

Shorter Dosing Intervals of Sublingual Immunotherapy Lead to More Efficacious Treatment in a Mouse Model of Allergic Inflammation

C. Rask, J. Brimnes & K. Lund

Department of Experimental Immunology, ALK-Abelló, Hørsholm, Denmark

Received 8 December 2009; Accepted in revised form 25 February 2010

Correspondence to: C. Rask, Experimental Immunology, ALK-Abelló, Bøge Allé 6-8, DK-2970 Hørsholm, Denmark. E-mail: crdkk@alk-abello.com

Abstract

Current day practice of sublingual immunotherapy (SLIT) includes varying modalities of treatment that differ with regard to formulation, dosing and administration regimens. The aim of this study was to explore the importance of the dosing intervals in SLIT. The immunological effect of increased SLIT dosing frequency was tested in a mouse model of allergic inflammation. Mice sensitized to *Pb_l p* were SLIT-treated with the same weekly cumulative dose administered with different administration frequencies. A SLIT sham-treated group was also included. All mice were challenged intranasally with *Pb_l p* extract following SLIT. Local and systemic cytokine production, eosinophil infiltration into airways and the development of *Pb_l p*-specific antibody responses were determined. Higher frequency of sublingual administration of allergen extract has a profound positive impact on the effect of SLIT, measured as induction of IgG and IgA antibodies. The once daily SLIT was the only treatment regimen being able to reduce all systemic Th2 cytokines and systemic IgE antibody responses when compared to sham-treated mice after the intra-nasal challenge period. The group receiving SLIT with the highest frequency of administration had the most pronounced effect of the treatment. In the same group, there was also a higher degree of protection against increase in IgE antibody levels after intra-nasal challenge with the allergen, our data demonstrate that a once daily regimen is more efficacious than regimens where SLIT, with the same weekly cumulative allergen dose, is administered with longer intervals but higher doses.

Introduction

Allergic rhinitis represents a major health problem worldwide [1]. Studies have revealed an average prevalence of 23% in western Europe [2]. Often, the current recommended treatment for allergic rhinitis constitutes allergen avoidance and pharmacotherapy. These treatments only affect the symptoms and have short-lasting efficacy. Allergen immunotherapy offers advantages of specific treatment with long lasting efficacy, and the ability to modify the course of the disease [3].

Successful specific immunotherapy has been performed by subcutaneous as well as sublingual administration of allergen extracts. The major benefits of sublingual immunotherapy (SLIT) compared to subcutaneous immunotherapy (SCIT) are the reduced risk of IgE-mediated severe

systemic adverse events and the convenience with allowing self-administration at home.

The mechanistic basis for the effect of allergen-specific immunotherapy is not yet completely known. A correlation between successful treatment and reduced Th2 responses, mainly IL-5, and eosinophil infiltration to airway mucosa has been shown both in clinical studies [4–6] and in animal models of allergic diseases [7–12]. The reduction of these inflammatory parameters has been suggested to be because of induction of regulatory T cells [13–17]. Moreover, non-IgE antibodies of mainly the IgG4 and IgA isotypes are induced following SCIT and SLIT and may, in addition to being robust markers of immunotherapy, be at least partly responsible for the clinical effect of the treatment in their capacity of blocking antibodies that compete with effector-cell bound IgE

antibodies for allergen binding [18–22]. SLIT can be prescribed as drops or tablets. The recommended dose and frequency of administration varies markedly between different SLIT products but little experimental evidence for the optimal dosing regimen exists. In this study, we addressed the contribution of allergen dose in combination with administration frequency on the efficacy of SLIT treatment. The effects of different SLIT regimens are measured by induction of allergen-specific antibodies, local and systemic cytokine responses and airway eosinophil infiltration. The same weekly cumulative dose of timothy grass allergen *Pbleum pratense* (*Pbl p*) extract was administered 7, 3 days or 1 day per week, respectively, to study the importance of frequency of SLIT. For this purpose, we used a recently developed murine model of allergic inflammation in which the mice react towards *Pbl p* [7]. This model shows many signs of the clinical and immunological features present in patients suffering from allergic rhinitis. In this study, we demonstrate that daily SLIT is the only regimen having the capacity to significantly down-regulate production of various Th2 cytokines. In addition, the induction of allergen-specific IgA is positively correlated with the dosing frequency. Moreover, daily administration of SLIT is the only regimen leading to more efficient protection against allergen-induced IgE antibody production compared to sham-treatment.

Materials and methods

Animals. Female Balb/cJ mice, 6–10-weeks old at the start of the experiment were used. The mice were bred in-house and were maintained on a defined diet, free of components that cross-react with antisera to *Pleum pratense* extract. The mice were housed in a specific pathogen-free environment with 12 h light, 12 h dark cycles. All experiments described in this report were conducted in accordance with Danish legislation.

Experimental design. Each experimental group consisted of 7–16 mice, depending on which parameter examined. The mice were sensitized by three intra-peritoneal (i.p.) injections of 5000 SQ alum-adsorbed *Pbl p* extract (ALK-Abelló) at days 0, 14 and 21. Blood samples were withdrawn at day 28, and mice were stratified into four groups based on their *Pbl p*-specific IgE, IgG2a and IgG1 titres, with descending priority. SLIT treatment was started at day 35 and administered over a period of 9 weeks. Groups of mice received SLIT with the same weekly cumulative dose of *Pbl p* extract, but with different frequency of administrations according to Fig. 1. A sham-treated control group which was sensitized as earlier and then SLIT treated with buffer was also included. SLIT was performed by holding the scruff of the mouse and carefully applying the 5 μ l of allergen extract solution under the tongue. The mouse was held by the scruff for 20 s to prevent the animal from immediately swallowing the allergen extract solution. Blood samples were withdrawn at day 56, 77 and 98, which correspond to after 3, 6 and 9 weeks of SLIT treatment. Mice were challenged intra-nasally (i.n.) day 98–112, with a daily dose of 5000 SQ *Pbl p* extract in 10 μ l buffer for 5 + 5 consecutive days. Mice were sacrificed at day 113, and blood, cervical lymph nodes (CLN), spleen and bronchoalveolar lavage (BAL) were collected.

