

ORIGINAL PAPER

Sublingual immunotherapy reduces allergic symptoms in a mouse model of rhinitis

J. Brimnes, J. Kildsgaard, H. Jacobi and K. Lund

Department of Experimental Immunology, ALK-Abelló A/S, Hoersholm, Denmark

Clinical and Experimental Allergy

Summary

Background Sublingual immunotherapy (SLIT) is a clinically effective treatment in both pollen and house dust mite-induced rhinitis and asthma. However, the mechanisms by which this is accomplished are not clear.

Objective The objective of the current study was to establish a mouse model of rhinitis in order to study the effect and mechanisms of SLIT.

Methods Mice were sensitized by intraperitoneal injections of alum-adsorbed *Phleum pratense* extract. Sensitized mice were SLIT-treated and subsequently challenged intranasally and analysed for clinical symptoms, antibody levels, eosinophilia and T cell response.

Results Intranasal challenge of sensitized mice led to the development of rhinitis characterized by significantly increased sneezing and influx of eosinophils into the nose. Levels of specific IgE were fivefold increased in nasopharyngeal lavage (NAL) fluid and more than doubled in serum. Furthermore, a T-helper type 2 (Th2) like T cell response was observed in local draining lymph nodes. SLIT treatment of sensitized mice reduced sneezing, eosinophilia and IgE levels in the NAL by more than 50%. Moreover, serum levels of IgE and IgG1 as well as T cell response in the draining lymph nodes were also significantly reduced. Treatment for a shorter time or with a lower dose only led to minor reductions of the clinical and immunological parameters, indicating that the effect of SLIT is time and dose dependent.

Conclusion In the present study, we have established a mouse model displaying the hallmarks of allergic rhinitis using a clinically relevant allergen. Using this model, we have demonstrated that SLIT treatment is able to reduce allergic symptoms in a time- and dose-dependent manner.

Keywords mouse model, rhinitis, sublingual immunotherapy

Submitted 4 May 2006; revised 23 August 2006; accepted 11 October 2006

Correspondence:

Jens Brimnes, Department of Experimental Immunology, ALK-Abelló A/S, Boege Alle 6-8, DK-2970 Hoersholm, Denmark.
E-mail: jeb@dk.alk-abello.com

Introduction

Over the past decades, there has been a significant increase in both the incidence and prevalence of atopic diseases in the western countries [1, 2]. Allergic rhinitis, which is the most common of the atopic diseases, affecting 15–20% of the population, is characterized by sneezing, watery nasal discharge, nasal obstruction and itching [1, 3]. The allergic reaction is triggered by allergen-mediated cross-linking of specific IgE on the surface of mast cells and basophils. This leads to the release of histamine and other mediators, causing an acute allergic

reaction. The acute reaction is followed by a late-phase reaction that peaks 6–24 h after exposure to the allergen and is characterized by an influx of eosinophils, neutrophils and CD4⁺ T-helper type 2 (Th2) cells producing IL-4, IL-5 and IL-13 [4].

Specific immunotherapy is recognized as an effective treatment of allergic rhinitis. Traditionally, specific immunotherapy has been conducted by repeated subcutaneous administrations of small amounts of specific allergen to the sensitized subject [5]. Although this form of treatment is an effective therapeutic option, over the last 15 years there has been an increasing interest in

developing more convenient alternatives to subcutaneous injections in order to improve safety and patient compliance. These include the oral, nasal, bronchial and sublingual route of administration. Especially, sublingual immunotherapy (SLIT) has been the focus of many clinical studies and has now been demonstrated to be a safe and clinically effective treatment in both pollen and house dust mite-induced rhinitis and asthma [6–8]. However, the mechanism of action of SLIT remains largely unknown. Among the immunological parameters investigated thus far in relation to SLIT, serum levels of IgE and IgG4 are by far the most frequent. Most studies demonstrate that the levels of allergen-specific IgE are either increased or remain unaffected by SLIT treatment, whereas the levels of allergen-specific IgG4, which are thought to reflect a modulation of the allergic Th2-response, are increased in patients actively treated with SLIT [9–11]. It has also been demonstrated that SLIT leads to a decrease in eosinophils and eosinophil cationic protein in the nasal lavage fluid [12, 13], indicating that the local inflammation in SLIT-treated patients is reduced. Only a few results regarding the effect of SLIT on the T cell response exist, and these suggest that SLIT may work by modulating the Th2-biased allergic immune response [14–16].

Mouse models are a valuable supplement to clinical and *in vitro* studies when investigating the mechanisms of allergic airway diseases. Moreover, such models may provide useful information concerning the effect and mechanisms of the current treatment of rhinitis, which is vital for the generation of new and more effective treatments of allergic rhinitis and asthma. Over the years, many different protocols have been applied to induce experimental allergic airway hyper-responsiveness (AHR) in mice. The most frequently used regimens consist of systemic sensitization, followed by challenge of the airways by either intranasal (i.n.) administration or by inhalation of aerosolized antigen. Depending on the challenge procedure, this leads to models displaying characteristics of either human rhinitis or asthma [17, 18]. Such mouse models have unveiled important mechanisms of allergic airway inflammation such as the link between IL-5 and the recruitment of eosinophils into the airways as well as the central role of IL-13 in allergic airway inflammation [19, 20].

In the present study, we have developed a murine model of rhinitis, in which the mice react towards the timothy grass allergen *Phleum pratense* (Phl p). This model presents many of the clinical and immunological features associated with human allergic rhinitis, including sneezing, increased AHR, an influx of eosinophils as well as elevated levels of IL-5 and antigen-specific IgE both locally and systemically. Using this model, we have studied the effect of SLIT and demonstrated that SLIT treatment is able to reduce allergic symptoms in a time- and dose-dependent manner.

Materials and methods

Animals

Female, 6–10-week-old Balb/cJ mice were bred in-house and maintained on a defined diet, not containing components cross-reacting with antisera to Phl p extract. The mice were housed in a specific pathogen-free environment under a 12-h light, 12-h dark cycle. All experiments described in this report were conducted in accordance with Danish legislation.

