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Are regulatory T cells required for the suppression of allergic manifestations in diseased humans upon allergen-specific immunotherapy?

Bachelor Thesis Biomedical Sciences

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Abstract

Allergies are characterized by an increase in $CD4^+$ Th2 cells. Regulatory T (Treg) cells are able to suppress inflammatory cells via multiple pathways and can therefore help induce tolerance against environmental allergens. This suggests a possible role for Treg cells in allergy treatment by specific immunotherapy (SIT). It is currently unknown whether Treg cells are really required for the suppression of allergic manifestations in diseased humans upon SIT treatment. Therefore, it was investigated whether Treg cells are required for successful SIT by reviewing several studies. The increase in Th2 cells associated to allergies was confirmed using ELISPOT analysis. When allergic patients were treated with SIT, changes in several cell subsets were observed. First, Th2/Th1 and Th2/Tr1 ratios were decreased by SIT treatment. An increase in the number of Th2 and Tr1 cells upon SIT treatment was demonstrated using ELISPOT analysis. The increase in Tr1 cells was more prevalent than the increase in Th2 cells. Co-culturing of Tr1 and effector T (Teff) cells showed that Tr1 cells are able to suppress Teff cells. It was also demonstrated that $Foxp3^+$ Treg cells are not increased by SIT, but that $Foxp3^-$ Treg cells are. Foxp3 expression was measured by either flow cytometry or qPCR. The number of $CD4^+CD25^+CD127^{low}$ Treg cells, which have been shown to represent $Foxp3^+$ Treg cells, was also measured by FACS. No changes in $CD4^+CD25^+CD127^{low}$ Treg cell frequencies were observed after SIT. It was also shown that $CD27^-$ allergen-specific $CD4^+$ T cells are decreased by SIT. These results together indicate that SIT increases adaptive Treg frequencies and can decrease $CD4^+CD27^-$ Th2 cells.

Contents

Introduction

Immune response in allergies

Type I hypersensitivity, otherwise known as allergy, is a widely reported problem (Larché et al., 2006). One of these type I allergies is allergic rhinitis, which can be caused by pollen exposure. Allergic rhinitisaffects 30% of the general population (Licari et al., 2014) and is characterized by multiple manifestations, such as rhinorrhea, nasal obstruction, nasal itching, sneezing and ocular symptoms (de Weger et al., 2011). Development of an allergy is caused by sensitization to a specific allergen. Sensitization is characterized by the presence of allergen-specific IgE. Differentiation and clonal expansion of allergen-specific $CD4^+$ Th2 cells, which produce IL-4 and IL-13, causes the induction of B-cell Immunoglobulin class-switching to the ε-immunoglobulin heavy chain and the production of allergen-specific IgE antibodies. Allergen-specific IgE binds to the high-affinity FcεRI on the surface of mast cells and basophils. Memory T and B cells will also be generated during this step (Palomares et al., 2010). A second encounter with the allergen will then cause a biphasic response. This response is characterized by an early and a late phase. The early phase peaks 15-30 minutes after the exposure. After activation by IgE binding, basophils and mast cells will release mediators, such as histamine, kinins, prostaglandin D2, cytokines, chemokines and leukotrienes. The direct effects of these mediators are: nasal itching, sneezing, rhinorrhea and nasal congestion. These manifestations are mainly mediated by histamine (Greiner et al., 2011; Alvaro et al., 2010). The mediators released by basophils and mast cells will also cause recruitment of cells that will induce the late phase of the allergy response. The late phase includes the activation of eosinophils, T lymphocytes and additional basophils. These inflammatory cells will also start producing mediators, which can cause rhinorrhea and nasal obstruction (Licari et al., 2014; Alvaro et al., 2010). Th2 cells produce IL-4, IL-5, IL-9 and IL-13, which are involved in production of IgE and IgG4, inhibition of Th1 cells, activation of Th2 cells and B cells, eosinophil differentiation, mast cell and basophil development, upregulation of IgE receptors, expression of adhesion molecules and mucus production (Alvaro et al., 2010).

Regulatory T cells in allergies

Regulatory T cells help inhibit abnormal immune responses (Palomares et al., 2010; Robinson et al., 2004). The next paragraph will discuss the function of regulatory T (Treg) cells in allergies.

Regulatory T cells are able to suppress the inflammatory process by inhibiting DCs, which activate Th1, Th2 and Th17 cells. Treg cells can directly inhibit Th2 cells and therefore limit the production of IL-4, IL-5, IL-9 and IL-13. Furthermore, Treg cells can inhibit the influx of effector T (Teff) cells into inflamed tissue by means of cytokine secretion and are involved in tissue repair. In addition, Treg cells inhibit inflammation by influencing mast cells, basophils and eosinophils. Treg cells have a direct effect on B cells and thereby suppress the production of allergen-specific IgE and IgG4 (Figure 1). They function via multiple factors, such as IL-10, TGF-β, CTLA-4, program death-1 or histamine receptor (Palomares et al., 2010).

There are two main types of Treg cells. The first are thymus-derived naturally occurring Treg cells and are defined by their $CD4^+CD25^+$ phenotype. The second are $CD4^+CD25^+$adaptive Treg cells. Natural Treg cells express Foxp3 and function via IL-10, TGF-β, CTLA4 and PD-1.The adaptive Treg cells, which include type 1 regulatory T (Tr1) cells, operate via IL-10 and TGF-β and do not express Foxp3 (Pellerin et al., 2014; Robinson et al., 2004).The exact factor that makes these cells suppressive is still largely unknown. *In vitro* suppression by both mouse and human $CD4^+CD25^+$T cells can be detected in the absence or blockade of IL-10, TGF-β, CTLA4 and PD-1. However, absence of Foxp3 did show a loss in suppression function of $CD4^+CD25^+$ T cells and a mutation of Foxp3 was associated with immune

dysregulations. $CD25^-$ T cells could also become suppressive when transfected with Foxp3. This indicates an important role for Foxp3 expression in the suppression of abnormal immune responses, such as allergic sensitization (Robinson et al., 2004).

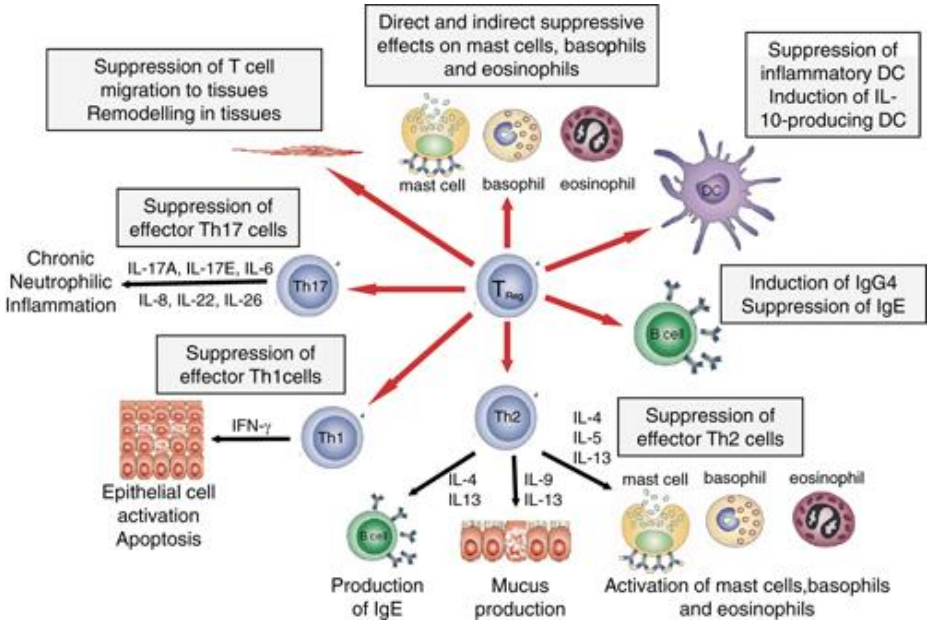

Figure 1. Function of regulatory T cells in the immune process. Red arrows indicate the suppressive effects of Treg cells. Black arrows indicate the activating effects of different effector cells (Palomares et al., 2010).

Treatment strategies in allergies

Allergic Rhinitis can be treated by means of several methods. The first is allergen avoidance. This method seems rather effective, since allergic rhinitis is mostly present during pollen season. However, complete avoidance of allergens seems rather difficult to maintain. The second possible treatment strategy is drug treatment. There are multiple drugs available to treat symptoms related to allergic rhinitis. H_1-antihistamines inhibit the action of histamine by blocking H_1-receptors and can thereby reduce allergic inflammation. They also decrease antigen presentation, expression of proinflammatory cytokines and cell adhesion molecules, and chemotaxis. Moreover, H_1-antihistamines are able to inhibit mast cell activation and histamine release in a concentration-dependent manner. Steroids, known for their anti-inflammatory function, block both the acute- and late-phase reactions. Steroids are able to reduce the number of antigen presenting cells and can impair the antigen processing function of these cells. Furthermore, steroids can reduce the influx of basophils into the nasal epithelium and can interfere with several eosinophil functions (licari et al., 2014). However, steroids and H_1-antihistamines are not curative (von Gunten et al., 2012; Alvaro et al., 2010) and steroids can cause various side effects if used for a long time (Alvaro et al., 2010). Therefore, research has focused on treatments that alter the underlying mechanisms of allergies. The abnormal immune response to allergens is one of these mechanisms. A treatment strategy directed against this aberrant immune response is allergen-specific immunotherapy (SIT). SIT is a method in which the patient is exposed to increasing amounts of allergen. This will eventually induce protective immunological changes (Alvaro et al., 2010).

Allergen-specific immunotherapy

There are currently two types of allergen-specific immunotherapy in clinical practice, subcutaneous immunotherapy and sublingual immunotherapy (SLIT). SIT focuses on changing the underlying pathology related to allergy. The cellular and molecular changes that take place during SIT consist of

four phases. The first phase is characterized by a decrease in degranulation of mast cells and eosinophils. The second phase is characterized by the development of allergen-specific Treg and regulatory B (Breg) cells, which produce IL-10 and are able to suppress allergen-specific effector T cell subsets. The antibody levels are regulated in the third phase. IgE levels will increase in the early stages of SIT and will decrease later on, while IgG4 levels will continue to increase over time. The fourth phase takes place after several months and partly results from the decrease in effector T cells induced in phase two. This phase is defined by a decrease in tissue mast cells and eosinophils and their mediators (Akdis and Akdis, 2014).