Bronchoalveolar lavage. BAL was performed 24 h after the last i.n. challenge using 700 μ l Hanks's Balanced Salt Solution (HBSS, Gibco[®], Carlsbad, CA, USA) complemented with protease inhibitor cocktail tablet (Roche[®], Basel, Switzerland) (Fig. 1). BAL was centrifuged at 400 g and cells were re-suspended in PBS, and total number of cells was counted using a microscope and Bürker chamber. Cell-free supernatants were stored at –20 °C until *Pbl p*-specific antibody levels were measured as described later. For differential counts, cytopsin preparations were fixed and stained with May-Grünwald (Merck, Darmstadt, Germany) Giemsa (Sigma-Aldrich Corp, St Louis, MO, USA)

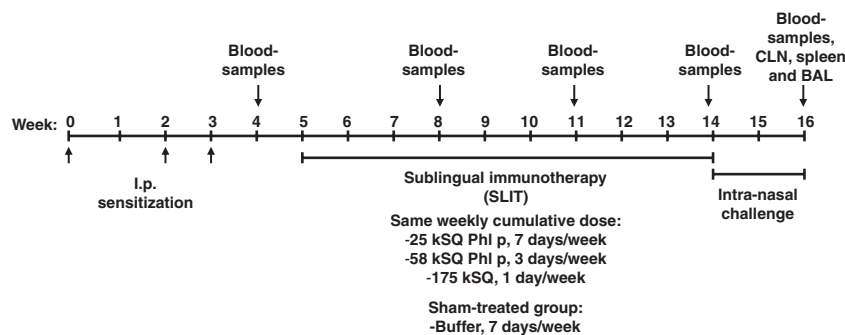


Figure 1 Experimental design. Mice were sensitized to *Pbleum pratense* (*Pbl p*) by three intraperitoneal (i.p.) injections of 5000 SQ allergen extract adsorbed to alum on day 0, 7 and 14. Starting 2 weeks after the third i.p. injection, mice were sublingual immunotherapy (SLIT)-treated for 9 weeks with different intervals and with different doses of *Pbl p* extract (indicated in the figure). After 9 weeks of SLIT, all mice were intra-nasally (i.n.) challenged for 5 + 5 consecutive days with 5000 SQ *Pbl p* extract. Mice were sacrificed, and blood, spleen and CLN were processed for further analyses. Bronchoalveolar lavage (BAL) was performed. Blood samples were withdrawn 1 week after the third i.p. injection and after 3, 6, and 9 weeks of SLIT.

staining, and number of eosinophils were identified by standard morphologic criteria and based on 200 cells counted per cytospin.

Measurement of cytokine production following *in vitro* re-stimulation. Single cell suspensions of CLN or spleens were prepared and washed three times in RPMI 1640 medium (Cambrex Bio Science, Baltimore, MD, USA). Cells were counted and dispersed in RPMI 1640 medium complemented with 50 µg/ml gentamycin (Invitrogen, Corp, Carlsbad, CA, USA), 1% Nutridoma (Roche Applied Science, Indianapolis, IN, USA) and 1.5 mmol/l monothioglycerol (Sigma-Aldrich Corp) and 1% Foetal Calf Serum (FCS) for lymph node cells. To each well of a 96-well flat-bottomed culture plate (Nalge Nunc International, Rochester, NY, USA), 3×10^6 cells were added. Cells were stimulated with *Pbl p* extract in a final concentration of 40 µg/ml at 37 °C in 5% carbon dioxide. Supernatants were collected after 5 days of stimulation, and cytokine levels were quantified by a mouse Th1/Th2 9-plex Ultra-sensitive kit (Meso Scale Discovery, Gaithersburg, MD, USA).

Measurement of *Pbl p*-specific antibodies. Sera were prepared from the blood samples by centrifugation at 1300 g for 10 min. The levels of *Pbl p*-specific IgE, IgA, IgG1 and IgG2a antibodies were measured on the ADVIA Centaur platform (Bayer Diagnostics, Tarrytown, NY, USA). Briefly, rat or goat anti-mouse antibodies covalently coupled to paramagnetic particles were used to capture the different serum antibody isotypes. Bound solid-phase antibodies were allowed to react with liquid-phase purified biotinylated major allergens that were detected as chemiluminescence using acridinium-ester-labelled streptavidin.

Pbl p-specific IgA antibodies in BAL were determined in a Magic Lite assay. Briefly, BAL fluids were diluted in wash buffer (0.06% gelatine and 0.1% Triton X in PBS, pH 6.65) and incubated with magnetic particles coupled to goat anti-mouse IgA (Indicia Biotechnology, Oullins, France). After washing, *Pbl p*-specific antibodies were detected by biotinylated *Pbl p* extract and streptavidin conjugated to acridinium ester. Visualization was carried out in a luminometer with automated injection of peroxide and hydrochloric acid. The technical characteristics of the assay are described elsewhere [23].

Statistical analysis. All statistical calculations were performed using the two-tailed non-parametric Mann-Whitney test using a software programme (GRAPHPAD Prism version 5.01; GraphPad Software Inc, San Diego, CA, USA).

Results

Kinetics of antibody responses during SLIT treatments

In this study, sensitized mice were stratified into four groups receiving the same weekly cumulative SLIT dose

or sham-treatment, followed by an i.n. challenge period (Fig. 1). The kinetics of *Pbl p*-specific IgE, IgA, IgG1 and IgG2a were followed during the experiment. The median values at each time-point are depicted in Fig. 2. Sensitization induced potent allergen-specific antibody responses of all isotypes except IgA, which is not surprising because systemic administration mainly gives rise to IgG and IgE antibody responses [24]. *Pbl p*-specific antibody responses in sham-treated mice decreased over time during the 9-week treatment period, which is a characteristic of this model [25] (and unpublished observations). However, in contrast to sham-treated mice, SLIT treatment with *Pbl p* extract rescued the drop in antibody levels resulting in no, or less pronounced, changes of antibody titres after 9 weeks of treatment when compared to immediately after sensitization. In contrast to the other isotypes, IgG1 levels were significantly reduced in all groups during the 9 week period of SLIT (Fig. 2). Levels of all allergen-specific isotypes increased dramatically because of the i.n. challenge with *Pbl p* extract. Differences in antibody levels immediately before and after the i.n. challenge in the various treatment groups are described in detail in the following paragraphs.

Pbl p-specific antibodies were most efficiently induced by daily SLIT with *Pbl p* extract

In clinical specific immunotherapy (SIT) studies, the most studied immunological parameter that is associated with treatment effects is induction of allergen-specific antibodies [26–30]. In analogy with clinical studies, we have used changes in allergen-specific antibody titres as indicators of the effect of treatment. *Pbl p*-specific serum antibodies of IgE, IgA, IgG1 and IgG2a isotypes induced after 9-week SLIT period, but before the i.n. challenge, are depicted in Fig. 3A, C, E and G, whereas antibody levels detected after the 2 weeks of i.n. challenge are shown in Fig. 3B, D, F and H. As also observed during the course of SCIT and SLIT in clinical trials, levels of allergen-specific IgE antibodies were increased by the active SLIT treatment, being most prominent in the 7×25 kSQ/week treatment group (Fig. 3A). In contrast, the *Pbl p*-specific IgE level in the buffer SLIT treatment group was significantly ($P < 0.01$) decreased during the 9-week period from a median of 64,500–27,900 Rlu (data not shown).