Induction of rhinitis

Mice were sensitized by three intraperitoneal (i.p.) injections of alum-adsorbed Phl p extract (ALK-Abelló, Hørsholm, Denmark) at days 1, 14 and 21. Each injection contained 5000 SQ units of Phl p extract, corresponding to approximately 1 µg of the major allergen Phl p 5. This was followed by daily i.n. challenge of 5 µL Phl p extract (100–500 SQ/µL) in each nostril (corresponding to a total dose of 1000–5000 SQ) for 1 or 2 weeks. At the end of the challenge period, the mice were analysed for clinical parameters. Subsequently, the mice were killed and blood, bronchoalveolar lavage (BAL) fluid, nasopharyngeal lavage (NAL) fluid, spleen and cervical lymph nodes were collected for analysis. The distribution of intranasally applied solutions was investigated by applying 5 µL of a 0.5% Evans Blue solution into each nostril. The mice were killed 5 min after the i.n. administration and the distribution of the dye in nasal tissue, trachea, lungs and muscle (negative control) was evaluated by visual inspection as well as by extraction of tissue in an acetone/0.5% Na₂SO₄ solution, followed by measurement of absorption at 620 nm.

Sublingual immunotherapy treatment

The effect of SLIT was assessed by daily sublingual treatment with either Phl p-extract or buffer after sensitization and before i.n. challenge. SLIT was performed by holding the scruff of the mice and carefully applying 5 µL of allergen solution under the tongue. The mice were held by the scruff for an additional 20 s to prevent the animal from swallowing the allergen solution. The mice were SLIT-treated with a daily dose of 25–125 kSQ, five days a week for 6–9 weeks, as indicated in the Results section. The distribution of sublingually applied solutions was investigated using a 0.5% Evans Blue solution. The mice were killed 5 min after the administration and the distribution of the dye in sublingual tissue, esophagus, stomach and muscle (negative control) was evaluated by visual inspection as well as by extraction of tissue in an acetone/0.5% Na₂SO₄ solution, followed by measurement of absorption at 620 nm.

Measurement of clinical parameters

Sneezes were counted manually in a blinded manner in the 10-min period following i.n. challenge with Phl p extract as described by Saito et al. [21]. AHR was measured *in vivo* 24 h after the last i.n. challenge using whole-body plethysmography (EMKA Technologies, Paris, France) by challenging the mice with increasing amounts of aerosolized methacholine (0–25 mg/mL). The mice were challenged with metacholine for 2 min, followed by 6 min of data recording.

Immunohistochemistry

Lungs were removed and fixed in 10% formalin in phosphate-buffered saline (PBS) overnight at 4 °C. Following this, the tissue was embedded in paraffin; sections were cut, stained with haematoxylin and eosin and evaluated by light microscopy. Detection of eosinophils in BAL and NAL fluid was performed by spinning down the cells of BAL and NAL and re-suspending them in PBS containing 1% BSA. Cytospins were prepared using 100 µL cell suspensions, corresponding to 1×10^5 cells per slide. The slides were air dried and stained with May–Grünwald Giemsa stain. For each slide, 200 cells were counted and differentiated and results were expressed as numbers of eosinophils per 200 cells.

Measurement of eosinophil peroxidase in bronchoalveolar lavage and nasopharyngeal lavage fluid

The presence of eosinophils in BAL and NAL fluid was also measured by detection of eosinophil peroxidase (EPO) by the method of Strath et al. [22]. In brief, the BAL and NAL fluids were spun down and the cell pellets were dissolved in Tris-HCl containing 0.1% Triton X-100 (Sigma, St Louis, MO, USA). Cell debris was centrifuged down and the supernatants were analysed in a 96-well microtitre plate by adding ortho-phenyl diamine and hydrogen peroxide (Sigma). After incubation for 30 min in the dark, the plates were read at 492 nm.

Measurement of specific antibodies

Allergen-specific IgG1 and IgG2a antibodies were measured in an indirect ELISA assay. NUNC maxisorp plates (Nunc, Roskilde, Denmark) were coated with Phl p extract (10 µg/mL in PBS, pH 7.2). After addition of diluted sera, the antibodies were detected with biotinylated goat anti-mouse IgG1 or biotinylated rat anti-mouse IgG2a antibodies (Southern Biotechnology, Birmingham, AL, USA and BD Biosciences, San Diego, CA, USA, respectively). Streptavidin-peroxidase (DAKO, Copenhagen, Denmark) was added and the plates were developed with TMB one (3,3', 5,5' tetramethylbenzidine) (Kem-En-Tec, Copenhagen, Denmark). Absorbencies were read at 450 nm.

Allergen-specific IgE and IgA antibodies were measured in a Magic Lite assay. Briefly, serum, BAL or NAL fluid was 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 or rat anti-mouse IgE (Indicia Biotechnology, Oullins, France). After washing, allergen-specific antibodies were detected by biotinylated Phl 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].

In some instances, the levels of Phl p-specific serum IgG1, IgG2a, IgE and IgA were measured on the ADVIA Centaur platform (Bayer Diagnostics, Tarrytown, NY, USA). Briefly, goat anti-mouse Ig (Southern Biotechnology) covalently coupled to paramagnetic particles was used to capture the different serum Ig-isotypes. Bound solid-phase Ig's were allowed to react with liquid-phase purified biotinylated major allergens that were detected as chemiluminescence using acridiniumester-labelled streptavidin.

T cell proliferation and cytokine production

Spleens or cervical lymph nodes were teased into a single-cell suspension and washed three times in RPMI-1640 (Bio Whittaker, Laden, Belgium). Cells were counted and adjusted to 1.67×10^6 cells/mL in RPMI-1640 containing 50 µg/mL gentamycin (Gibco, Carlsbad, CA, USA), 1% Nutridoma (Roche, Basel, Switzerland) and 1.5 mM monothioglycerol (Sigma). 3×10^5 cells were added to each well of a 96-well flat-bottomed culture plate (Nunc) and the cells were stimulated by Phl p extract (0, 5 and 40 µg/mL). The cells were cultured for 6 days at 37 °C and 5% CO₂. Proliferation was measured by adding 0.5 µCi of ³H-thymidine to each well for the last 18 h of the culture period, followed by harvesting the cells on a Tomtec 96-well plate harvester (Tomtec, Hamden, CT, USA) and counting the incorporated radiolabel using a Wallac Microbeta 1450 Liquid scintillation counter (Wallac, Wellesley, USA).