Function of regulatory T cells in SIT

Since allergies are associated with an increase in $CD4^+$ Th2 cells, it seems beneficial to suppress these Th2 cells during SIT. This could be achieved by activating Treg cells. That is, Treg cells can be generated during the second phase of SIT and can thereby inhibit effector T cells. It is currently unknown whether Treg cells are required for the suppression of allergic manifestations in diseased humansupon SIT treatment. Recent research has studied the effect of Treg cell depletion in mice. A study by Maazi et al. investigated the effect of $CD4^+CD25^+$ T cell depletion upon SIT using a mouse model of allergic asthma. Depletion of $CD4^+CD25^+$ T cells by anti-CD25 antibody treatment prior to SIT injections partially reversed suppression of airway hyperresponsiveness, but it did not affect the SIT-induced suppression of airway eosinophiliaor the level of IgE. They also demonstrated that specific depletion of $CD4^+Foxp3^+$ Treg cells reduced SIT-associated suppression of airway eosinophilia, but not of airway hyperresponsiveness and specific IgE levels (Maazi et al., 2012). This indicates that development of $Foxp3^+$ Treg cells upon SIT treatment may not be able to fully reverse the abnormal immune response related to allergies and that Treg cells are not critically required for tolerance induction against environmental allergens in SIT. However, since depletion of Treg cells upon SIT treatment is only investigated in the experimental mouse model, it is still to be determined if Treg cells are properly targeted by means of SIT treatment in diseased humans. It is hypothesized that Treg cells are not required for the suppression of allergic manifestations in diseased humans upon SIT. To investigate this, results acquired by different studies regarding SIT treatment in pollen-allergic subjects are discussed in this thesis.

Methods

1.

1.1 Study design

A study by Möbs et al. investigated the effect of birch pollen SIT on 15 birch pollen-allergic subjects. The first year of the study was completed by 13 of the 15 subjects (87%). The control groups consisted of 7 symptomatically-treated subjects allergic to birch pollen and 8 non-allergic subjects. The symptomatic treatment used for the allergic control group was not further specified. All allergic subjects had a positive skin prick test (SPT) reactivity to birch pollen extracts (ALK Prick SQ, ALK-Abelló, Hørsholm, Denmark), were positive for specific IgE to birch pollen extracts and rBet v 1 (Phadia ImmunoCAP System, Phadia, Uppsala, Sweden) and had a positive nasal provocation test (NPT) to birch pollen extracts (ALK-depot SQ, ALK-Abelló). NPT was performed with Rhinotest2000 (Allergopharma, Reinbek, Germany). SIT with *Betula verrucosa* pollen allergen extract was performed according to international guidelines and a safe-dose regimen. Patients received incremental, subcutaneously-injected, weekly doses of birch pollen allergen (ALK-depot SQ, ALK-Abelló) until a maintenance dose of 100,000 Standard Quality-Units per injection was reached; thereafter, it was given in monthly intervals. Birch pollen extract reactivity was evaluated before (m0) and 1 year (m12) after the initiation of SIT using a SPT. Patients were assessed for symptomatic changes before SIT

initiation (m0) and 1 month (m1), 3 months (m3), 6 months (m6) and 12 months (m12) after SIT initiation (Möbs et al., 2010).

1.2 Preparation of PBMCs

Citrate-phosphate-dextrose-adenine-containing peripheral blood samples were taken from birch pollen-allergic subjects and non-allergic controls at the defined time points. PBMCs were isolated by use of Pancoll (PAN-Biotech, Aidenbach, Germany) density gradient centrifugation. Samples were frozen at a concentration of 10^7 PBMCs/ml in 50% (v/v) heat-inactivated FCS (PAA Laboratories, Pasching, Austria) and 10% DMSO (Sigma, Schnelldorf, Germany), and stored in liquid nitrogen (Möbs et al., 2010).

1.3 ELISPOT assay

The presence and frequency of allergen-specific Th and Tr1 cells was measured by ELSISPOT analysis to investigate cellular immunological changes induced by birch pollen SIT. Frozen PBMCs were thawed and cultured in 24-well plates (Nunc, Langenselbold, Germany) at a concentration of 1×10^6/ml in medium consisting of RPMI 1640 (Lonza, Basel, Switzerland), 10% pooled human serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM l-glutamine (all from PAA Laboratories). PBMCs were stimulated with 10 µg/ml rBet v 1 (endotoxin level of Bet v 1 was 1.94 ng LPS/mg protein using LAL Test Kit QCL-1000, Bio-Whittaker, as specified by the manufacturer; Biomay, Vienna, Austria) or left unstimulated. On day 3, IL-2 (10 U/ml; Roche, Mannheim, Germany) and IL-7 (10 ng/ml; Miltenyi Biotec, Bergisch-Gladbach, Germany) was added for *in vitro* expansion of Bet v 1-specific T cells. PBMCs were restimulated with Bet v 1 (10 µg/ml) or PHA (1%; Sigma) as a positive control after 7 days. Plates were coated with anti-human IFN-γ or anti-IL-5 or anti-IL-10 monoclonal antibodies (mAb) overnight at 4°C, after which 2×10^5 PBMCs/well were added. Plates were incubated for 20 h at 37°C and 5% CO_2. ELISPOT analysis was performed according to the manufacturer's protocol (BD Biosciences, Heidelberg, Germany). Cytokine-producing spots were counted using an ELISPOT plate reader (A.EL.VIS, Hannover, Germany). The number of spots in the unstimulated wells was subtracted from those in the Ag-stimulated wells, after which the median number of spots was determined in triplicate (Möbs et al., 2010). .

1.4 Flow Cytometry

PBMCs were isolated from full blood by Ammonium-Chloride-Potassium lysis, which is used for the lysis of red blood cells. Cell surface staining was performed simultaneously for 20 min on ice under dark conditions using anti-human CD4-FITC, anti-human CD25-APC, and PE-conjugated mouse anti-human CD127 or appropriate isotype controls (all from BD Pharmingen, Heidelberg, Germany). Cells were washed and analyzed by flow cytometry (FACSCalibur, BD Biosciences) using CellQuest software. For the analysis of intracellular Foxp3 expression, Bet v 1-specific, IL-10–secreting Tr1 cells were isolated (see below), fixed in paraformaldehyde solution (1%), permeabilized in saponin and APC-conjugated Foxp3-specific Abs or isotype controls (both from eBioscience, San Diego, CA) were added for 30 min on ice under dark conditions. Afterwards, cells were washed and analyzed by flow cytometry as described before (Möbs et al., 2010).

1.5 Isolation and characterization of Bet v 1-specific Tr1 cells

After *ex vivo* stimulation of PBMCs with Bet v 1, IL-2/IL-7 (10 U/ml and 10 ng/ml respectively) was added. IL-10-secreting Tr1 cells were isolated using the IL-10 Secretion Assay Cell Enrichment and Detection Kit (Miltenyi Biotec), according to the manufacturer's protocol. $CD4^+CD25^-$ Teff cells were isolated by negative selection using the $CD4^+CD25^+$ Regulatory T cell Isolation Kit, human (Miltenyi

Biotec). $CD4^+CD25^-$ Teff cells were co-cultured at concentrations of 5, 7.5, and 9×10^4 cells/ml with IL-10-secreting Tr1 cells at ratios of 1:1, 3:1, or 9:1, respectively, in the presence of 4×10^5/ml autologous x-irradiated (2 × 31Gy) PBMCs and anti-CD3 (1 µg/ml; BD Pharmingen). To examine the role of IL-10 secretion by the Tr1 cells, unconjugated neutralizing anti–IL-10 mAb (10 µg/ml; JES3-19F1; BD Pharmingen) was added to co-cultures of Teff cells and Bet v 1-specific Tr1 cells. To analyze suppression of allergen-specific Teff cells by Tr1 cells, Bet v 1-specific Teff cell lines (1×10^5 cells/ml) were also co-cultured with autologous Tr1 cells ($1–5 \times 10^4$ cells/ml) at ratios of 2:1, 4:1, and 10:1 and Bet v 1 (10 µg/ml). Bet v 1-specific Teff cell lines were generated as described previously (Hertl et al., 1998) by repeated *in vitro* stimulation of PBMCs from birch pollen-allergic patients in the presence of 10 µg/ml Bet v 1 and 10 U/ml IL-2. [^{3}H]thymidine (0.5 µCi/well; Amersham Biosciences, Buckinghamshire, U.K.) was added for the final 18 h of culture and uptake was measured after 5 days to determine T cell proliferation (Möbs et al., 2010).

1.6 Statistics

Allergen-specific Ab concentrations and T cell frequencies were expressed as median ± range and depicted as box-and-whisker plots, since patients' data were distributed nonparametric. Statistical analysis was performed using SPSS. Comparisons of paired samples of patients before and at different time points during SIT were made using the Wilcoxon signed-rank test. Differences were considered statistically significant at P values < 0.05 (Möbs et al., 2010).

2.

2.1 Study design

A study by Grindebacke et al. investigated whether SIT might restore the Th2-suppressive capacity of $CD25^+$ T cells by comparing the function of $CD25^+$ T cells obtained from SIT patients and birch pollen-allergic controls on birch allergen-induced T cell responses during birch pollen season. The study included 11 birch pollen-allergic patients aged 17 to 38 who had received SIT with birch pollen extract for 6 months and 10 birch pollen-allergic controls aged 25 to 51. Each patient had a positive SPT result to *Betula verrucosa* (Soluprick SQ 10 HEP *B. verrucosa*, ALK-Abelló, Hørsholm, Denmark) and was positive for specific IgE to *B. verrucosa* as determined by ImmunoCAP (Phadia, Uppsala, Sweden). Peripheral blood (200 ml) was obtained by venous puncture and collected into preservative-free heparin tubes. SIT subjects received subcutaneously given incremental doses of Alutard SQ *B. verrucosa* at weekly intervals for 6–16 weeks. The starting dose was between 10 and 20 SQ of the allergen extract. When the maintenance dose of 1 ml of 100,000 SQ/ml Alutard SQ *B. verrucosa* (containing 12.3 µg of Bet v1-major allergen of birch pollen) was reached, injections were given at intervals of 6 weeks. SIT-treated patients received on average 74 µg of Bet v1 (range 53–140) distributed over 17–22 injections up to seasonal blood sampling. Patients were assessed for symptomatic changes during the study (Grindebacke et al., 2010).

2.2 Isolation and Characterization of Cells and Flow Cytometry

Lymphocytes were separated by Lymphoprep™ (Nycomed, Oslo, Norway) gradient centrifugation. $CD4^+$ cells were purified with Dynabead® CD4 Positive Isolation Kit (Dynal Biotech ASA, Oslo, Norway). In order to isolate $CD25^+$ Treg cells instead of activated $CD25^+$ effector cells, $CD4^+$ cells were incubated with suboptimal amounts of beads (3µL per 10^7 cells) instead of the bead/cell ratio recommended by the manufacturer (10µl per 10^7 cells). $CD25^-$ and $CD25^+$ fractions were recovered using a LS-magnetic column according to manufacturer's instructions (Milteny Biotech, BergischGladbach, Germany). PBMCs were depleted of $CD3^+$ cells (Dynabeads®), γ-irradiated (25 Gy), and used as APC. Cells were analyzed by flow cytometry using the following mAbs: PerCP-anti-CD4 (SK3), APC-anti-CD3 (SK7) and PerCP-, APC-, or PE-isotype control IgG_1 (Becton Dickinson,

Erembodegem, Belgium). PE-anti-CD25 (clone M-A251) was obtained from BD Pharmingen (San Diego, CA, USA) (Grindebacke et al., 2010).