Allergen-specific IgA antibody responses were significantly increased in all groups following 9 weeks of SLIT with *Pbl p* extract when compared to the buffer group. There was a clear dosing frequency-dependent induction of IgA, with the most prominent increase in the group of mice SLIT treated daily with 25 kSQ *Pbl p* extract. The daily SLIT treatment resulted in a significantly ($P < 0.005$) higher IgA antibody titre when compared to

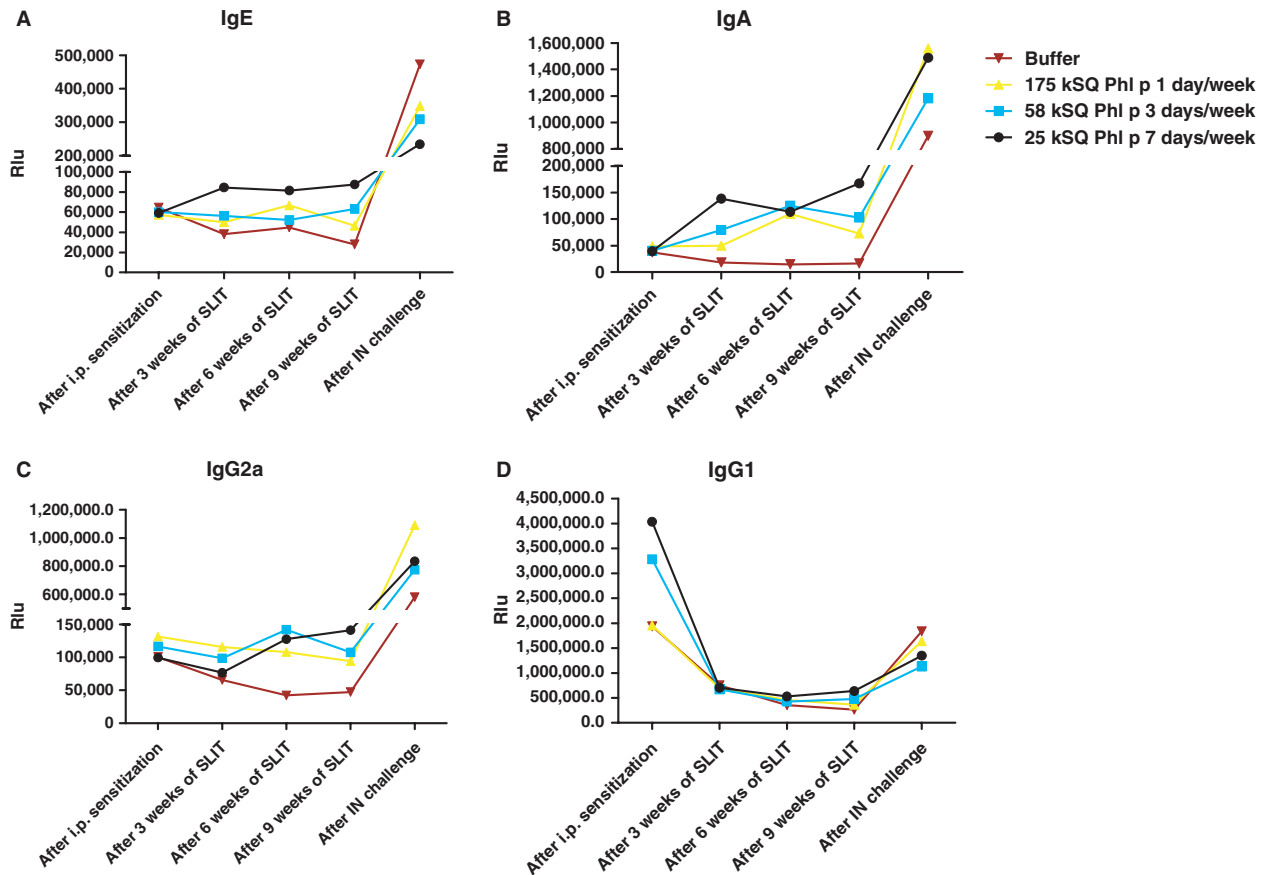


Figure 2 Overview of the kinetics of *Pbleum pratense* (*Pbl p*)-specific antibody responses during the experiment. Blood samples were collected 1 week after the third i.p. injection of alum-adsorbed *Pbl p* extract ($n = 16$ per group), 3 weeks ($n = 1$ pool of eight individual serum samples per group), 6 weeks ($n = 1$ pool of eight individual serum per group) and 9 weeks ($n = 8$ –15 per group) after sublingual immunotherapy (SLIT) and finally after the intra-nasal challenge at the end of the experiment ($n = 14$ –16 per group). *Pbl p*-specific IgE, IgA, IgG2a and IgG1 antibody responses were measured on the ADVIA Centaur platform. Group medians are depicted in the figure.

the group receiving one SLIT dose per week, with the same weekly cumulative dose of 175 kSQ *Pbl p* extract (Fig. 3C).

All active treatment groups showed significantly higher levels of allergen-specific IgG2a compared to the sham-treated group in serum taken after the 9-week SLIT period ($P < 0.05$ in all cases, Fig. 3G).

In similarity, IgG1 levels were also significantly higher in the two groups receiving the most frequent administrations of SLIT compared to the SLIT buffer group ($P < 0.01$ for both groups, Fig. 3E). Even though IgE, in similarity with the other isotypes, increased dramatically because of i.n. challenge in all groups, the IgE response ended up being significantly ($P < 0.05$) reduced in the once daily treated group when compared to sham-treated group after i.n. challenge (Fig. 3B). The increase was only 3-fold in the daily SLIT treatment group, whereas a 17-fold increase could be seen in the sham-treated group (median values). Intermediate effects were seen in the other treatment groups (5- and

8-fold for 3 days/week and 1 day/week treatment, respectively) demonstrating that among the different regimens used in this experiment, daily administration of SLIT provided the most efficient protection (>5-fold compared to sham-treated) against increase in allergen-induced IgE antibodies because of challenge.

