

An alternative allergen:adjuvant formulation potentiates the immunogenicity and reduces allergenicity of a novel subcutaneous immunotherapy product for treatment of grass-pollen allergy

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Abstract

Background Subcutaneous specific immunotherapy (SCIT) has proven sustained clinical efficacy against allergy. The recommended regimen for SCIT is a gradual up dosing over a period of weeks. Commonly, in commercial products for SCIT, the specific allergen is formulated with an adjuvant, most often in the form of aluminium hydroxide (AlOH). It has been shown that allergen-specific IgG antibodies are induced as a result of successful SIT. **Objective** To investigate the possibility of optimizing the formulation of AlOH-based grass-pollen allergy vaccines for SCIT in a way that allows for shorter up dosing regimens while maintaining the immunogenicity of the vaccine.

Methods Mice were immunized with various concentrations of *Phleum pratense* (Phl p) allergen extract and AlOH or a fixed dilution of the maintenance doses of one conventional and one alternatively formulated vaccine. The kinetics of Phl p-specific IgG antibody responses in serum and spleen T cell responses were determined. Allergenicity, measured as the ability of the formulations to activate human basophils, was also determined. In addition, human T cell responses and the expression of dendritic cell surface markers after vaccine challenge *in vitro* were analysed.

Results Specific IgG antibody responses were shown to depend on the AlOH concentration, but not on the allergen concentrations. The immunogenicity of the conventional formulation and the alternative formulation was shown to be similar with regard to the *in vivo*-induced IgG and T cell responses. In contrast, the allergenicity of the alternative formulation was significantly reduced compared with the conventional formulation.

Conclusion The optimization of the formulation allows for administration of a lower dose of allergen while maintaining the immunogenicity of the product and at the same time reducing allergenicity.

Clinical Relevance This study indicates that the optimization of the allergen and the adjuvant formulation could benefit the safety/efficacy profile and allow for shorter up dosing.

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Introduction

Gels of aluminium phosphate and aluminium hydroxide, commonly referred to as alum, have been used for decades as adjuvants for enhancing antibody response in humans, representing an acceptable compromise between adjuvanticity and safety profile [1, 2]. Unlike many other more novel adjuvant candidates, alum has shown not to exert its adjuvant effect via Toll-like

receptors (TLRs) [3, 4] and the mechanisms behind the adjuvant effect of alum have remained somewhat elusive. Adjuvant mechanisms were for a long period of time attributed to i) the depot effect of alum, resulting in slow release of adsorbed components prolonging the interaction between antigen and antigen-presenting cells (APCs) [5], ii) recruitment and activation of APCs due to induction of inflammation [6–8] and iii) the conversion of an antigen from soluble to particulate form

thus facilitating phagocytosis by APCs. More recently, an additional adjuvant mechanism of alum was proposed when it was demonstrated that alum is capable of inducing IL-1 β and IL-18 release in a caspase-1 dependent way in lipopolysaccharide (LPS) -primed human and murine DC *in vitro* [9] via the inflammasome. The inflammasome consists of the NOD-like receptor, NLRP3 together with the adaptor molecule apoptosis-associated speck-like protein (ASC), which binds to and activates procaspase-1 [9, 10]. Active caspase-1 catalyses the cleavage of proforms of IL-1 β and IL-18 into biologically active cytokines [11]. Despite the fact that there is consistency in the literature about IL-1 β and IL-18 maturation in a NLRP3-dependent manner due to alum-induced inflammasome activation, the importance of the inflammasome for the adjuvant effect of alum is under debate [9, 10, 12–14].