2.3 Measuring Foxp3 Expression by Quantitative Reverse Trancriptase Polymerase Chain Reaction

RNA was extracted from 10^5 cells using the RNeasy Micro kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and treated with DNase-I (DNA-free, Ambion, Austin TX, USA). Complementary DNA was prepared in a random hexamer-primed Superscript RT reaction (Invitrogen, Stockholm, Sweden) according to the manufacturer's protocol. Foxp3 messenger RNA (mRNA) levels were measured in duplicate using the LightCycler (Roche Diagnostics, Mannheim, Germany), with reagents from the LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics) and the primers: 5'-CAG CAC ATT CCC AGA GTT CCT-3' (forward) and 5' GCG TGT GAA CCA GTG GTA GAT-3' (reverse) (both from TIB MOLBIOL, Berlin, Germany). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as endogenous reference gene for relative quantification. GAPDH was detected by the following primers: 5'-GGC TGC TTT TAA CTC TGG-3' (forward) and 5'-GGA GGG ATC TCG CTC C-3' (reverse) (both from TIB MOLBIOL). Polymerase chain reaction cycling was performed under the following conditions: 95°C for 10 min, 45 cycles of 95°C for 15 s, 65°C for 7 s, and 72°C for 10 s. Data were collected using the LightCycler Data Analysis Software (Grindebacke et al., 2010).

2.4 Statistics

Data were analyzed by two-tailed Mann–Whitney *U* test and Wilcoxon-signed rank test using GraphPad Prism 3.00 (GraphPad, San Diego, CA, USA). *P* values of ≤0.05 were considered statistically significant: $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ (Grindebacke et al., 2010).

3.

3.1 Study design

A study by Francis et al. examined IL-10 production and $CD4^+CD25^+$ T cells after conventional grass pollen SIT. The study population consisted of 12 grass pollen-allergic subjects, 11 non-allergic subjects, and 10 subjects receiving grass pollen SIT. Allergic subjects had a positive SPT response to *Phleum pratense* (Soluprick; ALK Abelló, Horsholm, Denmark). SPTs, radioallergosorbent tests (RASTs), used to detect allergen-specific IgE in the blood, and total IgE measurements were performed before SIT and out of the pollen season. The SIT subjects had received grass pollen immunotherapy for at least 1.5 years. Peripheral blood was collected and stimulated with *Phleum pratense* for 6 days *in vitro*. The number of $CD4^+CD25^+$ T cells was measured thereafter. Patients were also assessed for symptomatic changes during the study (Francis et al., 2003).

3.2 Preparation of PBMCs and Flow Cytometry

PBMCs were isolated from heparinized blood by means of centrifugation over Histopaque (Sigma, Poole, United Kingdom), washed twice with RPMI-1640 (Gibco, Paisley, United Kingdom), and resuspended at 1×10^6 cells/ml in RPMI-1640 supplemented with 5% human sera (Sigma), 100 U/ml penicillin-streptomycin (Gibco), and 2 mmol/l L -glutamine (Gibco). Cells were incubated with either 20, 2, 0.2, or 0 μg/ml *P pratense* for 6 days. Proliferation was measured by adding 0.5 μCi of tritiated methylthymidine (Amersham, Aylesbury, United Kingdom) per well for the final 8 hours of culture. Cells were washed in FACS buffer and stained with CD25-FITC and CD4-Cy5 (Dako Cytomation, Ely, United Kingdom) for 20 minutes on ice for the analysis of CD4 and CD25 expression. Cells were washed and analyzed on a FACScalibur flow cytometer (Becton Dickinson) (Francis et al., 2003).

3.3 Statistics

Data were analyzed by the Wilcoxon matched-pairs signed-rank test for paired data or the Mann-Whitney *U* test for unpaired data with the GraphPad Instat program. *P* values <0.05 were considered significant (Francis et al., 2003).

4.

4.1 Study design

A study by Wambre et al. identified allergen-specific T cell responses linked with allergy or peripheral tolerance and examined changes in $CD4^+$ T cell responses to individual allergen-derived epitopes in SIT. The study population consisted of 12 timothy grass pollen-allergic subjects, 6 subjects who successfully responded to subcutaneous SIT and 5 non-allergic subjects. Subjects with DR04:01 or DR07:01 haplotypes were used. Subjects with TGP allergy had clinical symptoms, a positive SPT response, and positive IgE reactivity, as determined by the ImmunoCAP test (Phadia AB, Uppsala, Sweden), with TGP extracts (test score, ≥3). The SIT group had a clinical history, positive SPT responses, and IgE scores to TGP before SIT and underwent SIT for a minimum of 3 years (Wambre et al., 2014).

4.2 Generating a peptide library

A peptide library was generated based on the Phl p 1, Phl p 5a, and Phl p 5b sequences. The library consisted of overlapping peptides spanning the entire allergen, each 20 amino acids long with a 12-amino-acid overlap synthesized by Mimotopes (Clayton, Australia). Peptide-loaded DR04:01 and DR07:01 proteins were generated as previously described (Novak et al., 1999) and subsequently conjugated as tetramers by using R-PE streptavidin (Biosource International, Camarillo, Calif) (Wambre et al., 2014).

4.3 Tetramer-guided epitope mapping

For epitope mapping, Phl p 1-, Phl p 5-, and Phl p 5b-derived peptides were divided into pools of 5 peptides each. Cells were cultured with peptide pools for 14 days and then stained with pooled peptide tetramers. Cells from wells that resulted in positive staining were stained again with individual pMHCII tetramers from the positive pool. pMHCII tetramers loaded with irrelevant peptides were used as negative controls (Wambre et al., 2014).

4.4 *Ex vivo epitope-specific $CD4^+$ T cell analysis*

Forty million PBMCs in culture medium at a concentration of 150 million/ml were treated with dasatinib for 10 minutes at 37°C and stained with 20 μg/ml phycoerythrin (PE)-labeled tetramers at RT for 100 minutes. Thereafter, cells were labeled with anti-PE magnetic beads and enriched by using a magnetic column, according to the manufacturer's instructions (MiltenyiBiotec, Auburn, Calif). Frequency was calculated as previously described (Kwok et al., 2010). Magnetically enriched cells were stained with Abs against markers of interest or corresponding isotype-matched mAbs. Data acquisition was performed on a BD LSR II instrument and analyzed with FlowJo software (TreeStar, Ashland, Ore) (Wambre et al., 2014).

4.5 Intracellular cytokine staining

After culturing of $CD4^+$ T cells with specific immunodominant peptide for 14 days, cells were stained with corresponding PE-conjugated pMHCII tetramers for 60 minutes at 37°C. Cells were then restimulated with 50 ng/ml phorbol 12-myristate 13-acetate and 1 mg/mlionomycin in the presence of 10 mg/mlmonensin in 1 ml of complete medium for 6 hours at 37°C and 5% CO_2. Then, surface staining was performed, followed by fixation/permeabilization according to the manufacturer's protocol (eBioscience, San Diego, Calif). Cells were then stained with various combinations of Abs for IFN-γ, IL-17, IL-10 (all from BioLegend, San Diego, Calif), and IL-4 (eBioscience) or corresponding isotype-matched mAbs. After 30 minutes at 4°C, cells were washed and immediately analyzed by flow cytometry (Wambre et al., 2014).

4.6 Statistics

The nonparametric Mann-Whitney *U* test was used for unpaired comparisons between groups, whereas the nonparametric Wilcoxon matched-pairs test was used for paired comparison. All statistical analysis was performed with GraphPad Prism software 150, version 5.0a (GraphPad Software, La Jolla, Calif). It was not stated which *P* value was considered significant (Wambre et al., 2014).

Results

Th2 cells are increased in allergic subjects

It is thought that Th2 cells are increased during allergic reactions. This is confirmed by a study by Möbs et al. (Figure 2). Bet v 1stimulated PBMCs were coated with anti-human IFN-γ or anti-IL-5 or anti-IL-10 mAbs. Thereafter, frequencies of the different T cell subsets were analyzed by ELISPOT analysis. They showed that Bet v 1-specific IL-5-producing cells increase during the birch pollen season in allergic subjects (P= 0.046; Figure 2A). Bet v 1-specific IL-5 producing cells were identified as Th2 cells. The increase in Bet v 1-specific IL-5-producing Th2 cells was not observed in non-allergic subjects. There were no significant changes observed in the number of IFN-γ-producing cells and Il-10-producing cells in and out of the birch pollen season. IFN-γ-producing cells were identified as Th1 cells and IL-10-producing cells were identified as Tr1 cells. If these cytokines fully correlate to these cell types is however not further investigated. These results suggest that Bet v 1-specific IL-5-producing Th2 cells are increased in allergies and that the number of IFN-γ-producing Th1 cells and the number of IL-10-producing Tr1 cells are unaltered in allergies.

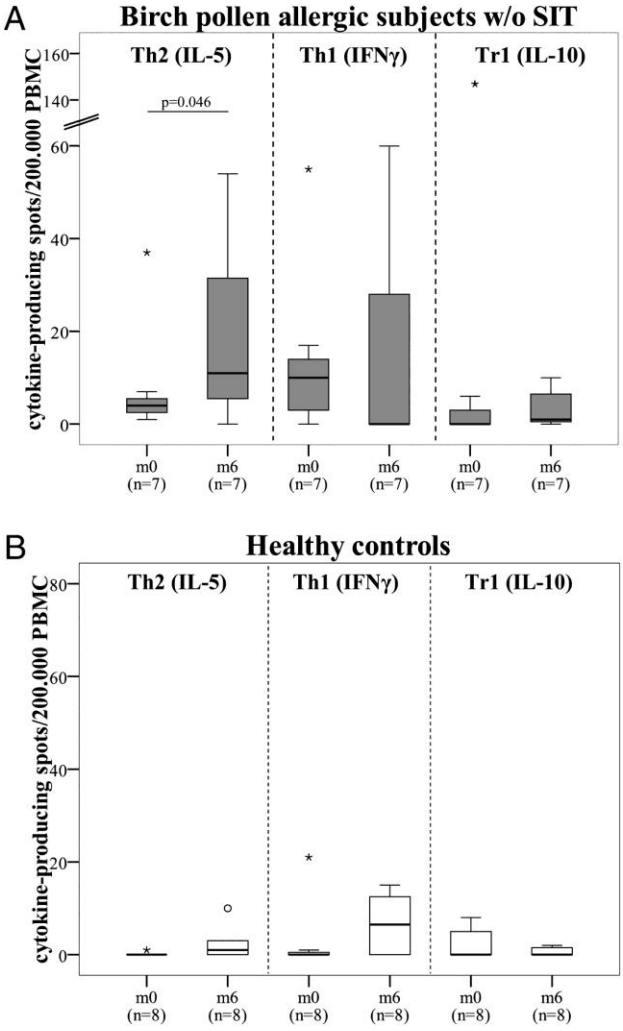

Figure 2. Effect of natural birch pollen exposure on allergen-specific T cell subsets. **(A)** Bet v 1-specific IL-5-producing Th2, IFN-γ-producing Th1, and IL-10–producing Tr1 cells in individuals allergic to birch pollen without (w/o) SIT. **(B)** Bet v 1-specific IL-5-producing Th2, IFN-γ-producing Th1, and IL-10–producing Tr1 cells in healthy controls. Frequencies were determined out of birch pollen season (m0) and during birch pollen season (m6) by ELISPOT analysis. The number of Bet v 1-specific IL-5-producing Th2 cells is elevated in allergic subjects during birch pollen season compared to non-allergic subjects. No major differences between IFN-γ-producing Th1 cells and Il-10-producing Tr1 cells were found (Möbs et al., 2010).