For measurements of cytokine levels, the cells were adjusted to 3×10^6 cells/mL and 900 µL cell suspensions were added to each well of a 24-well microtitre plate (Nunc) together with 100 µL medium or Phl p extract (40 µg/mL). Supernatants were harvested at day 5 and analysed for the presence of cytokines using a cytometric bead assay kit (BD Biosciences) according to the manufacturer's instructions.

Results

Establishment of rhinitis model

In order to be able to investigate the effect of SLIT on the course of rhinitis, a murine model of rhinitis had to be

established first. To this end, mice were sensitized by three i.p. injections of alum-adsorbed Phl p extract, followed by an i.n. challenge with buffer or different doses of Phl p extract (see Fig. 1 for experimental outline). Initial time- and dose-finding studies demonstrated that i.n. challenge with 5000 SQ Phl p extract was the most effective for the induction of rhinitis. Therefore, the mice have been intranasally challenged in this way throughout the study. The distribution of the intranasally administered solutions was investigated by applying 5 µL Evans Blue dye solution intranasally. The applied dye could only be found in the nasal cavity and no traces of the dye were detected in the lower airways (data not shown).

Figures 2a and b show that mice sensitized and challenged as described above displayed clear clinical symptoms of rhinitis, as the number of sneezes was increased two- to threefold compared with mice challenged with buffer. In addition, AHR was increased more than four times when challenged with the highest dose of metacholine. It can also be seen in Fig. 2 that i.n. challenge with buffer led to a low level of 'background-sneezing', indicating that i.n. instillation of a liquid *per se* leads to sneezes of a mechanical, non-allergic nature. This background sneezing was also observed when non-sensitized mice received buffer intranasally (data not shown).

Analysis of the NAL fluid showed a significant increased influx of eosinophils (Fig. 2c) as well as a fourfold increase in the levels of Phl p-specific IgE (Fig. 2d) – both markers of an allergic inflammation. In addition to the increased levels of eosinophils in the NAL, mice-challenged intranasally with Phl p extract also displayed inflamed nasal tissue, characterized by an influx of mononuclear cells into nasal mucosa (data not shown). In the BAL fluid, a non-significant trend towards increased influx of eosinophils and a significant increase in the levels of Phl p-specific IgE was observed. Together with the increased AHR mentioned above, this suggests involvement of the lungs. However, histological data did not

show any inflammation of the lungs, indicating that the allergic inflammation was primarily localized to the upper airways (data not shown).

In line with the results of NAL and BAL fluids, we observed a twofold increase in the levels of Phl p-specific IgE in the serum of intranasally challenged mice. In addition to IgE, the serum levels of IgG1 (which is also regarded as a marker of a Th2 type of response) and IgG2a were both increased twofold (data not shown).

The T cell response to the i.n. challenge was assessed by measuring the allergen-specific *ex vivo* proliferation and cytokine production of cells from the spleen and local draining cervical lymph nodes (LN) from sensitized and i.n.-challenged mice. As can be seen in Fig. 2e, the proliferation of spleen cells from sensitized mice, which were intranasally challenged with Phl p extract, did not differ from mice challenged with buffer. However, as can be seen in Fig. 2f, the levels of both IL-4 and IL-5 were significantly increased in spleen cell cultures from mice challenged with Phl p extract, indicating that a systemic Th2-like response is present. In contrast to the systemic T cell response, cells from the local draining lymph node of Phl p-challenged mice displayed a five times increased proliferation compared with buffer-challenged mice upon *in vitro* challenge with Phl p extract (Fig. 2g). Moreover, the levels of IL-4 in the LN cell cultures were more than twofold increased and the levels of IL-5 were increased more than threefold, whereas the levels of IFN-γ and IL-10 were not affected, indicating a strong local Th2-like response (Fig. 2h).

Taken together, these results show that we have established a murine model of rhinitis displaying the classic signs of the disease, such as increased sneezing, increased influx of eosinophils and inflammation in the nasal mucosa as well as increased levels of IgE.

Effect of sublingual immunotherapy treatment on the course of experimental rhinitis

Having established the mouse model of rhinitis, we applied this model to study the effect of SLIT on the course of rhinitis. To make sure that the sublingually administered solutions were not swallowed immediately, the mice were held in the scruff for 20 s. Using Evans Blue as a tracer dye, we demonstrated that dye was present in the stomach to an equal extent in mice that were allowed to swallow the allergen solution immediately and in mice that were held in the scruff for 20 s. In addition, we have previously demonstrated that there was a greatly diminished effect of the SLIT treatment in mice that were allowed to swallow the allergen solution immediately, indicating that contact with the sublingual mucosa is necessary for the effect of SLIT (data not shown).

As outlined in Fig. 1b, the effects of SLIT were investigated by treatment with either Phl p extract (active

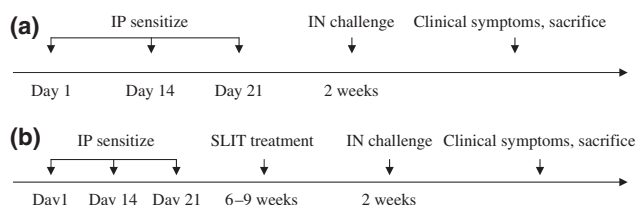


Fig. 1. Outline of the experimental design. (a) Rhinitis was induced by three intranasal (i.n.) injections of 5000 SQ Phl p extract adsorbed to alum at days 1, 14 and 21, followed by 2 weeks of daily i.n. challenges with 5000 SQ Phl p extract per mouse. Clinical symptoms were recorded immediately after the last i.n. challenge and 24 h later the mice were killed for collection of serum, nasopharyngeal lavage, bronchoalveolar lavage and tissues (b) Treatment of rhinitis by sublingual immunotherapy (SLIT) was performed by sublingual administration of Phl p extract (5 µL containing 25–125 kSQ) for 6–9 weeks. The mice were SLIT treated after sensitization and before i.n. challenge.