Aluminium-containing adjuvants primarily induce humoral immunity [2, 15–17] and have for decades been used successfully in prophylactic vaccines against infectious diseases [18, 19] as well as in therapeutic vaccines against allergic diseases, i.e. allergen-specific subcutaneous immunotherapy (SCIT) [20–23]. In contrast to symptomatic treatments, SCIT with alum-adsorbed allergen extract has been shown to confer long-term benefit even after discontinuation of treatment [24], as well as prevent new allergic sensitization [25] and progression from allergic rhinitis to asthma [26–28]. In spite of these clear advantages of SCIT treatment, the inconvenience of a relatively long up-dosing phase with frequent visits to physician before reaching maintenance dose to some extent affects patient compliance. The main reason behind the up-dosing phase is to decrease the risk of adverse events, especially severe systemic reactions that potentially, even though rarely, could be life-threatening [20]. The immediate allergic symptoms that occur in response to natural allergen exposure and adverse events connected with SCIT are based on the activation of allergic effector cells (mast cells and basophils). These cell types have surface-exposed allergen-specific IgE antibodies attached to their cell membranes via Fc ϵ RI and will degranulate and release mediators such as histamine in response to formation of IgE-allergen complexes. Effector-cell degranulation is allergen dose-dependent, and the incidence of severe adverse reactions of SCIT has been shown to be related to the maintenance allergen dose of the vaccine [29, 30].

Even though SCIT has been used successfully for many years, the exact molecular and cellular mechanistic basis for the effect is not yet completely known. However, a hallmark of SCIT is the induction of non-IgE allergen-specific antibodies primarily of the IgG1 and IgG4 isotypes which 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 [31–35].

In this article, we have investigated the possibility of developing an alternatively formulated grass-pollen vaccine for SCIT with maintained immunogenicity, i.e. IgG and T cell inducing capacity, but reduced with regard to allergenicity, i.e. its ability to activate effector cells in an allergen-specific manner. The clinical rationale for such a vaccine would be the possibility of faster up dosing, attributed to the reduced allergenicity and, hence, increased tolerability, but without loss of effect. One strategy was to optimize the formulation of allergen and aluminium hydroxide. By the use of an *in vivo* mouse model of vaccine immunogenicity and an *in vitro* effector-cell-based method for measuring allergenicity, we here demonstrate that by reducing the amount of allergen extract while maintaining the concentration of aluminium hydroxide compared with the original formulation, we have obtained a novel grass-pollen SCIT vaccine that complies with these criteria.

Materials and methods

Animals

Female Balb/c mice, 6–8 weeks old at the start of the experiments, were used. Mice were either purchased from Pipeline Biotech A/S (Trige, Denmark) (mice used in the experiment outlined in Fig. 1) or bred in house (mice used in the experiment outlined in Fig. 2 and 4). Mice were housed under specific pathogen-free environment under a 12 h light, 12 h dark cycle. All experiments described in this article were conducted in accordance with Danish legislation.

Materials

Grass-pollen extracts (ALK-Abelló, Hørsholm, Denmark) manufactured as described previously were used [36, 37]. The freeze dried allergen extracts were dissolved to a concentration 10 times the concentration of the final formulation. 1/10 volume of allergen solution was mixed with appropriate amount of Coca buffer (0.25% sodium hydrogen carbonate and 0.5% sodium chloride) and Alhydrogel[®] 1.3% (Al(OH)₃) (Brenntag Biosector, Fredrikssund, Denmark) was slowly added. Phenol was added to a final concentration of 0.5% in commercially produced SCIT products, but not in the non-GMP formulations (see below for use of commercial versus non-GMP produced formulations). In this article, three different batches of Phl p extract have been used. In all these Phl p extract batches, levels of endotoxin were lower or equal to 0.5 ng per 100 000 SQ Phl p extract.