Induction of *Pbl p*-specific local IgA antibody responses is dependent on the frequency of SLIT

BAL was performed at the end of the experiment, and local specific IgA antibody responses were quantified. IgA production was induced in all groups after the i.n. challenge. However, the IgA responses were significantly ($P < 0.05$) higher in those two groups who had received the most frequent SLIT administrations, 25 kSQ *Pbl p* 7 days/week with a median of 967,000 Rlu and 58 kSQ *Pbl p* 3 days/week with a median of 977,000 when compared to IgA levels in the buffer SLIT group with a median of 355,000 Rlu (Fig. 4).

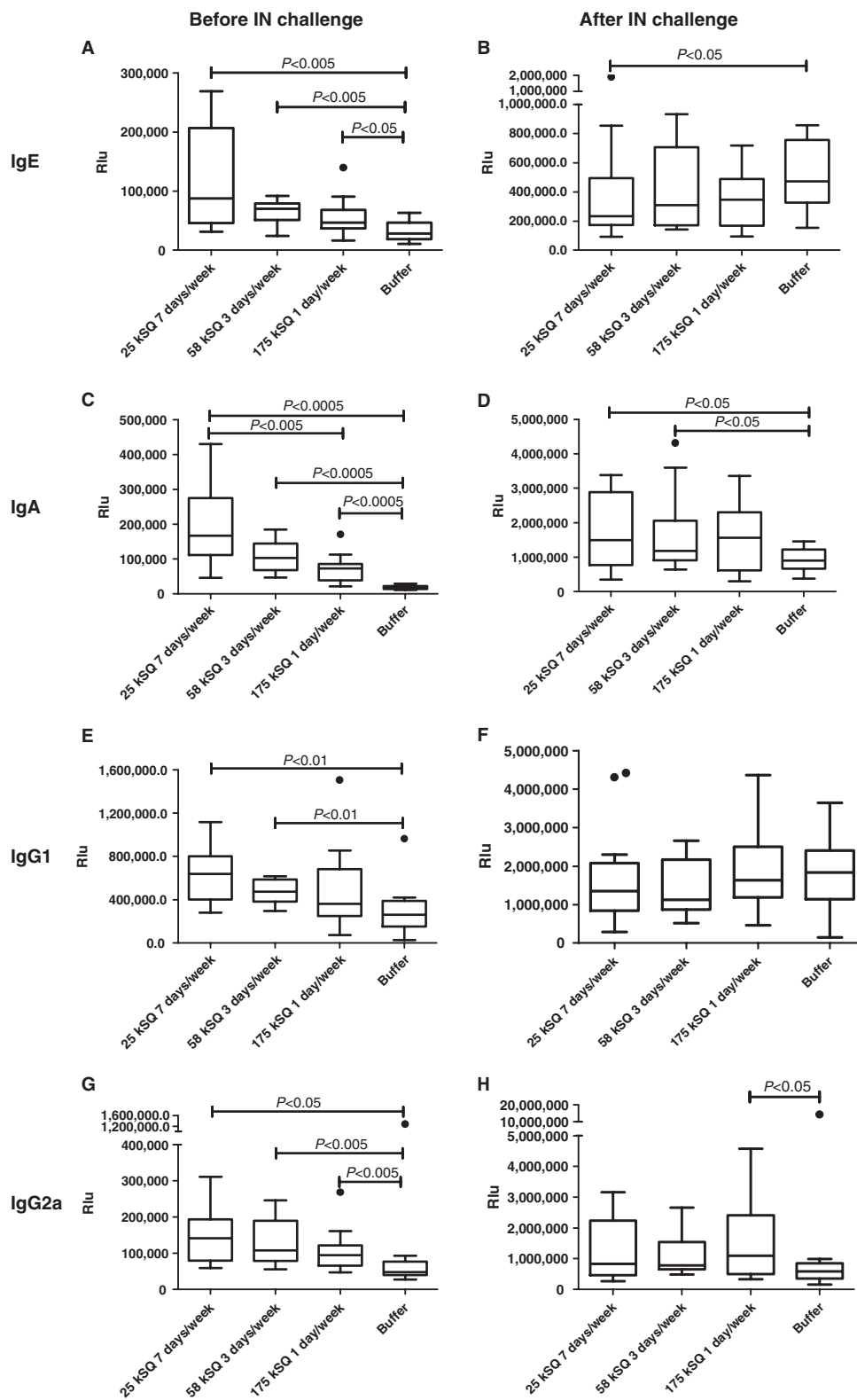


Figure 3 *Pleum pratense* (*Pbl p*)-specific antibody responses in serum after 9 weeks of sublingual immunotherapy (SLIT). *Pbl p*-specific antibodies of IgE (A and B), IgA (C and D), IgG1 (E and F) and IgG2a (G and H) isotypes were determined in blood samples taken after 9 weeks of SLIT (A, C, E and G) and after the intra-nasal challenge (B, D, F and H). All analyses were performed on the ADVIA Centaur platform. Boxes show the group median and the lower and upper quartiles with outliers (after 9 weeks of SLIT; $n = 8, 10, 15$ and 14 per group, and after intra-nasal challenge; $n = 16, 15, 16$ and 14 per group). Whiskers represent min and max value. Mann–Whitney test was used for statistical analyses.

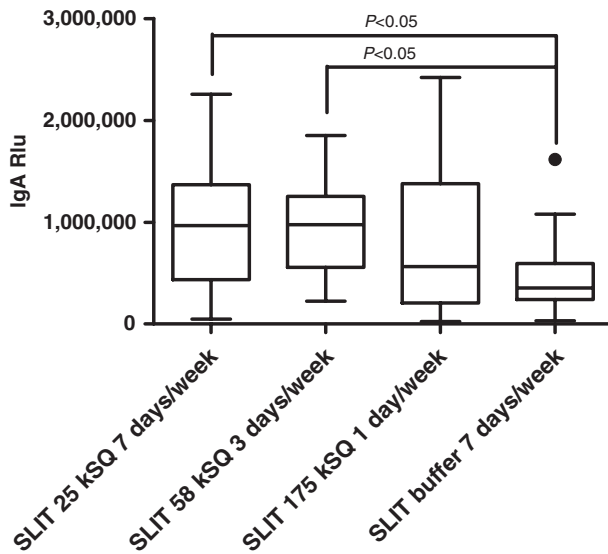


Figure 4 *Pbleum pratense* (*Pbl p*)-specific IgA antibody responses in BAL. *Pbl p*-specific IgA antibodies were determined in bronchoalveolar lavage (BAL) collected after the 2 weeks of intra-nasal challenge. Analyses were performed with Magic Lite assay. Boxes show the group median and the lower and upper quartiles with outliers ($n = 15, 15, 15$ and 14 per group). Whiskers represent min and max value. Mann-Whitney test was used for statistical analyses.