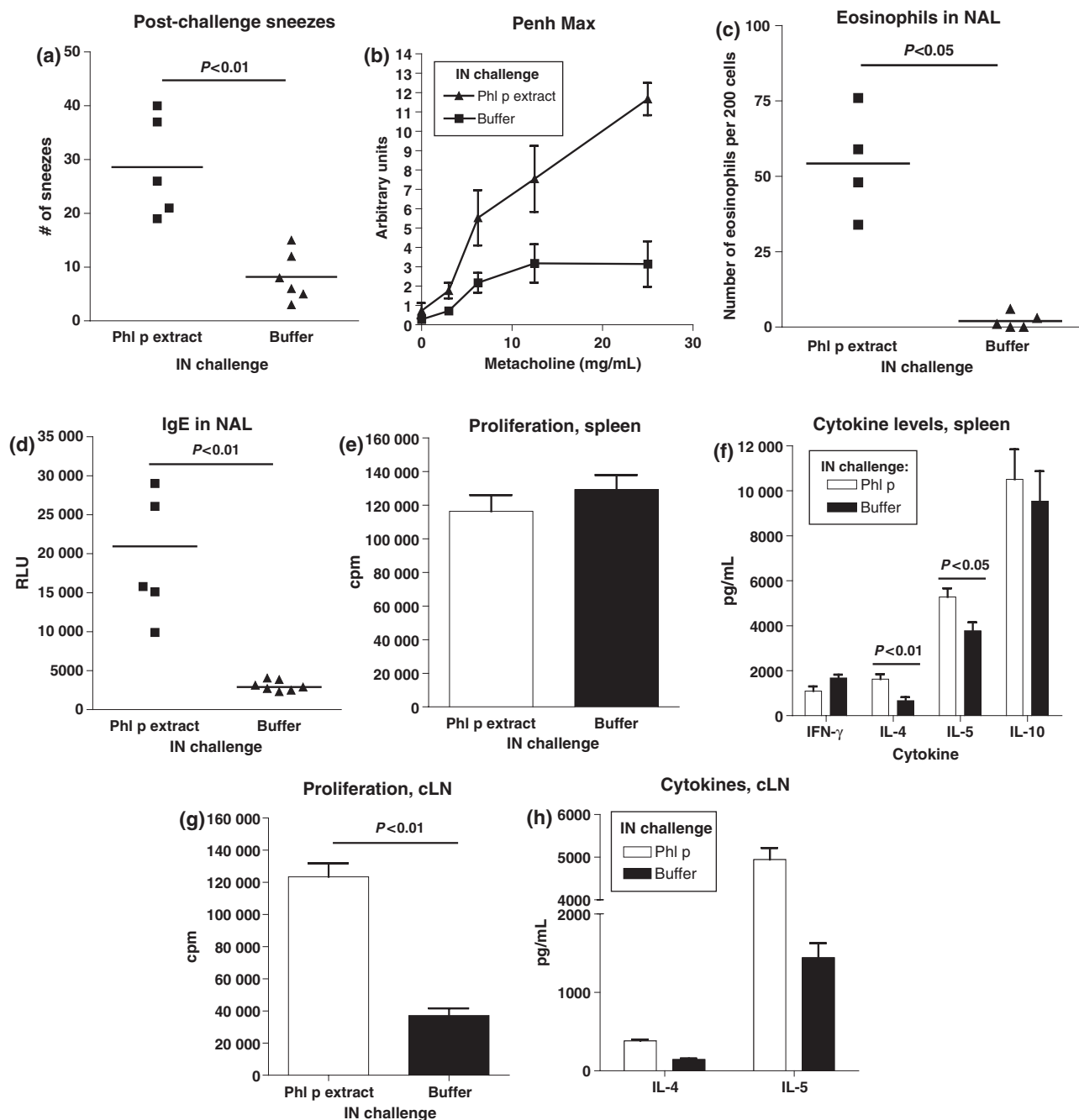


Fig. 2. Establishment of rhinitis. The response of sensitized mice to intranasal (i.n.) challenge with 5000 SQ Phl p extract (filled squares, open columns) or buffer (filled triangles, filled columns). (a) Number of sneezes recorded in a 10-min period immediately following i.n. challenge. (b) Airway hyper-responsiveness (AHR) upon metacholine challenge measured by whole-body plethysmography and expressed as enhanced pause (Penh) values. Each dot represents the mean value of the group ($n = 5$) and error bars indicate SEM. (c) Number of eosinophils per 200 cells in nasopharyngeal lavage (NAL) after i.n. challenge. (d) Levels of Phl p-specific IgE in NAL after i.n. challenge measured using the Magic Lite assay. (e) *Ex vivo* proliferation of spleen cells re-stimulated with Phl p extract. (f) *Ex vivo* cytokine production of spleen cells re-stimulated with Phl p extract. (g) *Ex vivo* proliferation of cLN cells re-stimulated with Phl p extract. (h) *Ex vivo* cytokine production of cLN cells re-stimulated with Phl p extract. Each dot in (a), (c) and (d) represents an individual mouse and lines indicate group medians. Each column in (e)-(h) represents the mean value of the group ($n = 6$), and error bars indicate standard error of mean.

treatment) or with buffer (placebo treatment). Both actively treated and placebo-treated mice were subsequently intranasally challenged with Phl p extract. As can be seen in Fig. 3, SLIT treatment of sensitized mice with 125 kSQ

Phl p extract for 9 weeks led to a significant reduction of the clinical signs of rhinitis. Panel A shows that post-challenge sneezes were reduced by more than 50% and panel B shows that also AHR upon metacholine challenge

was significantly decreased in Phl p SLIT-treated mice. Moreover, as can be seen in Figs. 3c and d, the levels of eosinophils and IgE in the NAL fluid were significantly reduced in mice that were SLIT treated with Phl p extract. Taken together, this demonstrates that SLIT treatment leads to a clear reduction of the local allergic inflammation.

The reduced levels of IgE observed locally in NAL were also reflected systemically in serum. The levels of both IgE and IgG1, which are both regarded as markers of a Th2-like response, were reduced by 50% in Phl p SLIT-treated mice. In contrast, IgG2a, which is associated with a Th1-like response, was not affected by the treatment (data not shown).