Th2/Th1 and Th2/Tr1 ratios are decreased by SIT

It is also demonstrated that Th2/Th1 and Th2/Tr1 ratios are decreased by SIT (Möbs et al., 2010; Figure 3). The ratio of Bet v 1-specific Th2/Th1 cells was unaltered within the first 6 months of SIT. However, a significant decrease was observed after 12 months of SIT (P= 0.016; Figure 3A).The ratio of allergen-specific Th2/Tr1 cells was significantly decreased after 3 months of SIT (P= 0.016; Figure 3B). This decrease persisted during the 12 months of SIT (p = 0.028; Figure 3B). The decrease was also apparent during birch pollen season (m6; P= 0.022; Figure 3B), despite the increased frequency of Bet v 1-specific Th2 cells at this time point (Figure 2A). This indicates that the number of Th2 cells and/or Th1 and Tr1 cells is altered during SIT.

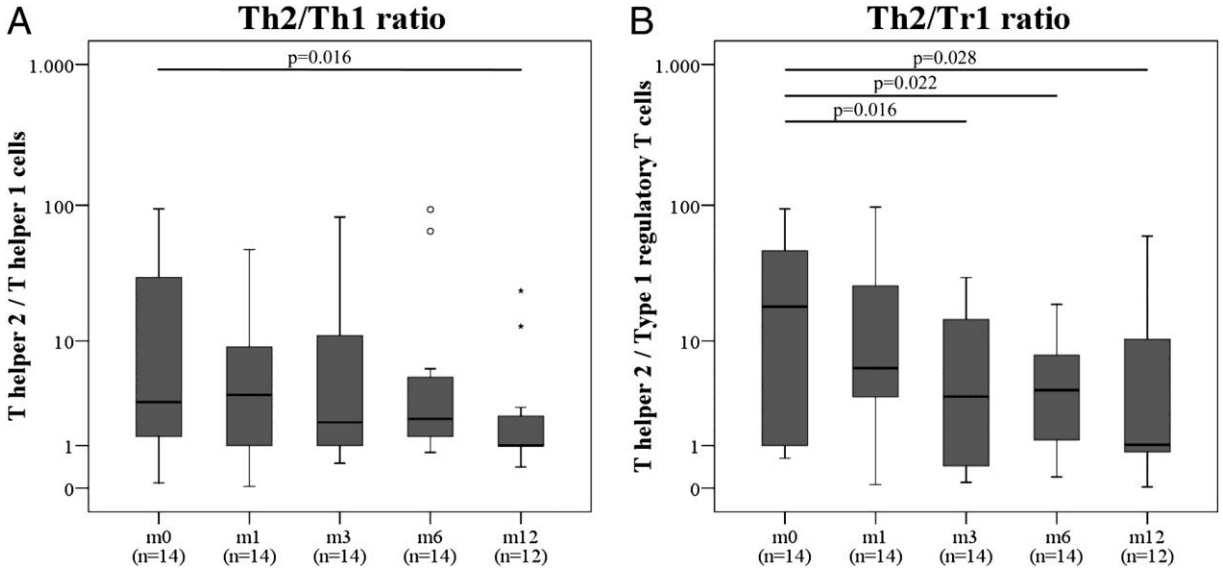

Figure 3. SIT-induced alterations in Bet v 1-specific T cell subsets. **(A)** Ratios of Bet v 1-specific Th2/Th1 cells in and out of birch pollen season. **(B)** Ratios of Th2/Tr1 cells in and out of birch pollen season. A decline in the Th2/Tr1 cell ratio is observed in SIT-treated allergic patients during birch pollen season (Möbs et al., 2010).

Tr1 and Th2 cells are increased with SIT

To investigate which cell numbers specifically change during SIT, the presence and frequency of allergen-specific Th and Tr1 cells were analyzed by ELISPOT assay (Möbs et al., 2010). IL-5-producing cells (Th2 profile) increase in allergic subject during birch pollen season (month 6) despite treatment with SIT (Figure 4A). Although there was no change in Bet v 1-specific IL-5-producing Th2 cell frequencies during the first 3 months of SIT, a significant increase was observed during the birch pollen season (m6; p= 0.025). This increase returned back to pretreatment values at month 12 (Figure 4A). This is in line with the previously found data, which showed that Bet v 1-specific IL-5-producing Th2 cells were increased during pollen season in allergic subjects (Figure 2A). However, the increase in Th2 cells in allergic subjects treated with SIT is much larger than the increase observed in allergic controls. SIT treatment did also result in increased Tr1 cell numbers during birch pollen season (m6; P= 0.003; Figure 4C). No significant changes in Th1 frequencies were observed (Figure 4B). This indicates that SIT increases both Th2 and Tr1 cell numbers in allergic subjects during pollen season. However, the increase in Tr1 cells seems more prevalent than the increase in Th2 cells.

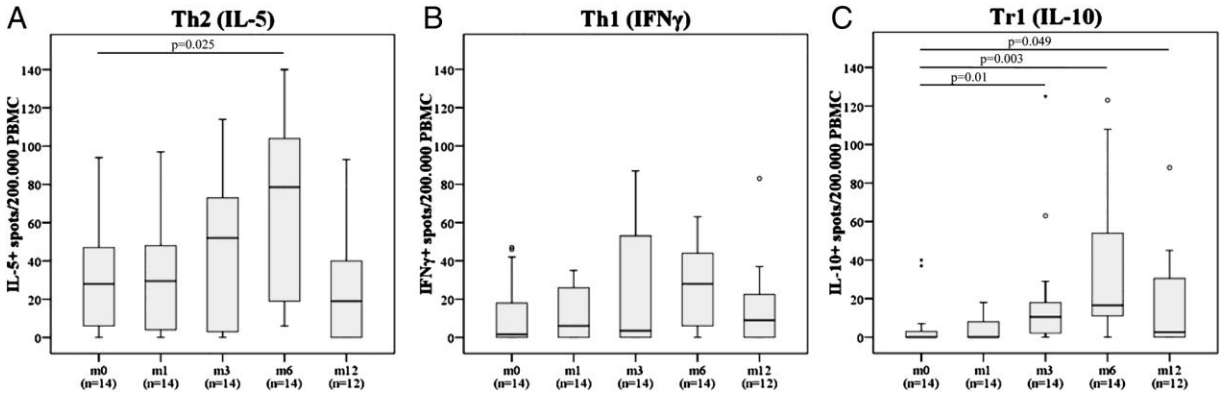

Figure 4. SIT induces Bet v 1-specific, IL-10–producing Tr1 cells. **(A)** Frequency of Bet v 1-specific Th2 cells. **(B)** Frequency of Th1 cells. **(C)** Frequency of Tr1 cells. Frequencies were determined during the first 12 months of SIT with birch pollen extract by ELISPOT analysis. Th2 (IL-5$^+$), Th1 (IFN-γ^+), and Tr1 (IL-10$^+$) cells are expressed as cytokine-producing spots per 2 × 10^5 PBMCs. Results at 6 months (m6) represent frequencies of T cells during birch pollen season. An increase in Tr1 cells was observed in allergic patient after SIT (Möbs et al., 2010).

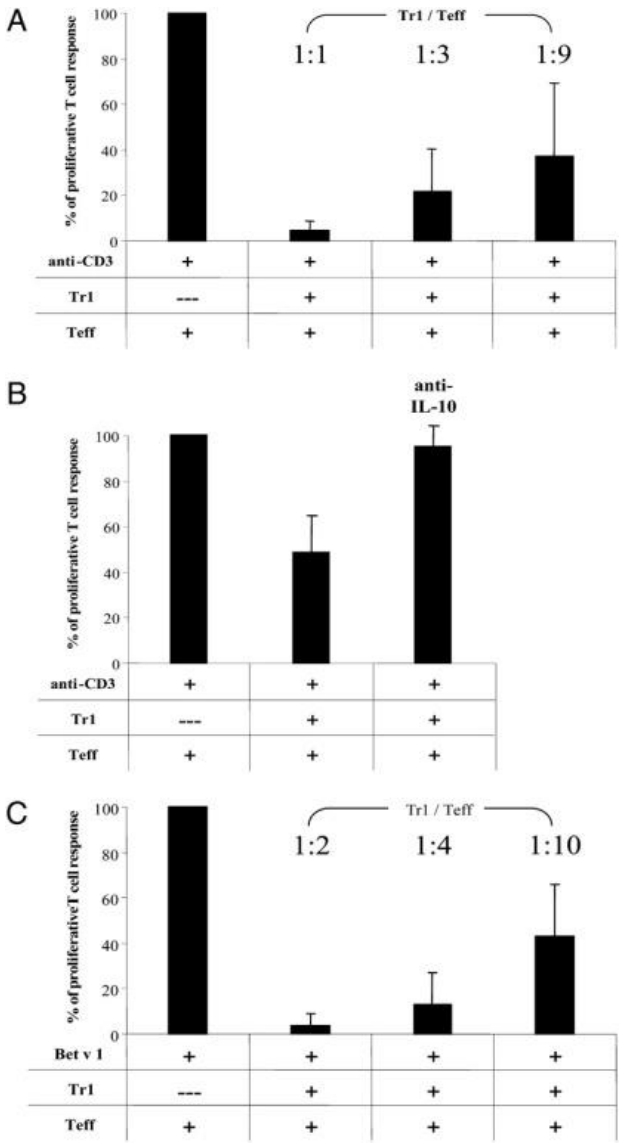

Figure 5. Suppressor activity of Bet v 1-specific Tr1 cells. **(A)** Bet v 1-induced IL-10-secreting Tr1 cells were co-cultured at different ratios with autologous $CD4^+CD25^-$Teff cells in the presence of x-irradiated autologous PBMCs and anti-CD3.Bet v 1-induced IL-10-secreting Tr1 cells best suppress $CD4^+CD25^-$Teff cells at a ratio of 1:1. **(B)** Tr1 cell-induced suppression of Teff cells is abolished by anti–IL-10 mAb. **(C)** Co-culture of Bet v 1-specific Tr1 cells and autologous allergen-specific Teff cell lines.$CD4^+CD25^-$Teff cells are best suppressed at a ratio of 1:2 (Tr1:Teff). For each individual patient, the extent of inhibition is expressed as the percentage of Teff cell proliferation after stimulation by anti-CD3 or Bet v 1 set as 100%. Bars depict mean percentage proliferation ± SD from three (*A*, *B*) or four (*C*) patients (Möbs et al., 2010).