Inflammatory responses in BAL, draining lymph nodes and spleen

There was a tendency towards dosing frequency-dependent suppression of eosinophil infiltration to BAL following i.n. challenge (Fig. 5), although these differences did not reach statistical significance. The same scenario was

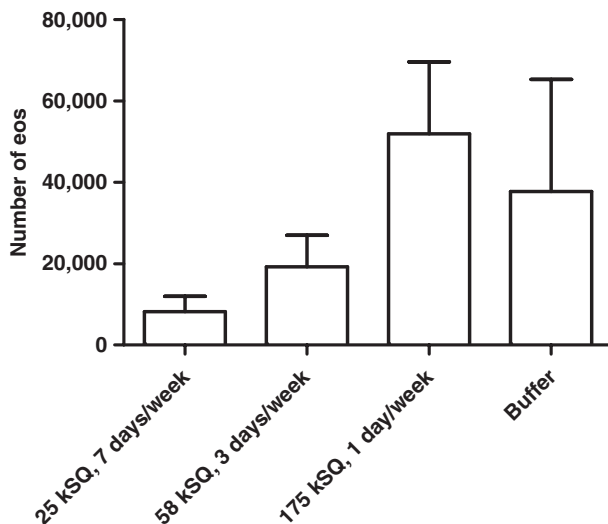


Figure 5 Total number of eosinophils in bronchoalveolar lavage (BAL). BAL cells were counted, and the total number of eosinophils was determined by differential counts on May-Grünwald Giemsa stained cytopsin glasses. Group means with SEM are shown ($n = 7$ per group).

seen when analysing the cytokine responses after *in vitro* re-stimulation of draining cervical lymph node cells, with a tendency towards dosing frequency-dependent down-regulation of all Th2 cytokines examined with borderline ($P = 0.06$) significance in the IL-4 and IL-10 responses (Fig 6).

The systemic spleen cell cytokine responses show a more clear-cut pattern with significant reduction of all cytokines evaluated in the group receiving daily SLIT with 25 kSQ *Pbl p* extract when compared to sham-treated mice (Fig. 7).

Discussion

The most studied immunological parameter that correlates with treatment in a dose-dependent manner in clinical studies of specific immunotherapy is the changes in allergen-specific antibody levels that occur during the course of treatment. Most commonly, in both SCIT and SLIT regimens, an initial rise in allergen-specific IgE production followed by a return to pretreatment levels combined with a blunting of the seasonal increase in the IgE production is observed [26–30]. Furthermore, gradual allergen-dose-dependent increase during the treatment period of allergen-specific non-IgE antibodies like IgG1 and IgG4 [27–33] is a hallmark of effective immunotherapy.

It has been demonstrated in many clinical studies [28, 34] and animal models of allergic diseases [7, 25] that the effect of SLIT, both clinical and immunological, depends on the individual allergen dose given with each administration. We have previously shown that the contact time between the sublingual mucosa and allergen extract is critical for the effect of SLIT in mice [25] which led us to hypothesize that the effective dose in a SLIT regimen may also rely on other parameters in addition to the allergen concentration itself. In this article, we explore the effect of the dosing frequency of SLIT in a mouse model of allergic inflammation using the local and systemic cytokine responses, allergic airway inflammation and induction of allergen-specific antibody levels as measurements for treatment effect.

Our data clearly demonstrate that the frequency of allergen has a profound impact on the effect of the treatment, with the impact on SLIT-induced IgA antibody levels and systemic Th2 responses being most prominent. In addition to earlier findings [7, 25] that higher allergen concentrations lead to better effect of SLIT including increased antibody responses, we here find that a regimen where lower allergen doses administered daily is more effective than a regimen where proportionally higher allergen doses were administered less frequently, even when the same weekly cumulative dose was used.

Even though antibody induction is the most commonly analysed parameter in clinical trials of SCIT and SLIT, T-cell proliferation, Th2 cytokine production and

