is that they were conducted in PBMCs populations in which allergen-specific CD4⁺ T cells are a minority. Furthermore, there is growing evidence that Th2 cells are heterogeneous, suggesting the pertinence of focusing on pathogenic subsets [27, 36]. Thus, we further monitored allergen-specific cells using pMHCII-tetramers, and analysed 'pro-allergic' Th2 cells, i.e. a subset of CD45RO⁺ CD27⁻ terminally differentiated CD4⁺ T cells, recently shown to contain most allergen-specific IL-5 secreting cells [27]. Our analyses showed a minor, albeit significant decrease in CD27⁻ Th2 cells in patients receiving active tablets, however, unrelated to clinical improvement. This observation was further corroborated using pMHCII-tetramers specific for Phl p 1-

and Phl p 5-reactive CD4⁺ T cells, with no variations observed in the frequency, nor in the polarization of pMHCII-tetramer-positive cells, irrespective of the treatment group and the time point considered (Fig. 4). The potential beginning trend in the down-regulation of CD27⁻ Th2 cells detected after 4 months of SLIT suggests that a longer treatment period might be needed to deplete the pool of circulating Th2 cells, as previously shown for long-lived IgE-producing B cells, which usually occurs only after 1 year or more of immunotherapy [4, 6, 7]. The latter is consistent with the recent observation that such CD27⁻ Th2 cells are depleted in the blood of patients having received SCIT for at least 3 years [27]. Interestingly, our previous studies on this cohort of patients failed to establish a correlation between changes in basophil activation and clinical efficacy [37]. In contrast, we observed that the expression of two markers of regulatory dendritic cells was up-regulated only in PBMCs from clinical responders [38].

In conclusion, changes in the polarization of allergen-specific CD4⁺ T in peripheral blood cannot be used to document the early onset (i.e. within 4 months) of SLIT efficacy. Whether the induction of Th1/Treg cells and the down-regulation of Th2 cells constitute markers associated with long-term immunotherapy efficacy and/or sustained protection after treatment cessation remain to be investigated.

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Conflict of interest

Hélène Moussu, Stéphane Horiot, Anne-Cécile Rimaniol, Olivier de Beaumont, Véronique Baron-Bodo, Philippe Moingeon are employees at Stallergenes S.A. The remaining authors declare no conflicts of interests.

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