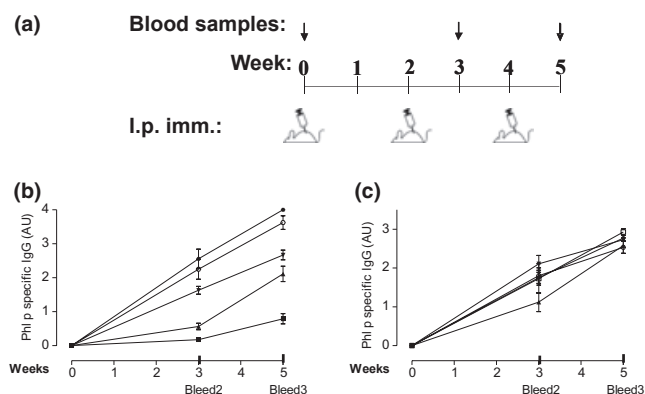


Fig. 1. IgG antibody response is dependent on AIOH concentration, but not on allergen concentration. Mice were immunized and serum samples were collected according to scheme in Figure a. In Figure b, mice were immunized with 100 000 SQ Phl p extract coupled to the following conc. of Al^{3+} in AIOH: 0 (■); 0.114 (▲); 0.3 (▼); 0.55 (○) and 0.8 (●) mg/mL. Serum samples from individual mice were analysed for amount of Phl p-specific IgG values by ELISA (AU = arbitrary units of mean $\pm$ 95% CI) and group means $\pm$ SEM for every group of mice are plotted in Figure b. In Figure c, mice were immunized with 5 000 (■); 10 000 (▲); 25 000 (▼); 50 000 (○) and 100 000 (●) SQ Phl p extract adsorbed to 1.14 mg/mL of Al^{3+} in AIOH. Serum samples from individual mice were analysed for amount of Phl p-specific IgG values (AU = arbitrary units of mean $\pm$ 95% CI) and group means $\pm$ SEM for every group of mice are plotted in Figure c.

The activity of the extract was given in standardized quality units (SQ-U) that are defined by the therapeutic effect related to optimal treatment in specific immunotherapy. In this study, various concentrations of *Phleum pratense* (Timothy grass) extract have been adsorbed to various concentrations of aluminium hydroxide (AIOH) as indicated in the Result section and figure legends. In addition, four different allergen-specific immunotherapy vaccines have been used, two of which being registered products and available in many European countries. The registered vaccines are in this article denoted 'conventionally formulated vaccines', and they consist of 100 000 SQ/mL of either a single grass specie, *Phleum pratense* (Phl p) extract or a mix of extracts from the following species (total concentration of 100 000 SQ/mL): *Arrhenatherum elatius*, *Dactylis glomerata*, *Poa pratensis*, *Festuca pratensis*, *Lolium perenne*, *Phleum pratense* and *Secale cereale*, adsorbed to 1.14 mg/mL of aluminium hydroxide (AIOH). The clinical maintenance dosing volume of these vaccines is 1.0 mL. The other two vaccine products used in this study are new formulations and available as Named Patient Products (NPP) in some European countries, and these formulations are denoted 'alternatively formulated vaccines' in this article. The alternatively formulated vaccines consist of 30 000 SQ/mL of either the single grass specie Phl p extract or a mix of extract from the following species (30 000 SQ/mL in total): *Dactylis*

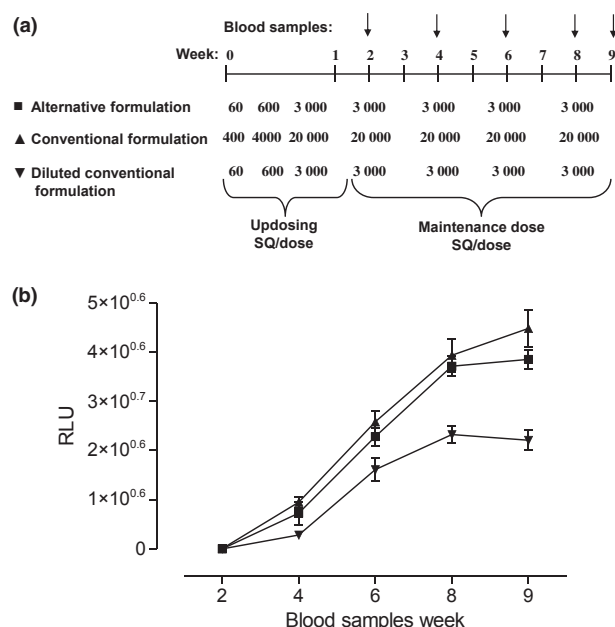


Fig. 2. IgG1 is induced to the same extent by immunization with an alternative or conventional vaccine formulation. Mice were i.p. immunized with 1/5th of the clinical dose of each formulation. Immunization schedule started with three up dosing steps (day 1, 3 and 5) followed by four immunizations with maintenance dose every second week. Alternative and conventional formulations contain 3 kSQ + Phl p + 0.114 mg of Al^{3+} in AIOH/dose (■) and 20 kSQ + Phl p + 0.228 mg of Al^{3+} in AIOH/dose (▲), respectively. 'Diluted conventional formulation' contains 3 kSQ Phl p + 0.0342 mg of Al^{3+} in AIOH/dose (▼). Blood samples were drawn every second week immediately before the next coming i.p. immunization (a). Kinetics of Phl p-specific IgG1 antibody responses in serum from immunized mice were followed and analysed on the ADVIA Centaur platform. Mean RLU (relative unit) values $\pm$ SEM are depicted in the figure (b).