Finally, in order to investigate the effect of SLIT treatment on the allergen-specific T cell response, the reactivity of spleen and cervical LN cells upon *ex vivo* re-stimulation with Phl p extract was examined. As can be seen in Fig. 3e, there was a trend towards a down-regulation of the Phl p-specific proliferation of spleen cells, although this was not statistically significant. In contrast, the proliferative response towards Phl p extract of cervical LN cells was significantly reduced in mice that were actively SLIT-treated compared with buffer-treated ones (Fig. 3g). The difference between cells from the spleen and LNs was also reflected in the cytokine profile as the levels of IL-4, IL-5 and IL-10 were down-regulated in cultures of cervical LN cells, whereas this was not the case in the spleen (Figs. 3f and h).

The effect of sublingual immunotherapy treatment is time and dose dependent

In order to investigate the dependency of the duration of the SLIT treatment, sensitized mice were SLIT treated with the same dose as above (125 kSQ) for 6 weeks instead of 9 weeks. In contrast to 9 weeks of treatment, 6 weeks of treatment did not lead to a reduction of the clinical signs of experimental rhinitis. Although a trend towards a reduction of sneezes and AHR could be observed, this was not significant and the levels of eosinophils in the NAL fluid were not significantly affected by the SLIT treatment (Fig. 4a). However, contrary to the lack of effect on the clinical parameters, the levels of IgE in both NAL fluid and serum were significantly reduced in actively SLIT-treated mice (Figs. 4b and c), indicating that although 6 weeks of treatment did not have a statistically significant effect on the clinical parameters, it did have some effect on the immune system. SLIT treatment with 125 kSQ Phl p extract for 6 weeks did not affect the allergen-specific *ex vivo* T cell response. This was true for both the systemic T cell response in the spleen and the local T cell response in the cervical LN, although a trend towards a down-regulated response could be observed in the latter (data not shown). Finally, the dose dependency of SLIT treatment was investigated by lowering the dose

five times, i.e. SLIT treating the mice for 6 weeks with 25 kSQ. This did not have any impact on the course of experimental rhinitis in our mouse model as neither the clinical nor the immunological parameters were modified compared with buffer-treated mice.

Taken together, the above data indicate that the effect of SLIT treatment is both dose and time dependent.

Discussion

In the present study, we have established a murine model of rhinitis, which, in terms of immunological and clinical parameters, is reminiscent of the symptoms associated with allergic rhinitis in humans. In line with previously published work [17, 21, 24], the protocol applied in the present study consisted of a sensitization phase in which the mice received i.p. injections of the allergen adsorbed to alum, followed by i.n. administration of the allergen. Our results show that this leads to clear clinical and immunological signs of allergic airway disease. In contrast to the majority of mouse models of allergic airway disease in which ovalbumin serves as the target allergen, Timothy grass (*P. pratense*) was chosen as the target allergen in the present model. We believe that this makes the current model more clinically relevant as the Phl p grass-pollen extract more closely resembles a naturally occurring, complex allergen source than e.g. ovalbumin, especially when studying the aetiology and treatment of inhalation allergies. The importance of using naturally occurring grass-pollen allergen extract for model development is underscored by the fact that 8–35% of young adults are sensitized towards grass-pollen allergens [25].

Whereas it has been demonstrated in several different mouse models that inhalation of aerosolized allergen leads to inflammation in both the upper and lower airways [17], it has been a matter of debate whether i.n. administration of allergen leads to inflammation in the upper airways only or whether this also causes inflammation in the lower airways. It has been demonstrated that when the mice were heavily anaesthetized, up to 30% of the allergen solution can be found in the lower airways where it causes inflammation [26]. Other studies, however, have shown that although the intranasally administered allergen does not leave the upper airways, the mice do develop inflammation in the lower airways [24, 27]. In the present study, in order to avoid the direct contact of allergen with the lower airways during i.n. challenge, only a small volume (5 µL compared with 20–30 µL in most other studies) of the allergen extract was instilled into each nostril of slightly anaesthetized mice. Although experiments using Evans Blue showed no distribution of the dye to the lungs, we observed some response in the lower airways characterized by increased levels of eosinophils and specific IgE in the BAL fluid. However, as we did not observe any histological signs of inflammation, the