Tr1 cells suppress effector T cells

Tr1 cells have been demonstrated to suppress the function of effector T cells. To evaluate the mechanisms by which Bet v 1-specific Tr1 cells suppress Teff cells, IL-10–secreting Bet v 1-stimulated Tr1 cells were co-cultured with anti-CD3 mAb-stimulated $CD4^+CD25^-$Teff cells (Möbs et al., 2010). Proliferation was determined by [^{3}H]thymidine uptake. Stimulation with anti-CD3 mAb did not alter proliferation of Bet v 1-specific IL-10-producing cells (Tr1 profile) from allergic subjects (no Ag: 118 ± 129 cpm; anti-CD3: 129 ± 117 cpm; n = 3). However, stimulation with anti-CD3 did increase proliferation of $CD4^+CD25^-$Teff cells from the same subjects (no Ag: 58 ± 7 cpm; anti-CD3: 15,320 ± 14,988 cpm). This response was suppressed up to 95% when equal numbers of Bet v 1-specific Tr1 cells were added (Teff alone: 15,320 ± 14,988 cpm; Teff + Tr1: 586 ± 846 cpm; Figure 5A). The suppressive effect of the Tr1 cells was almost completely abolished after addition of mAb against IL-

10 (Teff alone: 4784 ± 3651 cpm; Teff + Tr1: 2478 ± 2501 cpm; Teff + Tr1 + anti–IL-10: 4423 ± 3434 cpm; *n* = 3; Figure 5B). Second, *in vitro* experiments were performed with Bet v 1-specific Teff cells from allergic subjects (no Ag: 57 ± 19 cpm; Bet v 1: 9771 ± 4380 cpm; *n = 4*). Co-culture of Bet v 1-stimulated Teff cells with autologous allergen-specific Tr1 cells suppressed allergen-induced Teff cell proliferation (Teff alone: 9771 ± 4380 cpm; Teff + Tr1: 468 ± 750 cpm; Figure 5C). This suppression was dependent on the ratio of Teff/Tr1 cells (Figure 5C). The expression of Foxp3 in the Bet v 1-specific Tr1 cells was analyzed by flow cytometry to examine whether Foxp3$^+$Treg cells contributed to the suppression. No Foxp3 expression was observed in the Bet v 1-specific IL-10–secreting Tr1 cells after staining with a mAb against Foxp3 (Figure 6). Thus, suppression of Teff cells by SIT-induced Bet v 1-specific Tr1 cells is dependent on the Teff/Tr1 ratio and IL-10 and is independent of Foxp3 expression.

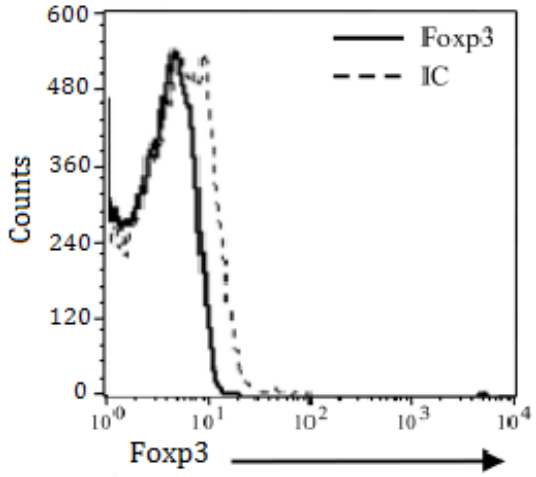

Figure6. Foxp3 expression of Tr1 cells. Bet v 1-specific, IL-10-producing Tr1 cells were isolated by passage over magnetic columns. Enriched Tr1 cells were analyzed by addition of APC-conjugated Foxp3-specific Abs. Flow cytometric analysis of Bet v 1-specific IL-10 producing Tr1 cells (*solid line*) did not reveal intracellular expression of Foxp3. IC, isotype control (*dashed line*)(Möbs et al., 2010).

CD25$^+$ Treg cell frequencies are unaltered upon SIT

Several studies have investigated whether SIT alters Treg frequencies (Möbs et al., 2010; Grindebacke et al., 2009). The study by Möbs et al. examined whether the number of CD4$^+$CD25$^+$CD127^{low} Treg cells changed during SIT by means of FACS analysis. CD4$^+$CD25$^+$CD127^{low}T cells have been shown to represent Foxp3$^+$ Treg cells (Liu et al., 2006). No changes in CD4$^+$CD25$^+$CD127^{low}Treg cell frequencies were observed after SIT (Figure 7). A study by Grindebacke et al. measured Foxp3 mRNA expression, which is a marker for natural Treg cells, during birch pollen season by means of qPCR. CD25$^+$cells expressed 14-fold higher ratios of Foxp3/GAPDH compared to CD25$^-$cells. There were no differences in Foxp3 expression between SIT patients and controls in both cell types (Figure 8A). There were also similar numbers of CD25$^+$ and CD25^{high} T cells (putative CD25$^+$Treg) observed in both the SIT patients and allergic controls (Figure 8B). These results indicate that SIT does not seem to elevate the frequency of CD25$^+$Treg cells.

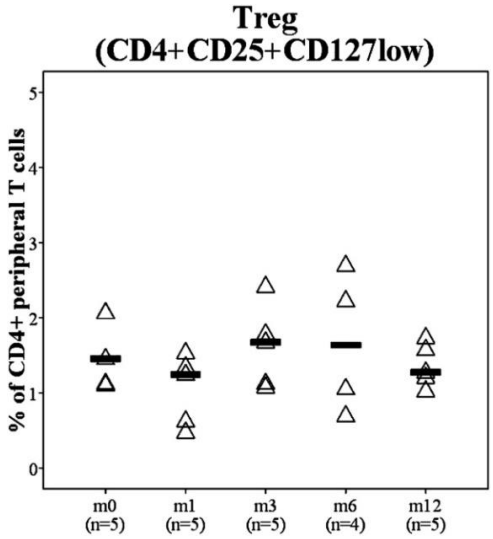

Figure7. (A) The number of $CD4^+CD25^+CD127^{low}$ cells during SIT. $CD4^+CD25^+CD127^{low}$ Treg cells are not altered by SIT. Treg cells were detected by FACS analysis as a subpopulation of $CD4^+CD25^+CD127^{low}$ cells of total peripheral blood $CD4^+$ lymphocytes (Möbs et al., 2010).

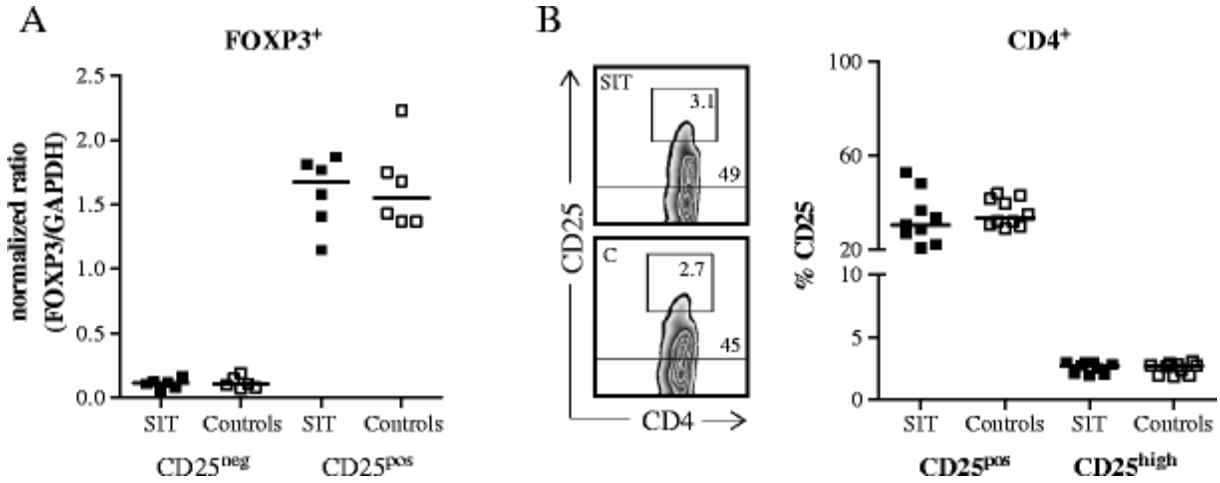

Figure 8. Foxp3 expression in $CD25^-$ and $CD25^+$ T cells in SIT patients and birch pollen-allergic controls as determined by qPCR. **(A)** Normalized ratios of Foxp3/GAPDH in the $CD25^-$ and $CD25^+$ T-cell fractions in SIT patients and allergic controls. **(B)** Representative zebra plots show the gates for $CD25^+$ and $CD25^{high}$T cells and the proportions of $CD25^+$ and $CD25^{high}$ T cells within the $CD4^+$ T-cell fraction isolated from peripheral blood from SIT patients and allergic controls. Median values are depicted as a *horizontal line*. The purity of the $CD4^+$ cells was >90%, and the purity of the $CD25^+$ cells was >94%. The proportion of $CD25^{high}$ T cells within the $CD4^+$T-cell fraction was 2.6% (median) in SIT patients and 2.7% (median) in allergic controls. The proportion of $CD25^{high}$ T cells within the $CD25^+$ T-cell fraction was 33% (median) in SIT patients and 35% (median) in allergic controls (Grindebacke et al., 2009).

$CD4^+CD25^+$Treg cell frequencies are increased in allergen-stimulated cultures of SIT-treated subjects

A study by Francis et al. measured the number of $CD4^+CD25^+$ T cells in SIT patients and allergic controls (Francis et al., 2003). $CD4^+CD25^+$ T cell numbers were significantly increased in *P pratense*-stimulated cultures of SIT subjects compared to the number of $CD4^+CD25^+$ T cells in cultures of allergic controls and non-allergic controls (Figure 9). However, the number of $CD4^+CD25^+$ T cells was not increased in the unstimulated cultures of SIT-treated patients (data not shown), which suggests that allergen exposure after SIT treatment elicits $CD4^+CD25^+$ T cells, but that SIT treatment itself does not elevate the number of $CD4^+CD25^+$ T cells. The relationship between CD25 expression and T-cell activation was also examined by measuring proliferation. $CD4^+CD25^+$ T cells correlated with proliferation in both the allergic ($P < 0.05$) and healthy groups ($P < 0.01$). However, no correlation

was observed in the subjects receiving SIT. This suggests that not all $CD4^+CD25^+$ T cells in SIT subjects were activated after *P pratense* stimulation, which indicates that the increased $CD4^+CD25^+$ T cell subpopulation might represent a regulatory T cell population.