glomerata, *Festuca pratensis*, *Lolium perenne*, *Phleum pratense* and *Secale cereale*, adsorbed to 1.14 mg/mL of AIOH. The clinical maintenance dosing volume of the alternatively formulated vaccine is 0.5 mL. The aluminium hydroxide gel, Alhydrogel[®] 1.3% ($\text{Al}(\text{OH})_3$), was used for allergen extract adsorption. In this study, Alhydrogel[®] 1.3% ($\text{Al}(\text{OH})_3$) is referred to as AIOH, and the concentrations and/or amounts are expressed as mg aluminium ion (Al^{3+})/mL or mg Al^{3+} . Non-GMP AIOH-adsorbed allergen formulations were used in all mouse IgG studies.

In basophil activation assays as well as in human and mouse T cell stimulation assays (see below), commercially available vaccine formulations were used. The SQ unit of the alternative formulation is followed by a plus (+)-sign. One SQ is equal to one SQ+ with regard to the allergen extract content. The sign refers to the fact that the ratio between the adjuvant AIOH and the allergen is increased in the alternatively formulated vaccine as compared with the conventionally formulated vaccine.

Immunizations

Mice were immunized i.p. with ALOH-allergen formulations mixed to various ratios as indicated in figure legends.

Antibody analysis

Sera were prepared from each individual blood-sample and Phl p-specific IgG antibodies were analysed either by ELISA or on the ADVIA Centaur platform (Siemens Healthcare Diagnostics, Deerfield, IL, USA) as indicated in figure legends.

ELISA analysis: NUNC Maxisorp plates were coated with allergen extract (Phl p extract, 1 µg/well) and blocked for unspecific binding (2% casein buffer, 200 µL/well). Six dilutions in 0.5% bovine serum albumin (BSA) buffer (starting at 1 : 200, fourfold, 100 µL/well) of serum samples were applied followed by detection with HRP labelled rabbit anti-mouse IgG (Jackson 315-036-045, diluted 1 : 10,000 in 0.5% BSA buffer, 200 µL/well) and TMB ONE (Kem-En-Tec, 100 µL/well). Each step was separated by a wash (three times without soak in phosphate buffered saline (PBS) buffer pH = 7.2 with 0.05% Tween). OD was determined as an endpoint value at 450 nm (Bio Kinetics Reader EL-340). All OD values were adjusted for plate to plate variation.

ADVIA Centaur analysis: Briefly, goat anti-mouse IgG1 (Southern Biotechnology) covalently coupled to paramagnetic particles was used to capture the serum IgG1 antibodies. Mouse serum was diluted 1 : 1000-fold. Bound solid-phase IgG1 antibodies were allowed to react with liquid-phase purified biotinylated Phl p extract that were detected by chemiluminescence using acridiniumester-labelled streptavidin [38].

In vitro proliferation of mouse spleen cells

At the end of experiment, mice were killed and spleens were collected. Single cell suspensions of spleens from individual mice 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, St Louis, MO, USA). To each well of a 96-well flat-bottomed culture plate (Nalge Nunc International, Rochester, NY, USA), 300 000 cells were added. Cells were stimulated with Phl p extract in a final concentration of 1 000, 3 000, 9 000 or 27 000 SQ/mL at 37°C in 5% carbon dioxide for 6 days. ³H-thymidin was added 16 h prior to harvest of cells. Filters with

harvested cells were counted on beta counter (Wallac model 1450; PerkinElmer, Waltham, MA).