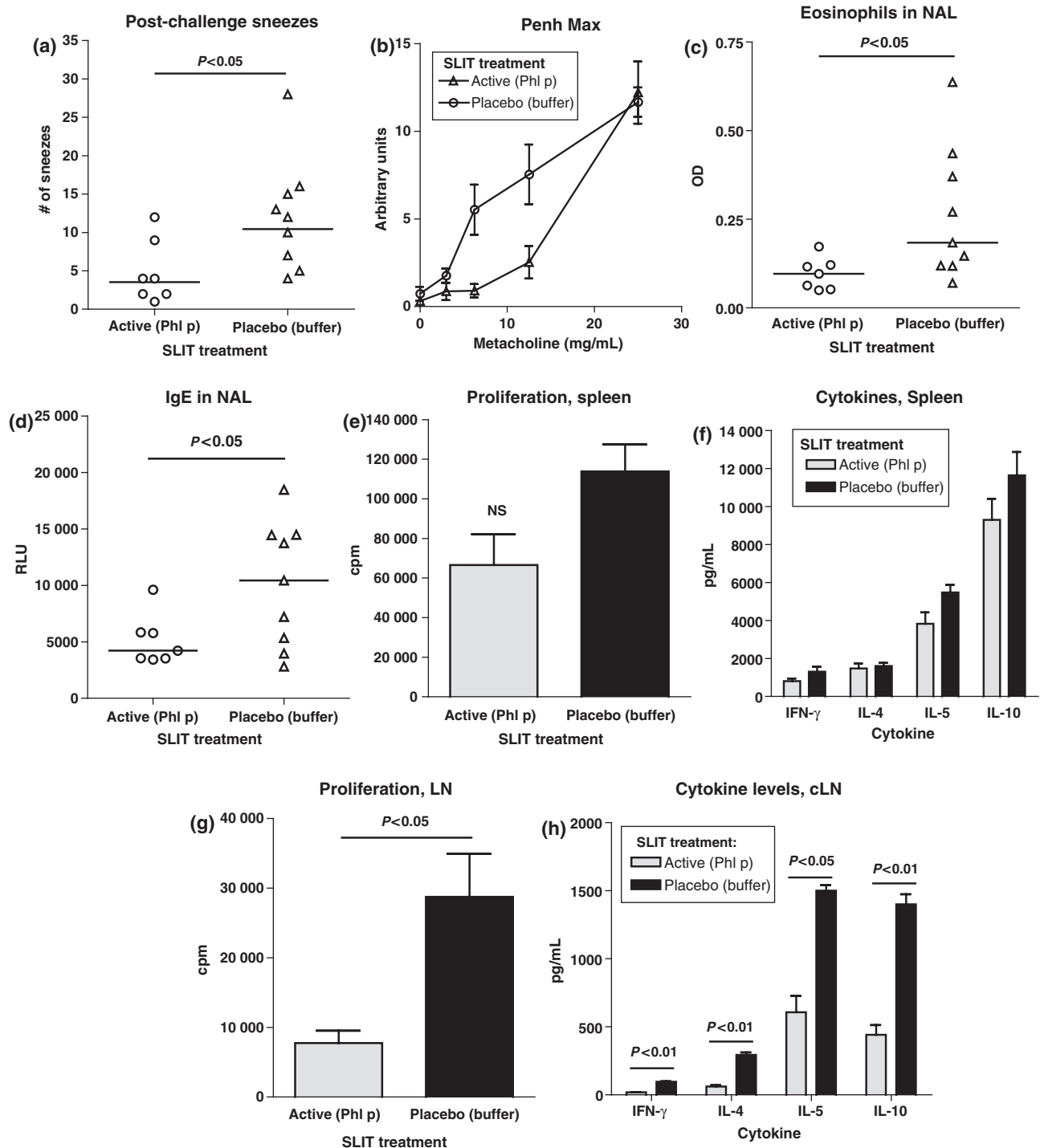
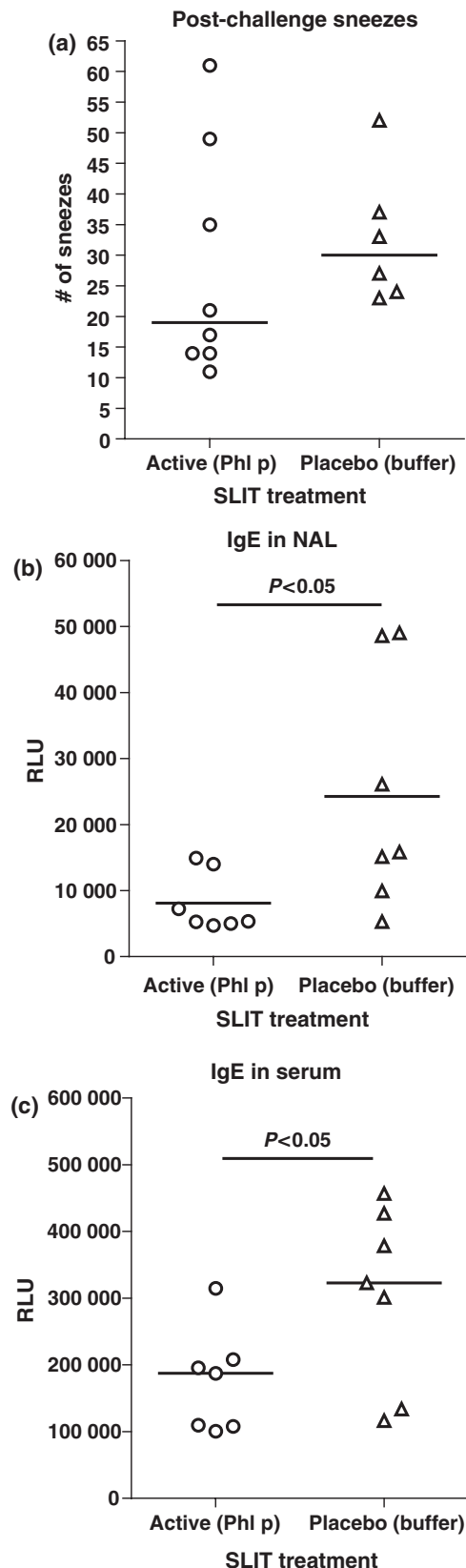


Fig. 3. Sublingual immunotherapy (SLIT) treatment. The effect of 125 kSQ Phl p SLIT treatment for 9 weeks. Sensitized mice were SLIT treated with 125 kSQ Phl p extract (open circles, grey columns) or placebo treated with buffer (open triangles, hatched columns) for 9 weeks before intranasal (i.n.) challenge. (a) Number of sneezes recorded in a 10-min period immediately following i.n. challenge. (b) Airway hyper-responsiveness (AHR) upon metacholine challenge measured by whole-body plethysmography and expressed as enhanced pause (Penh) values. Each dot represents the mean value of the group ($n = 8$), and error bars indicate SEM. (c) Level of eosinophils in nasopharyngeal lavage (NAL) after i.n. challenge measured by eosinophil peroxidase ELISA. (d) Levels of Phl p-specific IgE in NAL after i.n. challenge, measured using the Magic Lite assay. (e) *Ex vivo* proliferation of spleen cells re-stimulated with Phl p extract. (f) *Ex vivo* cytokine production of spleen cells re-stimulated with Phl p extract. (g): *Ex vivo* proliferation of cLN cells re-stimulated with Phl p extract. (h) *Ex vivo* cytokine production of cLN cells re-stimulated with Phl p extract. Each dot in (a), (c) and (d) represents an individual mouse and lines indicate group medians. Each column in (e)–(h) represents the mean value of the group ($n = 6$) and error bars indicate standard error of mean.



involvement of the lower airways was considered limited. Taken together, these data suggest that inflammation of the upper airways may not be present without a low grade inflammation in the lower airways. This is in line with the notion that allergic rhinitis and asthma may be a part of a systemic disease complex with a common aetiology, albeit with different clinical manifestations, which is also known as the united airways hypothesis [24, 28].

Parallel to the increased antibody response, i.n. challenge led to an increase of the allergen-specific T cell response beyond the level induced by the systemic sensitization. This was most pronounced in the draining LNs, where both the increased proliferation and the increased levels of IL-4 and IL-5 indicate an activation of Th2-like cells in the local tissue following challenge. In spleen cell cultures, the i.n. challenge only led to an increased production of IL-4 and IL-5, indicating that the systemic effect of the i.n. challenge was less pronounced than the effect observed in the draining LNs. The increased Th2-like T cell response observed in the present study is in line with the limited data existing on the role of T cells in the pathogenesis of allergic rhinitis showing an increase in the numbers and recruitment of CD4⁺ Th2 cells [21, 29]. Furthermore, the importance of CD4⁺ Th2 cells in the pathogenesis of allergic airway disease has been highlighted in several asthma models [30].