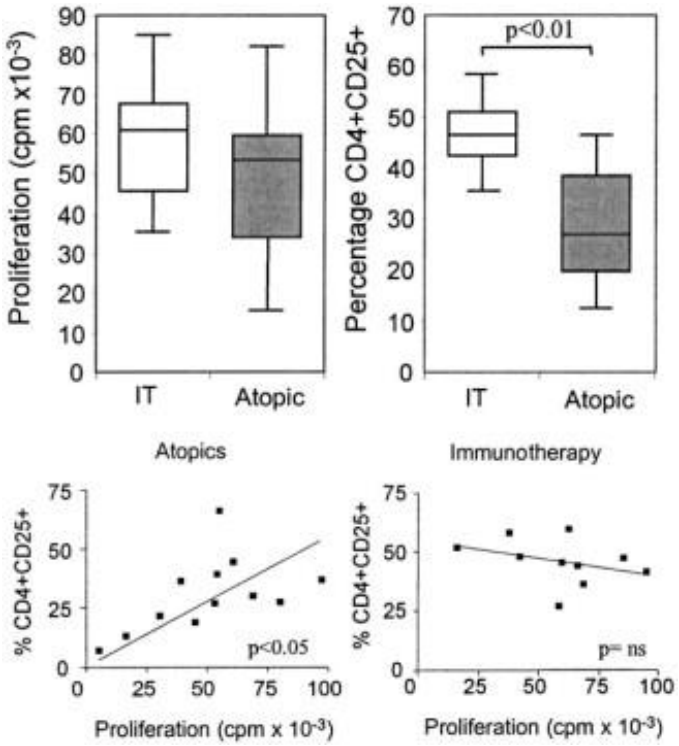

Figure 9. Expression of CD4 and CD25 by allergen-stimulated cells. After 6 days of culture with 20 µg/ml *P pratense*, cells were analyzed for the expression of CD4 and CD25 by means of flow cytometry. The results show the percentage of $CD4^+CD25^+$ cells from the total population of lymphocytes. $CD4^+CD25^+$ T cells were significantly increased in SIT subjects. In addition, proliferation (in counts per minute) of $CD4^+CD25^+$ cells obtained from the same donors is shown. $CD4^+CD25^+$ T cells correlated with proliferation in both the allergic ($P < 0.05$) and healthy groups ($P < 0.01$) (Francis et al., 2003).

Phl p 1 epitopes elicit the greatest TGP-specific $CD4^+$ T cell response

Wambre and colleagues examined the effectiveness of several timothy grass pollen (TGP) epitopes by studying allergen-specific T cell responses (Figure 10). They used a tetramer-guided epitope mapping approach to determine $CD4^+$ T cell epitopes within TGP major allergens. Since most subjects expressed a DR04:01 haplotype, experiments focused on HLA-DRB1*04:01. A total of 8 immunogenic DR04:01-restricted $CD4^+$ T-cell epitopes from group 1 (Phl p 1) and group 5 (Phl p 5a and Phl p 5b) TGP major allergens were identified. These were then loaded on pMHCII tetramers to track TGP allergen-specific $CD4^+$ T cells *ex vivo*. The non-allergic subjects showed a low TPG epitope-specific T cell frequency. There were no differences observed in the numbers of specific Th cells between the different epitopes in healthy controls (Figure 10). The allergic group, however, showed a 20- to 30-fold greater Tcell number specific for two specific epitopes: Phl p $1_{120\text{-}139}$ and Phl p $1_{244\text{-}263}$ (average, 122.5 ± 95.9 and 86.3 ± 63.1 vs 5.4 ± 1.6 and 3.7 ± 1.6 specific T cells per 10^6 $CD4^+$ T cells, respectively; $P < .005$) than the healthy controls (Figure 10A). The group 5 TGP-derived epitopes did not elicit major reponses (average, 11.5 ± 8.7 specific T cells per 10^6 $CD4^+$ T cells). Phl p $1_{120\text{-}139}$- and Phl p $1_{244\text{-}263}$- specific $CD4^+$ T cells dominate the DR04:01-restricted T cells in allergic subjects (Figure 10B). Thus, most $CD4^+$ T cells seem Phl p 1-specific in allergic subjects.

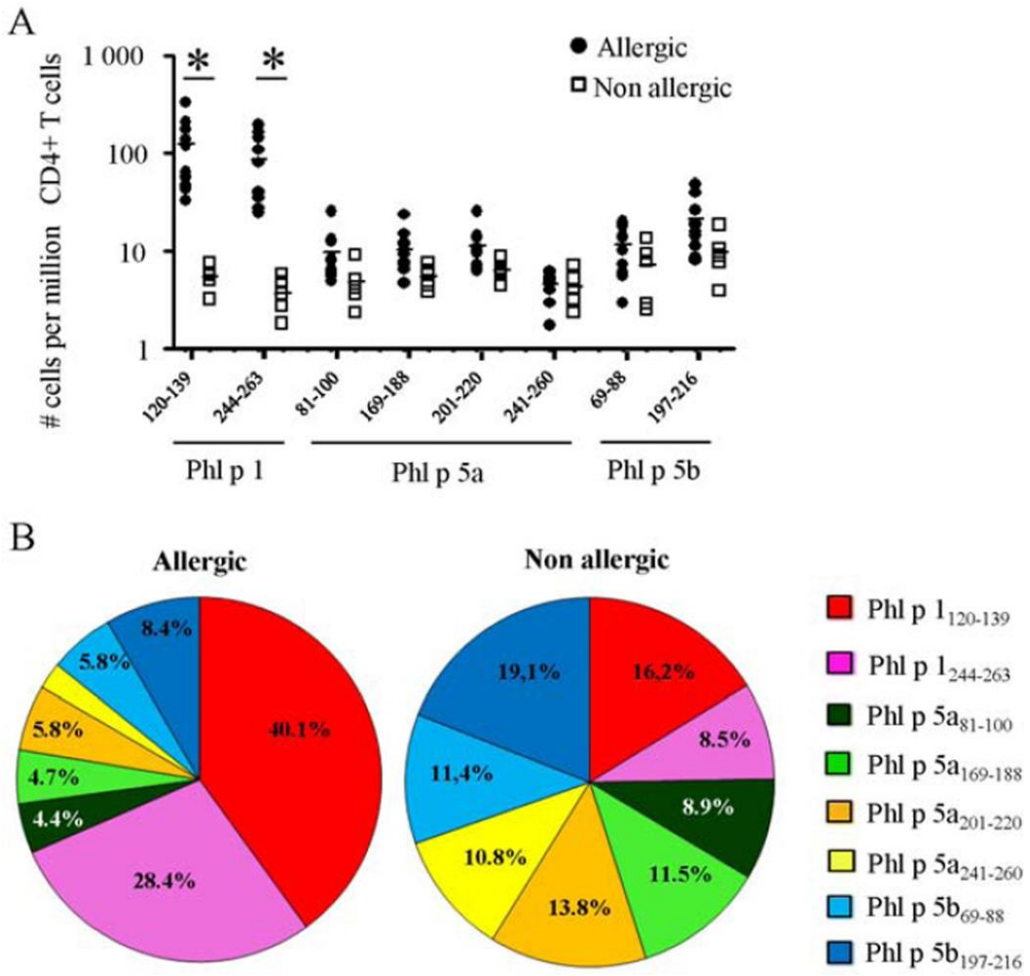

Figure 10. (A) *Ex vivo* frequencies of group 1 and group 5 TGP-specific T cells in allergic subjects (solid circles) and non-allergic subjects (open squares). Allergic patients show an increased T-cell immune response to Phl p 1 epitopes. $*P < .001$. **(B)** Contribution of each epitope to the global DR04:01-TGP allergen-specific T-cell response in allergic (n = 10) and non-allergic (n = 5) subjects. Phl p 1-specific $CD4^+$ T cells dominate the DR04:01-restricted T cells in allergic subjects. Data are presented as mean values from each group in pie charts (Wambre et al., 2014).

Ratios of T cell subpopulations are restored upon SIT

To investigate whether differences in the number of epitope-specific T cells correlate with differences in phenotype, cytokine profiles of DR04:01-restricted TGP epitope-specific T cells were examined. Phl p $1_{120-139}$, Phl p $1_{244-263}$,Phl p $5a_{81-100}$, Phl p $5a_{169-188}$, and Phl p $5a_{201-220}$ elicited strong IL-4 responses (Figure 11A and 11D), whereas Phl p $5a_{241-260}$, Phl p $5b_{69-88}$, and Phl p $5b_{197-216}$ elicited IFN-γ and IL-10 responses in the same subject (Figure 11B and 11D). TGP epitope-specific T cells in non-allergic subjects were dominated by IFN-γ–producing $CD4^+$ T cells (Th1 profile) and IL-10–producing $CD4^+$ T cells (Tr1 profile) (Figure 11C). Epitope-specific T cells from allergic subjects showed different responses to different epitopes. To investigate the effect of SIT on epitope-specific T cells, cytokine profiling and phenotyping was performed in SIT-treated subjects. It was demonstrated that all DR04:01-restricted TGP epitope-specific $CD4^+$ T cell responses observed in SIT-treated patients were dominated by IFN-γ–producing $CD4^+$ T cells (Th1 profile) and IL-10–producing $CD4^+$ T cells (Tr1 profile) (Figure 12A). These results were consistent with *ex vivo* phenotyping showing increased numbers of allergen-specific Th cells displaying CXCR3 expression, a marker associated with Th1 cells (Acosta-Rodriguez et al., 2007; Bonecchi et al., 1998), after SIT. Phenotyping also demonstrated a decrease in expression of CRTH2 and CCR4, which are markers associated with Th2 cells (Cosmi et al.,

2000; Sallusto et al., 1998), after SIT (Figure 12B). This indicates that SIT increases the relative number of Th1 cells and decreases the relative number of Th2 cells. These data together indicate that Phl p $1_{120-139}$,Phl p $1_{244-263}$,Phl p $5a_{81-100}$, Phl p $5a_{169-188}$, and Phl p $5a_{201-220}$ are Th2 epitopes and that Phl p $5a_{241-260}$, Phl p $5b_{69-88}$, and Phl p $5b_{197-216}$ are Th1 epitopes. The Phl p $1_{120-139}$ and Phl p $1_{244-263}$seem to be dominant Th2 epitopes, while the Phl p $5a_{81-100}$, Phl p $5a_{169-188}$, and Phl p $5a_{201-220}$ seem to be nondominant Th2 epitopes. That is, Phl p 1 epitope-specific $CD4^+$ T cells dominated in allergic subjects.The data also demonstrate that SIT restores the allergy-induced increase in Th2 cells by increasing the number of Th1 and Tr1 cells and decreasing the number of Th2 cells.