Human blood samples

Blood samples used for purification of T cells and generation of monocyte-derived DC; Buffy coats were obtained from atopic donors sensitized to *Phleum pratense* with an immunoCAP class ≥ 2 (transfusion centre, Mainz, Germany). Specific sensitization was verified by detection of allergen-specific IgE in the sera (ImmunoCAP[®] specific IgE blood test, Thermo Fisher Scientific, Waltham, MA, USA). The study was approved by the local Ethics Committee. Informed consent was obtained from all subjects before the study.

Blood samples used for basophil activation test; blood samples were obtained from 13 grass allergic patients tested positive for Phl p-specific IgE (CAP class ≥ 2) and shown to release histamine towards purified major grass allergens Phl p 1 and/or Phl p 5 (> 10 nm histamine at 5 ng/mL of major allergen). All patients had a clinical history indicating grass pollen as the major cause of their allergy symptoms and none of the patients received SIT before the study. Vaccines were analysed in concentrations normalized according to the recommended therapeutic dose. Blood donors were recruited and informed according to the project description approved by the local Ethics Committee (H-Ø-2004-1-14G).

Generation of monocyte-derived DC

Peripheral blood mononuclear cells (PBMC) of Phl p allergic donors (IgE class ≥ 2) were isolated by Ficoll Paque 1077 g/mL (PAA Laboratories GmbH, Cölbe, Germany) density centrifugation from heparinized blood. CD14⁺ cells were enriched by incubation of 5×10^6 PBMC in a 12-well-plate (Greiner, Frickenhausen, Germany) with 1 mL/well Iscove's modified Dulbecco's medium containing L-glutamine and 25 mM Hepes (IMDM; PAA Laboratories GmbH) and 3% autologous heat-inactivated plasma at 37°C for 45 min. Cell culture plates were washed twice with pre-warmed PBS to remove non-adherent cells. The remaining monocytes (purity $> 90\%$) were incubated with 1.5 mL/well IMDM, 1% autologous plasma, 1000 U/mL IL-4 (Strathmann Biotech GmbH, Hannover, Germany) and 200 U/mL granulocyte-macrophage colony-stimulating factor (GM-CSF, Leukine[®]; Immunex Corp., Seattle, WA). Cells were fed with fresh medium on day 3. On day 6, the resulting immature DCs were pulsed with conventionally or alternatively formulated vaccines. Final dilutions of the vaccines are indicated in the figures and dilutions refer to the absolute maintenance dose given clinically. As controls, DCs are pre-pulsed with a control

formulation containing AIOH only or Phl p allergen extract only. The control formulation containing AIOH only was used in a concentration equal to the AIOH content in the lowest dilution of the conventional formulation. Phl p allergen doses correspond to the allergen doses present in the lowest dilution of the products (1 : 25). Pulsed DCs were matured by stimulation with 1000 U/mL TNF- α , 2000 U/mL IL-1 β (Strathmann Biotech GmbH) and 1 μ g/mL prostaglandin E₂ (Cayman Chemical, Ann Arbor, MI) to generate mature DC [39] or received no further treatment (immature DC). DCs were harvested after 48 h, washed twice with PBS and analysed for expression of surface markers or co-cultured together with autologous T cells (see below).

Cell surface marker expression

Surface markers of pre-pulsed immature DCs were identified with the following mouse anti-human mAbs: phycoerythrin (PE)-conjugated CD80 (L307.4), CD83 (HB15e), CD86 [2331 (FUN-1)], FITC-conjugated human leucocyte antigen (HLA)-DR (L243) and mouse IgG isotype controls (all from BD Biosciences, Heidelberg, Germany). Surface phenotyping was performed by staining 1×10^5 DC with specific mAbs for 15 min at 4°C. After being washed with PBS containing 0.1% BSA, the cells were analysed in a by flow cytometry (FACSCalibur, Becton Dickinson, Mountain View, CA) equipped with CELLQuest Software.

Purification of T cells

Autologous CD4⁺ T cells were obtained from PBMC using antibody-coated paramagnetic MicroBeads (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) according to the instructions of the manufacturer (purity > 98% CD4⁺ T cells as measured by flow cytometry).