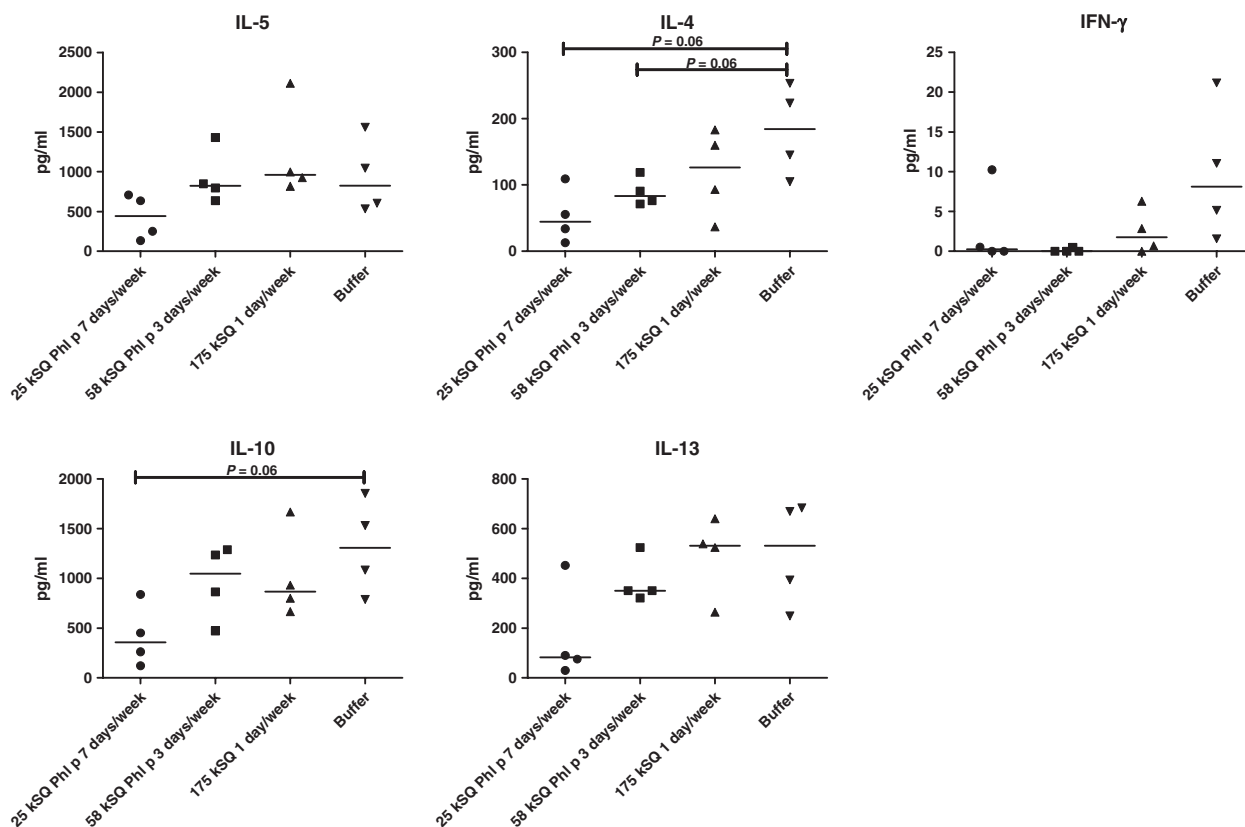


Figure 6 Cytokine responses in cervical lymph node cells. Single cell suspensions from cervical lymph node cells (CLN) were *in vitro* re-stimulated with 40 $\mu\text{g/ml}$ *Pbium pratense* (*Pbl p*) extract for 5 days. Cytokine levels in the supernatants were quantified by a mouse Th1/Th2 9-plex Ultra-sensitive kit. Scatter plot with median values are shown. CLN from two mice were pooled, generating four data points per group. Mann-Whitney test was used for statistical analyses.

levels of eosinophils in respiratory mucosa have also been shown to be associated with successful immunotherapy [4–6, 35]. In this sense, our mouse models resemble the clinical situation, and we have been able to show that all Th2 cytokines measured systemically are reduced after daily administration of SLIT when compared to sham-treatment, whereas no or limited effect was observed in groups administered less frequently. Moreover, we have demonstrated tendencies towards reduced BAL eosinophilia as well as locally produced Th2 cytokines in the group receiving the most frequent SLIT dosing regimen. Induction of regulatory T cells has been suggested after SCIT [14, 15] as well as SLIT [13, 17, 36] in human beings. However, induction of regulatory T cells was not investigated in this study and for the time being, we can neither prove nor rule out that these types of cells play a role in our mouse model.

As mentioned earlier, the kinetics of the IgE antibody response in allergic patients successfully treated with SIT shows an initial increase of serum IgE levels with a blunting of pollen-induced IgE during the seasons. This pattern is indeed also observed in the mouse model of allergic inflammation used in the

current article, being most pronounced in the daily SLIT-treated group when compared to sham-treated. SLIT-induced IgE response in animals treated daily with a low dose of *Pbl p* extract was better protected (>5-fold) against subsequent boosting of IgE levels by intranasal challenge, than animals treated less frequently with higher allergen doses. In addition, it was only the daily treated SLIT group that shows a significantly reduced IgE response compared to sham-treatment after the intra-nasal challenge period.

In the murine model of allergic inflammation used in this study, a dramatic increase of all allergen-specific isotypes could be observed after the intra-nasal challenge with the allergen. All groups, including the buffer SLIT-treated mice, were challenged by this route, and it is known that intra-nasal administration of antigen efficiently induces systemic and local antibody production [37]. After the intra-nasal challenge, IgA was the only parameter still being significantly higher in the SLIT group treated daily with 25 kSQ *Pbl p* extract. Thus, more frequent SLIT administration was superior to the other treatment regimens in priming the mucosal immune system for subsequent robust IgA production.

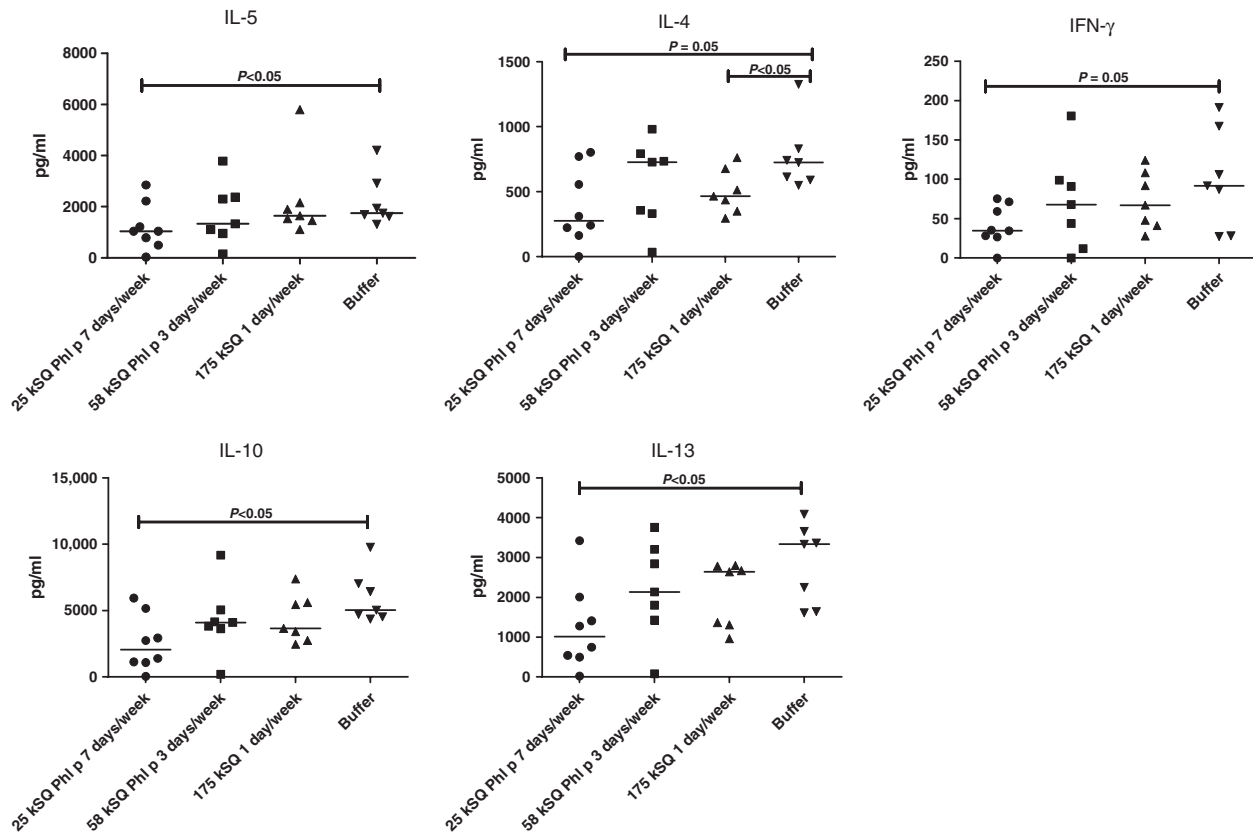


Figure 7 Cytokine responses in spleen cells. Single cell suspensions from spleen were *in vitro* re-stimulated with 40 $\mu\text{g}/\text{ml}$ *Plebeum pratense* (*Pbl p*) extract for 5 days. Cytokine levels in the supernatants were quantified by a mouse Th1/Th2 9-plex Ultra-sensitive kit. Scatter plot with median values are shown ($n = 8, 7, 7$ and 7 per group). Mann–Whitney test was used for statistical analyses.