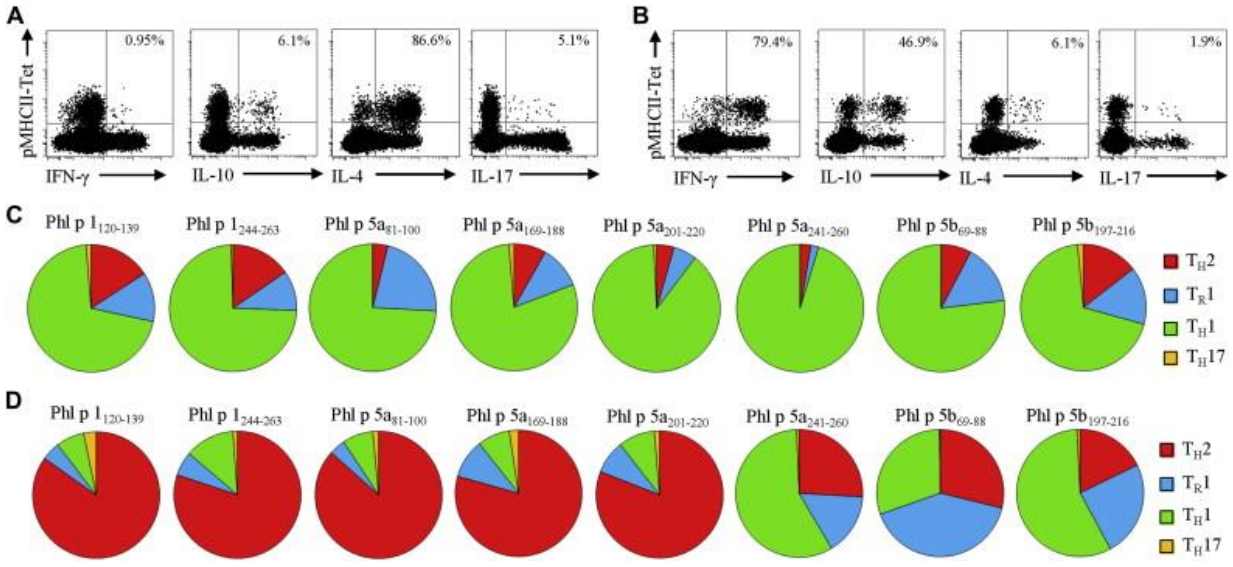

Figure 11. (A) Cytokine expression of Phl p $1_{120-139}$-specific T cells in DR04:01-restricted allergic subjects. **(B)** Cytokine expression of Phl p $5b_{197-216}$-specific T cells in DR04:01-restricted allergic subjects. **(C)** Cytokine profiles of DR04:01-restricted TGP epitope-specific T cells in non-allergic subjects. IFN-γ–producing $CD4^+$ T cells (Th1 cells) and IL-10–producing $CD4^+$ T cells (Tr1 cells) dominate the T cell subsets in non-allergic subsets. **(D)** Cytokine profiles of DR04:01-restricted TGP epitope-specific T cells in allergic subjects. IL-4-producing $CD4^+$ T cells (Th2 cells) dominate the Phl p 1- and the Phl p $5a_{81-100}$, Phl p $5a_{169-188}$, and Phl p $5a_{201-220}$-specific T cell subsets. Data are representative of at least 5 subjects per group and are presented as mean values from each group in pie charts.

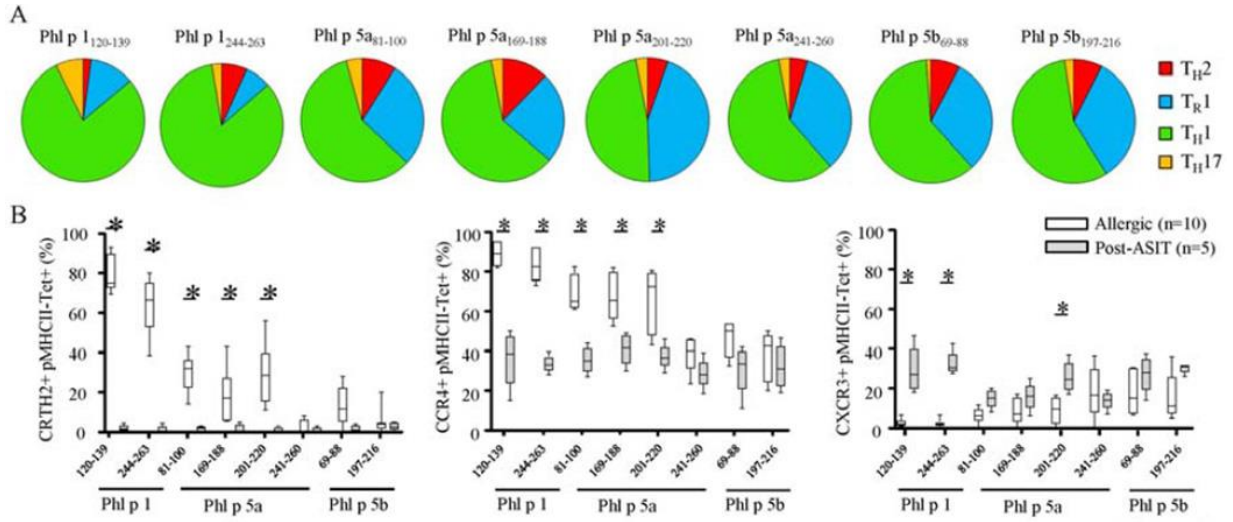

Figure 12. (A) Cytokine profiles of DR04:01-restricted TGP epitope-specific T cells in SIT-treated subjects. Data are representative of at least 4 subjects per group. All DR04:01-restricted TGP epitope-specific $CD4^+$ T cell responses are dominated by IFN-γ-producing $CD4^+$ T cells (Th1 cells) and IL-10–producing $CD4^+$ T cells (Tr1 cells) after SIT. **(B)** *Ex vivo* phenotype of DR04:01-restricted TGP epitope-specific $CD4^+$ T cells in allergic subjects and SIT patients. CRTH2 and CCR4 expression are decreased after SIT and CXCR3 expression is increased after SIT. Differences between groups were analyzed by using the Mann-Whitney *U* test. $*P < .01$.

$CD27^-$ allergen-specific $CD4^+$ T cells are mostly affected by SIT

Changes in TGP-specific T cell numbers induced by SIT were next assessed by comparing the DR04:01-restricted TGP epitope-specific $CD4^+$ T cell responses between allergic subjects and SIT-treated patients. The absolute number of group 1 (Phl p 1) and group 5 (Phl p 5a and Phl p 5b) TGP-reactive $CD4^+$ T cells was significantly lower in SIT-treated patients (32.4 ± 7.9) compared to untreated allergic subjects (280 ± 181.6 cells per 10^6 $CD4^+$ T cells, $P < .001$). A 20- to 30-fold decrease in the absolute number of Phl p $1_{120-139}$– and Phl p 1_{244-26}-specific T cells was observed in SIT-treated patients (average, 7.7 ± 2.2 and 4.2 ± 1.6 vs 122.5 ± 95.9 and 86.3 ± 63.1 cells per 10^6 $CD4^+$ T cells, respectively; $P < .001$) when compared with allergic controls (Figure 13A). The relative number of allergen-specific $CD4^+$ T cells specific for nondominant Th2 epitopes only decreased by 2-fold after treatment with SIT, while the relative number of allergen-specific $CD4^+$ T cells specific for Th1/Tr1 epitopes were unaltered (average, 12.1 ± 9.2 vs 8.9 ± 5.7 cells per 10^6 $CD4^+$ T cells; $P > .05$) compared with those seen in allergic controls. This indicates that SIT decreased the number of allergen-specific Th2 cells, but not the number of Th1 or Tr1 cells (Figure 13B). Frequencies of $CD27^-$ and $CD27^+$ DR04:01-restricted TGP allergen-specific T cells were also determined by means of flow cytometry. Former research has demonstrated that $CD27^-CD4^+$ T cells represent allergen-specific Th2 cells and that $CD27^+CD4^+$ T cells represent allergen-specific Th1 and Tr1 cells (Wambre et al., 2012). It has also been shown that $CD27^+$ allergen-specific $CD4^+$ T cells are more prevalent in non-allergic subjects (Wambre et al., 2014). Flow cytometry showed a decrease in $CD27^-$ allergen-specific $CD4^+$ T cells with SIT. No significant changes were observed in $CD27^+$cells in SIT patients when compared with allergic subjects (Figure 13C). These alterations result in a change in the ratio of allergen-specific Th2/Th1 and Th2/Tr1 cells. The previously subdominant $CD27^+$ allergen-specific $CD4^+$ T cells become dominant after deletion of allergen-specific $CD4^+CD27^-$ T cells by SIT. Foxp3 expression was also measured by means of *ex vivo* phenotyping, but no significant changes were observed in the percentage of $Foxp3^+$ cells within allergen-specific $CD4^+$ T cells after treatment with SIT (data not shown), which indicates that the number of $Foxp3^+$ Treg cells is not increased upon SIT. These results together indicate that the number of $CD27^-CD4^+$ Th2 cells is decreased upon SIT and that the number of natural Treg cells is unaltered upon SIT. This last finding is in conflict with the results acquired by cytokine profiling and phenotyping. That is, these experiments suggested that SIT treatment did increase the number of Th1 and Tr1 cells.