Co-culture of T cells and autologous pulsed DC

For the proliferation assay, 1×10^5 CD4⁺ T cells were co-cultured in triplicates with 1×10^4 pulsed mature DC in 200 μ L IMDM and 5% heat-inactivated autologous plasma. After 5 days of culture, the cells were pulsed with 37 kBq/well of [³H]TdR ([methyl-³H]thymidine, ICN, Irvine, CA) for 6 h. [³H]TdR incorporation was measured in a beta counter (1205 Betaplate, LKB Wallac, Turku, Finland). Preliminary experiments have shown that this 1 : 10 ratio of DC:T cell co-culture leads to optimal proliferative responses.

Basophil activation assay

Activated human basophils express the degranulation activation marker CD63 on their cell surface [40, 41].

Basophils in whole blood from grass allergic donors were incubated with increasing doses of the conventionally or alternatively formulated vaccines or grass extract in the presence of IL-3 (1 ng/mL). Activation-induced expression of CD63 was measured by flow cytometry as previously described [42]. Percentages of basophils with increased CD63 expression compared with non-stimulated basophils were determined.

Statistics

Data are expressed as group mean values $\pm$ SEM, and statistical analysis was conducted using two-tailed non-parametric Mann-Whitney test, Wilcoxon signed rank test or two-tailed ANOVA with Bonferroni correction using a software programme (GraphPad Prism version 5.01; GraphPad Software Inc, San Diego, CA, USA); p values below 0.05 were considered significant.

Results

The magnitude of the allergen-specific IgG antibody responses is driven primarily by the ALOH concentration

The capacity of an allergen-aluminium hydroxide (AIOH) formulation to induce allergen-specific IgG antibody responses in serum upon i.p. injection was studied. Mice were immunized at three occasions every second week with a formulation containing a fixed concentration (100 000 SQ) of Phl p allergen extract adsorbed to increasing AIOH, or increasing concentrations of Phl p extract adsorbed to a constant concentration (1.14 mg/mL) of AIOH. Blood samples were drawn before immunization and 1 week after second and third immunization (Fig. 1a), and the levels of Phl p-specific IgG antibodies were determined. The kinetics of the response is depicted in Fig. 1b and c. A clear dose-response curve dependent on the concentration of AIOH could be demonstrated (Fig. 1b). The specific IgG responses following i.p. immunization with 100 000 SQ Phl p extract adsorbed to 0, 0.114 and 0.3 mg/mL AIOH were significantly reduced as compared to the same amount of Phl p extract adsorbed to the highest concentrated AIOH tested (0.8 mg/mL). In contrast, the antibody response following increasing concentrations of Phl p allergen showed no significant difference between the 5 000 SQ/mL and higher concentrated doses after three i.p. immunizations when the concentration of AIOH was held constant at a concentration of 1.14 mg/mL (Fig. 1c). On the basis of these data, we could conclude that, within the concentration ranges studied here, the IgG-inducing capacity in mice is dependent on the AIOH, but not the Phl p allergen, concentration.