In the present study, we have demonstrated that SLIT treatment is able to reduce allergic symptoms in the above-described mouse model of rhinitis. To our knowledge, this is the first time that the effect of SLIT has been studied in an animal model of rhinitis, making comparison with similar studies difficult. However, our results are in line with what has been observed in humans, as SLIT treatment has been demonstrated to reduce efficiently both symptom score and medication requirements in rhinitis patients [6]. Furthermore, the fact that SLIT treatment in the present rhinitis model is able to reduce the allergic symptoms both in the upper (sneezes) and in the lower (AHR) airways is in accordance with evidence for a preventive effect of subcutaneous immunotherapy on the development of asthma [31] and it is conceivable that a similar effect can be obtained by SLIT.

That the effect of SLIT is indeed obtained through exposure of the grass pollen extract to the oral/sublingual

Fig. 4. Sublingual immunotherapy (SLIT) treatment. The effect of 125 kSQ Phl p SLIT treatment for 6 weeks. Sensitized mice were SLIT treated with 125 kSQ Phl p extract (open circles) or placebo treated with buffer (open triangles) for 6 weeks before intranasal (i.n.) challenge. (a) Number of sneezes recorded in a 10-min period immediately following i.n. challenge. (b) Levels of Phl p-specific IgE in nasopharyngeal lavage (NAL) after i.n. challenge, measured using the Magic Lite assay. (c) Levels of Phl p-specific IgE in serum after i.n. challenge, measured using the Magic Lite assay. Each dot represents an individual mouse, and lines indicate group medians.

mucosa rather than by contact with the intestinal mucosa (traditional oral tolerance) is strongly supported by the fact that the effect of SLIT was greatly diminished in mice that were allowed to swallow the allergen solution immediately.

Regarding our understanding of the underlying immunological mechanisms of SLIT treatment, it is now well established that SLIT treatment leads to a systemic increase of allergen-specific IgG4, whereas systemic, allergen-specific IgE is either increased or not affected [9–11, 32]. Furthermore, in humans, SLIT treatment reduces the influx of eosinophils into the eyes and nose upon allergen challenge. Most of these results could be reproduced in the present murine model of rhinitis, but in contrast to the human studies, we observed that SLIT treatment of allergen-sensitized mice reduced the level of allergen-specific IgE both locally and systemically. This is consistent with the results from other animal models showing that i.n. [33], sublingual [34] and oral [35] administration is able to down-regulate specific IgE.

In the present study, we show that SLIT treatment down-regulates the allergen-specific Th2-like response of T cells from the local draining cervical LNs. Although, a tendency towards a down-regulation of the systemic T cell response in the spleen was observed, this was not significant, indicating that the effect on the T cell response may predominantly be local. In line with this, a previously published study showed that cervical LNs possess an intrinsic capacity to mediate immunological tolerance [36]. This tolerance was associated with the generation of regulatory T cells in the draining LNs [37]. In the present work, both IFN- γ and IL-10 were down-regulated in addition to IL-4 and IL-5, suggesting that neither a regulatory nor a Th1 response is induced by the SLIT treatment. The down-regulation of Th2-, Th1- and T-regulatory cytokines suggests that deletion of allergen specificity may be the mechanism by which the effect of SLIT is mediated in this model of rhinitis. However, identification of the mechanisms underlying the effect of SLIT needs extensive further research.

Our results show that the effect of SLIT is both time and dose dependent. Treatment with 125 kSQ for 9 weeks was able to ameliorate both clinical symptoms, eosinophilia, allergen-specific IgE as well as the local T cell response. A shorter treatment period still had an effect on the levels of IgE, whereas there was no effect on the clinical symptoms. The fact that the levels of specific IgE were reduced without effect on the clinical symptoms questions a direct connection between these two parameters. Interestingly, the lack of correlation between clinical symptoms and levels of IgE is also observed in humans, as SLIT treatment of allergic patients leads to an increase in the levels of specific IgE, while the clinical symptoms are reduced [32]. Further lowering the SLIT dose led to a complete neutralization of the effect of the treatment. Such time and dose

dependency has also been observed in humans. In a large, tablet-based SLIT study, it was shown that 8 weeks or more of pre-seasonal treatment were significantly more effective than 6 weeks, with respect to reduction of symptom score and medication requirements [32]. Moreover, this study demonstrated that the highest dose was the most effective one.

In conclusion, in the present study, we have demonstrated that it is possible to establish a murine model of rhinitis in which the mice recognize the clinically relevant allergen Phl p, and that displays the hallmarks of human allergic rhinitis. Using this model, we have demonstrated that SLIT treatment leads to the reduction of both the clinical and immunological signs of rhinitis in a time- and dose-dependent manner.

Acknowledgements

Tobias Næsborg, Sandra Urioste, Lis Sørensen and Annette Giselsson are gratefully acknowledged for their expert technical assistance.

Conflict of interest statement: All authors are employees of ALK-Abelló A/S.

References

- 1 Kay AB. Allergy and allergic diseases. First of two parts. *N Engl J Med* 2001; **344**:30–7.
- 2 Gupta R, Sheikh A, Strachan DP, Anderson HR. Burden of allergic disease in the UK: secondary analyses of national databases. *Clin Exp Allergy* 2004; **34**:520–6.
- 3 Durham S. ABC of allergies. Summer hay fever. *BMJ* 1998; **316**:843–5.
- 4 Robinson DS, Larche M, Durham SR. Tregs and allergic disease. *J Clin Invest* 2004; **114**:1389–97.
- 5 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.
- 6 Wilson DR, Lima MT, Durham SR. Sublingual immunotherapy for allergic rhinitis: systematic review and meta-analysis. *Allergy* 2005; **60**:4–12.
- 7 Dahl R, Stender A, Rak S. Specific immunotherapy with SQ standardized grass allergen tablets in asthmatics with rhinoconjunctivitis. *Allergy* 2006; **61**:185–90.
- 8 Kleine-Tebbe J, Ribet M, Herold DA. Safety of a SQ-standardised grass allergen tablet for sublingual immunotherapy: a randomized, placebo-controlled trial. *Allergy* 2006; **61**:181–4.
- 9 Bousquet J, Scheinmann P, Guinépain MT *et al.* Sublingual-swallow immunotherapy (SLIT) in patients with asthma due to house-dust mites: a double-blind, placebo-controlled study. *Allergy* 1999; **54**:249–60.
- 10 Lima MT, Wilson D, Pitkin L *et al.* Grass pollen sublingual immunotherapy for seasonal rhinoconjunctivitis: a randomized controlled trial. *Clin Exp Allergy* 2002; **32**:507–14.
- 11 La Rosa M, Ranno C, Andre C, Carat F, Tosca MA, Canonica GW. Double-blind placebo-controlled evaluation of sublingual-swallow