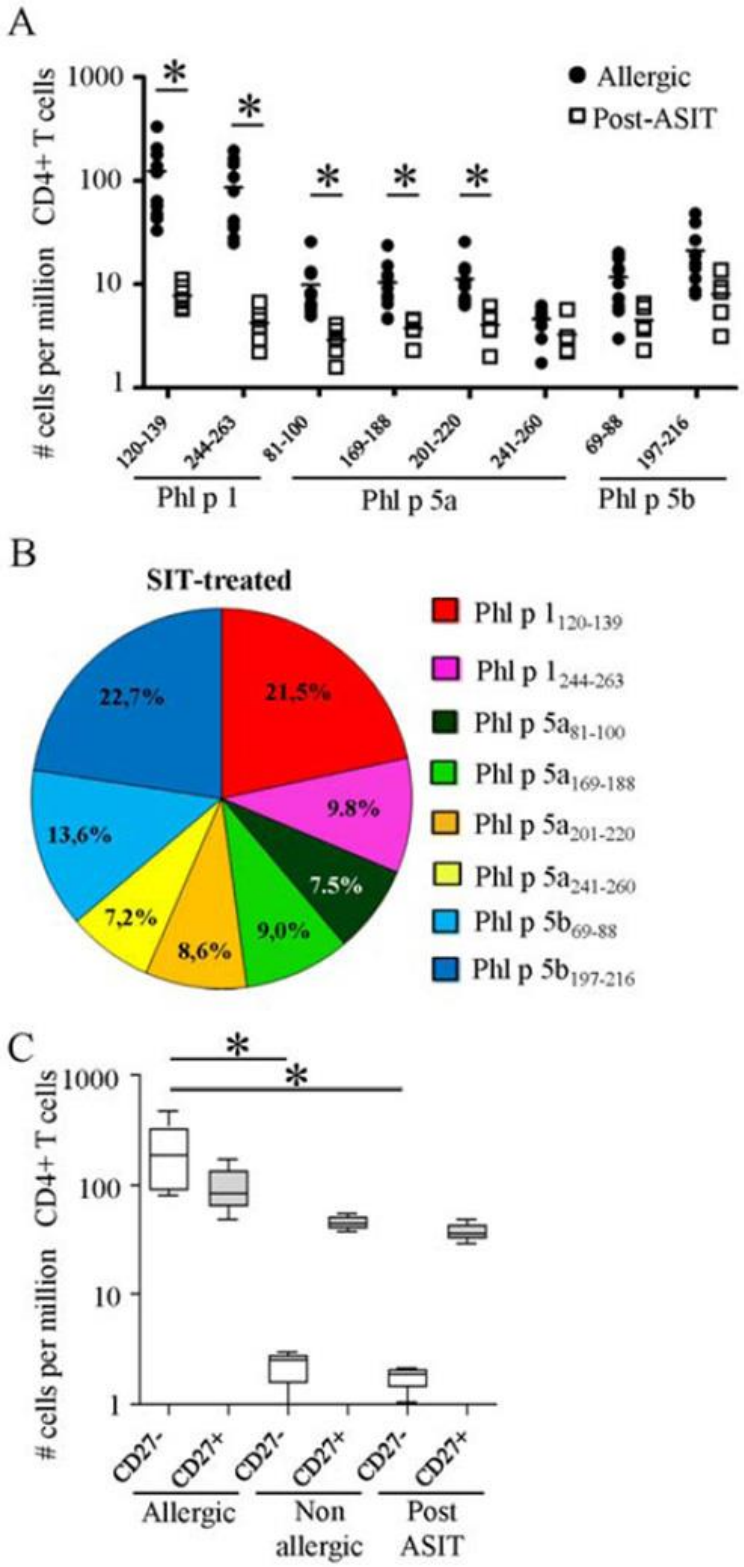

Figure 13. (A) *Ex vivo* frequencies of DR04:01-restricted TGP-specific CD4+ T cells in allergic subjects and SIT patients. Phl p 1 and Phl p 5a specific T cells are significantly decreased by SIT. **(B)** Contribution of each DR04:01-restricted TGP epitope to the global allergen-specific CD4+ T-cell response in SIT patients. **(C)** Overall frequencies of $CD27^-$ and $CD27^+$ DR04:01-restricted TGP allergen-specific T cells in allergic subjects (n = 10), non-allergic subjects (n = 5), and subjects after SIT (n = 6) as determined by flow cytometry. $CD27^-$ T cells are increased in allergic subjects compared to non-allergic subjects. SIT suppresses this increase. *$P < .01$ (Wambre et al., 2014).

Discussion

This thesis focused on determining whether Treg cells are required for the suppression of allergic manifestations in diseased humans using allergen-specific immunotherapy. The second phase of SIT treatment was therefore further explored. Several studies have led to more insight regarding the optimization of SIT in allergic subjects. The increase in Th2 cells during allergic reactions was confirmed using ELISPOT analysis (Möbs et al., 2010). When allergic patients were treated with SIT, changes in several cell subsets were observed. First, Th2/Th1 and Th2/Tr1 ratios were decreased by SIT treatment. Both the number of IL-5-producing Th2 cell and IL-10-producing Tr1 cells seemed increased upon SIT. However, the increase in the number of Tr1 cells, which were shown to suppress Th2 cell functions, seemed more prevalent (Möbs et al., 2010). $CD25^+$ Treg frequencies were shown to be unaltered upon SIT (Grindebacke et al., 2009). However, other studies suggest that SIT does increase $CD4^+CD25^+$ Treg cell frequencies (Francis et al., 2003). In contrast, a study by Wambre et al. showed that SIT decreases $CD4^+CD27^-$ Th2 cells and can even cause deletion of these cells as determined by staining for Th cell subsets specific for the dominant Phl p 1 epitopes. This study also showed unaltered numbers of allergen-specific Th1 and Tr1 cells upon SIT (Wambre et al., 2014). The studies by Möbs et al., Grindebacke et al. and Francis et al. also showed a reduction of symptoms after SIT by use of assessment scores.

The results of these studies are somewhat conflicting. The difference in results can be caused by multiple factors. First, since humans are used as experimental subjects, differences in results might result from (genetic) differences between subjects. Second, subjects different from the ones in the allergic control groups were treated with SIT. Lastly, treatment strategies differed between the studies. The studies by Möbs et al. and Grindebacke et al. used birch-pollen allergic subjects, while the studies by Francis et al. and Wambre et al. used grass-pollen allergic subjects. SIT treatment for the birch pollen-allergic subjects consisted of *Betula verrucosa* allergen. The allergic controls in the study by Möbs et al. were also symptomatically treated. The duration of SIT treatment especially differed between the studies. The subjects used in the study by Möbs and colleagues were exposed to SIT for 1 year, after which PBMCs were taken and stimulated with Bet v 1. The SIT group used in the study by Grindebacke and colleagues were treated with SIT for only 6 months. The SIT-treated subjects in the study by Francis et al. were exposed to SIT for at least 1.5 years, after which PBMCs were taken and PBMCs were exposed to *Phleum pratense* for 6 days. The study by Wambre and colleagues used subjects who were treated with SIT for at least 3 years.

One of the main conflicting results concerned changes in Th2 cell numbers upon SIT. The study by Möbs et al. stated that SIT increased the number of Th2 cells and Tr1 cells (Möbs et al., 2010). However, the study by Wambre et al. showed that SIT decreased the number of $CD4^+CD27^-$ Th2 cells and that SIT may increase the number of allergen-specific Th1 and Tr1 cells (wambre et al., 2014). These conflicting findings can be caused by multiple factors. First, the studies were based on different allergies. The difference in allergy types suggests that SIT may alter different cells in different allergies. However, this seems rather unlikely. The duration of SIT treatment also differed between the two studies. Subjects in the study by Möbs et al. were treated with SIT for 1 year and those in the study by Wambre et al. for at least 3 years. This suggests that the number of Th2 cells and Tr1 might increase within the first year of SIT, but that the number of these cells decreases after long-term SIT treatment. The studies also differed in PBMC treatment. The study by Möbs et al. exposed PBMCs to *Betula verrucosa*, while Wambre and colleagues exposed PBMCs to allergen-specific peptide tetramers. However, since epitopes are part of the allergen, this should not radically change the results. Another possible cause is the use of different analysis methods. Möbs et al. used ELISPOT analysis to measure IL-5 and indicated that IL-5 secretion correlated to Th2 cells. It was not investigated whether IL-5 levels correlated to $CD4^+$ T cells. Since they measured IL-5 production in PBMCs, IL-5 production may not have fully correlated to Th2 cells. However, since Th2 cells are the main IL-5 producing allergen-specific subset, measurements should correspond to Th2 cells. The

study by Wambre and colleagues measured CD27, of which the expression differs between Th2 and Th1 and Tr1 cells, and used a method in which CRTH2 and CCR4, markers associated to Th2 cells, were measured. They also measured changes $CD4^+$ T cell responses specific for Th2 cell epitopes. Since multiple factors were measured, the results should correlate to Th2 cells. These findings together suggest that the number of Th2 and Tr1 cells increases within the first year of SIT treatment, but that the number of $CD4^+CD27^-$ Th2 cells decreases after the first year of SIT treatment. However, since the study by Wambre et al. did not measure T cell frequencies during the 3 years of SIT treatment, further research will be needed to confirm this statement.

Results acquired by Grindebacke and colleagues also did not correspond to results acquired by Francis and colleagues. The study by Grindebacke et al. demonstrated that $CD25^+$ Treg frequencies were unaltered upon SIT treatment (Grindebacke et al., 2009). However, the study by Francis et al. showed that SIT did increase $CD4^+CD25^+$ Treg cell frequencies (Francis et al., 2003). The difference in results acquired by these studies can be caused by the fact that the study by Grindebacke et al. measured Foxp3 expression, which is a marker for natural Treg cells, while the study by Francis et al. only measured the number of $CD4^+CD25^+$ T cells. No changes in Foxp3 expression were observed after SIT (Grindebacke et al., 2009), which indicates that SIT does not increase natural Treg cells. The study by Wambre et al. confirmed this finding. However, Grindebacke et al. also measured the frequency of $CD25^+$ cells, which did not seem to be increased with SIT. This last finding was in conflict with results obtained by Francis and colleagues. They discovered an increase in $CD4^+CD25^+$ T cells in *P pratense*-stimulated cultures of SIT-treated subjects. The increase was not present in SIT patients without *P pratense* stimulation. The increase in $CD4^+CD25^+$ T cells after *P pratense* stimulation in SIT-treated subjects did not correlate to allergen-induced proliferation, which indicates that the increased $CD4^+CD25^+$ T cell subpopulation might represent a regulatory T cell population. However, the lack in correlation found between $CD4^+CD25^+$ T cells and proliferation in SIT-treated patients may also have been caused by cell death due to the absence of growth medium. That is, proliferation may have correlated to $CD4^+CD25^+$ T cells during the first days of *P pratense* stimulation, but the large number of proliferating cells may have caused the medium to diminish. The conflicting findings can be caused by the fact that subjects in the study by Grindebacke and colleagues were treated with SIT for 6 months, while those in the study by Francis and colleagues were treated with SIT for 1.5 years. This suggests that the number of $CD25^+$ cells may increase exclusively after long-term treatment with SIT. Previous research has shown that at least 3 years of treatment is required to obtain the long-term benefits of SIT (Durham et al., 1999; Naclerio et al., 1997). There was also a difference in cell isolation strategy between the studies. The study by Grindebacke et al. used suboptimal amounts of beads in order to isolate $CD25^+$ Treg cells instead of activated $CD25^+$ effector cells and therefore excluded $CD25^+$ effector cells from their measurements. This indicates that the number of $CD4^+CD25^+$Teff cells might be increased upon SIT, but that the number of $CD4^+CD25^+$ Treg cells is unaltered upon SIT. Lastly, the study by Francis and colleagues exposed isolated PBMCs to *Phleum pratense* for 6 days *in vitro*, while Grindebacke and colleagues did not expose PBMCs to allergen. Since the increase in $CD4^+CD25^+$ T cells was only present after PBMCs were stimulated, the difference in PBMC exposure may have led to the conflicting results. This indicates that SIT treatment only elicits an increase in $CD4^+CD25^+$ T cells upon allergen exposure. These findings together suggest that allergen exposure in SIT-treated subjects elevates the number of $CD4^+CD25^+$ T cells. It is unclear whether these are $CD4^+CD25^+$Teff cells or $CD4^+CD25^+$Treg cells. However, since the second cell type was also found to be increased in the study by Möbs et al., the latter is more likely.

In conclusion, SIT seems to increase $Foxp3^-$ adaptive Treg frequencies within the first year of SIT upon allergen exposure. SIT can also cause a decrease in $CD4^+CD27^-$Th2 cells after long-term treatment. This suggests that Treg cells might indeed not be required for the suppression of allergic manifestations in diseased humans upon SIT and that allergen-specific $CD27^-CD4^+$Th2 cell deletion might be an important contributor to successful SIT. However, since data differ between studies, further research will be needed. It seems especially important to investigate the difference in

immediate and long-term effects of SIT treatment in diseased humans, since data suggest that the immediate and long-tem effects of SIT may differ. Future research should also use the same subjects pre-SIT as post-SIT, since changes in immune cells can then be completely assigned to SIT treatment.

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