Evidence for the induction of systemic and mucosal secretory IgA (SIgA) following SLIT is beginning to evolve both clinically [29, 38–40] and experimentally [9, 25]. The primary role of SIgA antibodies is to prevent commensal and pathogenic microorganisms to cross the epithelial barrier. This is performed by antigen exclusion at mucosal surfaces and by virus and endotoxin neutralization within epithelial cells without causing tissue damage [41]. The role of IgA in allergic diseases is not completely understood. In theory, one could assume that high levels of allergen-specific IgA would prevent allergen absorbance and thereby sensitization and subsequent development of allergy. Supporting this theory, low levels of total and allergen-specific SIgA and transient IgA deficiency have been associated with an increased risk of allergy [42–44]. It has also been shown that non-atopic children have higher levels of allergen-specific IgA compared to allergic children and that these changes are equalized following SLIT [38]. In addition, the cytokine TGF- β , which have been demonstrated to have various anti-inflammatory effects, is also associated with IgA production [45, 46]. It is plausible that both of these immunological features contribute to the positive effects of SLIT.

In conclusion, we show that the induction of possible blocking antibodies following the SLIT period is positively correlated with SLIT administration frequency. Moreover, it is the daily SLIT regimen group that exclusively, when compared to sham-treatment, shows significantly reduced systemic Th2 cytokines and IgE antibody responses after the intra-nasal challenge with *Pbl p* extract, representing a pseudo pollen-season. Increasing the allergen dose was not sufficient to effectively compensate for a lower administration frequency when the cumulative weekly dose was held constant. We believe that it is likely that the frequency dependency also holds true for clinical SLIT treatment. However, this issue needs to be addressed in well-designed clinical studies in the future.

Acknowledgment

The skilful technical assistance of Sandra Urioste (ALK-Abelló) is greatly appreciated. We also thank Annette Giselsson and Astrid Thomsen (ALK-Abelló) for their professional antibody analyses at the ADVIA Centaur platform.