- immunotherapy with standardized *Parietaria judaica* extract in children with allergic rhinoconjunctivitis. *J Allergy Clin Immunol* 1999; **104**:425–32.
- 12 Marcucci F, Sensi L, Frati F *et al*. Effects on inflammation parameters of a double-blind, placebo controlled one-year course of SLIT in children monosensitized to mites. *Allergy* 2003; **58**:657–62.
 - 13 Marcucci F, Sensi L, Frati F *et al*. Sublingual tryptase and ECP in children treated with grass pollen sublingual immunotherapy (SLIT): safety and immunologic implications. *Allergy* 2001; **56**:1091–5.
 - 14 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.
 - 15 Ippoliti F, De Santis W, Volterrani A *et al*. Immunomodulation during sublingual therapy in allergic children. *Pediatr Allergy Immunol* 2003; **14**:216–21.
 - 16 Reich M, Zwacka G, Markert UR. Nonspecific plasma proteins during sublingual immunotherapy. *Chem Immunol Allergy* 2003; **82**:99–108.
 - 17 Hellings PW, Ceuppens JL. Mouse models of global airway allergy: what have we learned and what should we do next? *Allergy* 2004; **59**:914–9.
 - 18 Herz U, Renz H, Wiedermann U. Animal models of type I allergy using recombinant allergens. *Methods* 2004; **32**:271–80.
 - 19 Wang J, Palmer K, Lotvall J *et al*. Circulating, but not local lung, IL-5 is required for the development of antigen-induced airways eosinophilia. *J Clin Invest* 1998; **102**:1132–41.
 - 20 Wills-Karp M, Luyimbazi J, Xu X *et al*. Interleukin-13: central mediator of allergic asthma. *Science* 1998; **282**:2258–61.
 - 21 Saito H, Howie K, Wattie J *et al*. Allergen-induced murine upper airway inflammation: local and systemic changes in murine experimental allergic rhinitis. *Immunology* 2001; **104**:226–34.
 - 22 Strath M, Warren DJ, Sanderson CJ. Detection of eosinophils using an eosinophil peroxidase assay. Its use as an assay for eosinophil differentiation factors. *J Immunol Methods* 1985; **83**:209–15.
 - 23 Woodhead JS, Weeks I. Magic Lite design and development. *J Biolumin Chemilumin* 1989; **4**:611–4.
 - 24 McCusker CT. Use of mouse models of allergic rhinitis to study the upper and lower airway link. *Curr Opin Allergy Clin Immunol* 2004; **4**:11–6.
 - 25 Burney P, Malmberg E, Chinn S, Jarvis D, Luczynska C, Lai E. The distribution of total and specific serum IgE in the European Community Respiratory Health Survey. *J Allergy Clin Immunol* 1997; **99**:314–22.
 - 26 Tomaki M, Zhao LL, Lundahl J *et al*. Eosinophilopoiesis in a murine model of allergic airway eosinophilia: involvement of bone marrow IL-5 and IL-5 receptor alpha. *J Immunol* 2000; **165**:4040–50.
 - 27 McCusker C, Chicoine M, Hamid Q, Mazer B. Site-specific sensitization in a murine model of allergic rhinitis: role of the upper airway in lower airways disease. *J Allergy Clin Immunol* 2002; **110**:891–8.
 - 28 Passalacqua G, Ciprandi G, Canonica GW. The nose-lung interaction in allergic rhinitis and asthma: united airways disease. *Curr Opin Allergy Clin Immunol* 2001; **1**:7–13.
 - 29 Kanaizumi E, Shirasaki H, Sato J, Watanabe K, Himi T. Establishment of animal model of antigen-specific T lymphocyte recruitment into nasal mucosa. *Scand J Immunol* 2002; **56**:376–82.
 - 30 Wills-Karp M. Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu Rev Immunol* 1999; **17**:255–81.
 - 31 Moller C, Dreborg S, Ferdousi HA *et al*. Pollen immunotherapy reduces the development of asthma in children with seasonal rhinoconjunctivitis (the PAT-study). *J Allergy Clin Immunol* 2002; **109**:251–6.
 - 32 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.
 - 33 Winkler B, Baier K, Wagner S *et al*. Mucosal tolerance as therapy of type I allergy: intranasal application of recombinant Bet v 1, the major birch pollen allergen, leads to the suppression of allergic immune responses and airway inflammation in sensitized mice. *Clin Exp Allergy* 2002; **32**:30–6.
 - 34 Holt PG, Vines J, Britten D. Sublingual allergen administration. I. Selective suppression of IgE production in rats by high allergen doses. *Clin Allergy* 1988; **18**:229–34.
 - 35 Sato MN, Carvalho AF, Silva AO, Maciel M Jr, Fusaro AE, Duarte AJ. Oral tolerance induced to house dust mite extract in naive and sensitized mice: evaluation of immunoglobulin G anti-immunoglobulin E autoantibodies and IgG–IgE complexes. *Immunology* 1998; **95**:193–9.
 - 36 Wolvers DA, Coenen-de Roo CJ, Mebius RE *et al*. Intranasally induced immunological tolerance is determined by characteristics of the draining lymph nodes: studies with OVA and human cartilage gp-39. *J Immunol* 1999; **162**:1994–8.
 - 37 Unger WW, Hauet-Broere F, Jansen W, van Berkel LA, Kraal G, Samsom JN. Early events in peripheral regulatory T cell induction via the nasal mucosa. *J Immunol* 2003; **171**:4592–603.