References

- 1 Bousquet J, van CP, Khaltaev N. Allergic rhinitis and its impact on asthma. *J Allergy Clin Immunol* 2001;108:S147–334.
- 2 Bauchau V, Durham SR. Prevalence and rate of diagnosis of allergic rhinitis in Europe. *Eur Respir J* 2004;24:758–64.
- 3 Bousquet J, Lockey R, Malling HJ. Allergen immunotherapy: therapeutic vaccines for allergic diseases. A WHO position paper. *J Allergy Clin Immunol* 1998;102:558–62.
- 4 Durham SR, Ying S, Varney VA *et al*. Grass pollen immunotherapy inhibits allergen-induced infiltration of CD4+ T lymphocytes and eosinophils in the nasal mucosa and increases the number of cells expressing messenger RNA for interferon-gamma. *J Allergy Clin Immunol* 1996;97:1356–65.
- 5 Furin MJ, Norman PS, Creticos PS *et al*. Immunotherapy decreases antigen-induced eosinophil cell migration into the nasal cavity. *J Allergy Clin Immunol* 1991;88:27–32.
- 6 Wilson DR, Irani AM, Walker SM *et al*. Grass pollen immunotherapy inhibits seasonal increases in basophils and eosinophils in the nasal epithelium. *Clin Exp Allergy* 2001;31:1705–13.
- 7 Brimnes J, Kildsgaard J, Jacobi H, Lund K. Sublingual immunotherapy reduces allergic symptoms in a mouse model of rhinitis. *Clin Exp Allergy* 2007;37:488–97.
- 8 Neimert-Andersson T, Thunberg S, Swedin L *et al*. Carbohydrate-based particles reduce allergic inflammation in a mouse model for cat allergy. *Allergy* 2008;63:518–26.
- 9 Razafindratsita A, Saint-Lu N, Mascarell L *et al*. Improvement of sublingual immunotherapy efficacy with a mucoadhesive allergen formulation. *J Allergy Clin Immunol* 2007;120:278–85.
- 10 Santeliz JV, Van NG, Traquina P, Larsen E, Wills-Karp M. Amb a 1-linked CpG oligodeoxynucleotides reverse established airway hyperresponsiveness in a murine model of asthma. *J Allergy Clin Immunol* 2002;109:455–62.
- 11 Taher YA, van Esch BC, Hofman GA, Henricks PA, van Oosterhout AJ. 1 α ,25-dihydroxyvitamin D₃ potentiates the beneficial effects of allergen immunotherapy in a mouse model of allergic asthma: role for IL-10 and TGF- β . *J Immunol* 2008;180:5211–21.
- 12 Wiedermann U, Jahn-Schmid B, Lindblad M *et al*. Suppressive versus stimulatory effects of allergen/cholera toxoid (CTB) conjugates depending on the nature of the allergen in a murine model of type I allergy. *Int Immunol* 1999;11:1131–8.
- 13 Bohle B, Kinaciyani T, Gerstmayr M, Radakovics A, Jahn-Schmid B, Ebner C. Sublingual immunotherapy induces IL-10-producing T regulatory cells, allergen-specific T-cell tolerance, and immune deviation. *J Allergy Clin Immunol* 2007;120:707–13.
- 14 Francis JN, Till SJ, Durham SR. Induction of IL-10+ CD4+ CD25+ T cells by grass pollen immunotherapy. *J Allergy Clin Immunol* 2003;111:1255–61.
- 15 Jutel M, Akdis M, Budak F *et al*. IL-10 and TGF- β cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. *Eur J Immunol* 2003;33:1205–14.
- 16 O'Hehir RE, Sandrini A, Anderson GP, Rolland JM. Sublingual allergen immunotherapy: immunological mechanisms and prospects for refined vaccine preparation. *Curr Med Chem* 2007;14:2235–44.
- 17 Radulovic S, Jacobson MR, Durham SR, Nouri-Aria KT. Grass pollen immunotherapy induces Foxp3-expressing CD4+ CD25+ cells in the nasal mucosa. *J Allergy Clin Immunol* 2008;121:1467–72.
- 18 Cooke R, Bernhard J, Hebal S, Stull A. Serological evidence of immunity with coexisting sensitization in hay fever type of human allergy. *J Exp Med* 1993;3:733–51.
- 19 Garcia BE, Sanz ML, Gato JJ, Fernandez J, Oehling A. IgG4 blocking effect on the release of antigen-specific histamine. *J Invest Allergol Clin Immunol* 1993;3:26–33.
- 20 Lichtenstein LM, Holtzman NA, Burnett LS. A quantitative in vitro study of the chromatographic distribution and immunoglobulin characteristics of human blocking antibody. *J Immunol* 1968;101:317–24.
- 21 Mothes N, Heinzkill M, Drachenberg KJ *et al*. Allergen-specific immunotherapy with a monophosphoryl lipid A-adjuvanted vaccine: reduced seasonally boosted immunoglobulin E production and inhibition of basophil histamine release by therapy-induced blocking antibodies. *Clin Exp Allergy* 2003;33:1198–208.
- 22 Till SJ, Francis JN, Nouri-Aria K, Durham SR. Mechanisms of immunotherapy. *J Allergy Clin Immunol* 2004;113:1025–34.
- 23 Woodhead JS, Weeks I. Magic Lite design and development. *J Biolumin Chemilumin* 1989;4:611–4.
- 24 Bergquist C, Lagergard T, Lindblad M, Holmgren J. Local and systemic antibody responses to dextran-cholera toxin B subunit conjugates. *Infect Immun* 1995;63:2021–5.
- 25 Kildsgaard J, Brimnes J, Jacobi H, Lund K. Sublingual immunotherapy in sensitized mice. *Ann Allergy Asthma Immunol* 2007;98:366–72.
- 26 Aberer W, Hawranek T, Reider N, Schuster C, Sturm G, Kranke B. Immunoglobulin E and G antibody profiles to grass pollen allergens during a short course of sublingual immunotherapy. *J Invest Allergol Clin Immunol* 2007;17:131–6.
- 27 Dahl R, Kapp A, Colombo G *et al*. Sublingual grass allergen tablet immunotherapy provides sustained clinical benefit with progressive immunologic changes over 2 years. *J Allergy Clin Immunol* 2008;121:512–8.
- 28 Durham SR, Yang WH, Pedersen MR, Johansen N, Rak S. Sublingual immunotherapy with once-daily grass allergen tablets: a randomized controlled trial in seasonal allergic rhinoconjunctivitis. *J Allergy Clin Immunol* 2006;117:802–9.
- 29 Malling HJ, Lund L, Ipsen H, Poulsen L. Safety and immunological changes during sublingual immunotherapy with standardized quality grass allergen tablets. *J Invest Allergol Clin Immunol* 2006;16:162–8.
- 30 Smith H, White P, Annala I, Poole J, Andre C, Frew A. Randomized controlled trial of high-dose sublingual immunotherapy to treat seasonal allergic rhinitis. *J Allergy Clin Immunol* 2004;114:831–7.
- 31 Ebner C, Siemann U, Bohle B *et al*. Immunological changes during specific immunotherapy of grass pollen allergy: reduced lymphoproliferative responses to allergen and shift from TH2 to TH1 in T-cell clones specific for Pbl p 1, a major grass pollen allergen. *Clin Exp Allergy* 1997;27:1007–15.
- 32 Fanta C, Bohle B, Hirt W *et al*. Systemic immunological changes induced by administration of grass pollen allergens via the oral mucosa during sublingual immunotherapy. *Int Arch Allergy Immunol* 1999;120:218–24.
- 33 Jarolim E, Poulsen LK, Stadler BM *et al*. A long-term follow-up study of hyposensitization with immunoblotting. *J Allergy Clin Immunol* 1990;85:996–1004.
- 34 Marcucci F, Sensi L, Di CG, Incorvaia C, Frati F. Dose dependence of immunological response to sublingual immunotherapy. *Allergy* 2005;60:952–6.
- 35 Savolainen J, Jacobsen L, Valovirta E. Sublingual immunotherapy in children modulates allergen-induced in vitro expression of cytokine mRNA in PBMC. *Allergy* 2006;61:1184–90.
- 36 O'Hehir RE, Gardner LM, de Leon MP *et al*. House Dust Mite Sublingual Immunotherapy – The Role for TGF- β and Functional Regulatory T Cells. *Am J Respir Crit Care Med* 2009;180:936–47.
- 37 Rask C, Fredriksson M, Lindblad M, Czerkinsky C, Holmgren J. Mucosal and systemic antibody responses after peroral or intranasal immunization: effects of conjugation to enterotoxin B subunits and/or of co-administration with free toxin as adjuvant. *APMIS* 2000;108:178–86.

- 38 Bahceciler NN, Arikan C, Taylor A *et al.* Impact of sublingual immunotherapy on specific antibody levels in asthmatic children allergic to house dust mites. *Int Arch Allergy Immunol* 2005;136:287–94.
- 39 Francis JN, James LK, Paraskevopoulos G *et al.* Grass pollen immunotherapy: IL-10 induction and suppression of late responses precedes IgG4 inhibitory antibody activity. *J Allergy Clin Immunol* 2008;121:1120–5.
- 40 Platts-Mills TA, von Maur RK, Ishizaka K, Norman PS, Lichtenstein LM. IgA and IgG anti-ragweed antibodies in nasal secretions. Quantitative measurements of antibodies and correlation with inhibition of histamine release. *J Clin Invest* 1976;57:1041–50.
- 41 Brandtzaeg P. Induction of secretory immunity and memory at mucosal surfaces. *Vaccine* 2007;25:5467–84.
- 42 Bottcher MF, Haggstrom P, Bjorksten B, Jenmalm MC. Total and allergen-specific immunoglobulin A levels in saliva in relation to the development of allergy in infants up to 2 years of age. *Clin Exp Allergy* 2002;32:1293–8.
- 43 Payette K, Weiss NS. Salivary IgA levels in atopic children. *Ann Allergy* 1977;39:328–31.
- 44 van Asperen PP, Gleeson M, Kemp AS *et al.* The relationship between atopy and salivary IgA deficiency in infancy. *Clin Exp Immunol* 1985;62:753–7.
- 45 Mayer L. Review article: local and systemic regulation of mucosal immunity. *Aliment Pharmacol Ther* 1997;3:81–5.
- 46 Pilette C, Nouri-Aria KT, Jacobson MR *et al.* Grass pollen immunotherapy induces an allergen-specific IgA2 antibody response associated with mucosal TGF-beta expression. *J Immunol* 2007;178:4658–66.