The conventionally formulated vaccine, which has a therapeutic maintenance volume of 1.0 mL and a maintenance dose of 100 000 SQ Phl p adsorbed to 1.14 mg/mL of AIOH, is an alum-adsorbed SCIT product which has marketing authorizations in several European countries. Considering the data shown in Fig. 1, we hypothesized that it would be possible to develop a new product more suitable for a fast up-dosing regimen but with maintained immunogenicity, by reducing the allergen concentration and maintaining the AIOH concentration, compared with the original formulation. This new alternatively formulated SCIT product is formulated with 30 000 SQ + Phl p extract and 1.14 mg AIOH per mL and is currently available in some European countries as a Named Patient Product (NPP). The maximum treatment maintenance volume of 0.5 mL results in a maintenance dose of 15 000 SQ + Phl p and 0.57 mg AIOH. The ability of the conventional and new alternative formulations to induce Phl p-specific IgG1 antibody responses in mice after immunization was compared. In mice, all maintenance volumes were scaled down to 1/5th compared with the corresponding clinically relevant volume, as it is not possible due to ethical reasons to inject a volume of 1.0 mL i.p. in mice. Thus, mice received a maintenance dose of 20 000 SQ Phl p/0.228 mg of AIOH in a volume of 200 µL for the conventional formulation and a maintenance dose of 3 000 SQ + Phl p/0.114 mg AIOH in a volume of 100 µL for the alternative formulation, respectively. Although the absolute amounts of AIOH differ between the two formulations, the concentrations are identical. Following a three-step up-dosing schedule, mice were immunized every second week at five occasions with a 1/5 × maintenance dose of each of the vaccines, and blood samples were drawn after each immunization (Fig. 2a). The kinetics of the serum IgG1 antibody response showed no significant difference at any time-point between groups of mice immunized with the conventional or the alternative formulation, respectively. To verify the dependency of AIOH for immunogenicity, one group of mice was immunized with a conventional formulation diluted with respect to both the allergen and AIOH concentrations. This test-vaccine which had the same allergen concentration and dose as the alternatively formulated vaccine but reduced with regard to AIOH concentration was denoted 'Diluted conventional formulation'. As shown in Fig. 2b, the immunogenicity of the Diluted conventional formulation was significantly reduced ($P < 0.05$) compared with the alternative formulation, confirming that AIOH concentration is crucial for the ability of the vaccine to induce IgG antibody responses, at least for this particular concentration of grass-pollen allergen.

Formulation of Phl p extract with AIOH stimulates the T cell immune response in vivo but does not affect the allergen-specific stimulation in vitro

Antigen uptake and presentation is a pre-requisite for induction and isotype switching of IgG antibodies [43, 44]. To examine whether these processes are directly affected by AIOH, human DCs were pulsed *in vitro* with Phl p extract in the absence or presence of AIOH and subsequently co-cultured with autologous T cells using a recently described allergen-specific *in vitro* set-up [43, 44]. Monocyte-derived DCs were pre-pulsed with either conventionally or alternatively formulated vaccines or controls (AIOH formulation without allergen and Phl p allergen without AIOH). The conventionally and the alternatively formulated vaccines induced up-regulation of the CD83 maturation marker on DCs to the same extent. In addition, pre-pulsing with a control formulation containing AIOH but no Phl p extract induced levels of CD83-expression similar to preparations containing both allergen and AIOH, showing that the effect is due to AIOH (Fig. 3a). Neither the DC co-stimulatory molecules, CD80 or CD86, nor the activation marker, HLA-DR were affected by incubation with any of the formulations investigated (data not shown). Thereafter, the ability of human monocyte-derived DCs pulsed with either the conventional or the alternative formulation to induce T cell proliferation was measured. T cell proliferation was more efficiently induced *in vitro* by DCs pulsed with the conventionally formulated vaccine as compared to DCs pulsed with the alternatively formulated vaccine ($P < 0.0005$). Co-culture of T cells with DCs pre-pulsed with Phl p extract in the absence of AIOH induced proliferation to a similar extent as the corresponding dose of Phl p in an AIOH-formulated vaccine, but DCs pre-pulsed with the control formulation containing AIOH only gave much lower level of proliferation (Fig. 3b). Together, the proliferation data indicate that the allergen concentration is the most important factor for *in vitro* T cell stimulation. We now hypothesized that T cells stimulated with the different SCIT formulations *in vivo* instead of *in vitro* might reveal additional effects of AIOH (and allergen) on T cells compared with the *in vitro* situation. A mouse model similar to the one outlined in Fig. 1 was used to test this hypothesis. Mice were i.p. immunized with 1/5th of the clinically relevant maintenance doses as before. Two additional groups, in which the allergen dose corresponds to the dose present in the alternatively formulated vaccine, but with either a lower dose of AIOH (Diluted conventional formulation) or no alum at all were included. Following a three-step up-dosing schedule, mice were immunized twice, once every second week. Proliferation of isolated spleen cells following *in vitro* stimulation with Phl p allergen was

determined at the end of the experiment (Fig. 4), showing that the magnitudes of T cell proliferation observed after i.p. immunization with the conventionally and the alternatively formulated vaccines were almost identical. In contrast, the T cell proliferation was significantly reduced ($P < 0.005$ and $P < 0.01$ for *in vitro* stimulation of 9 000 and 27 000 SQ/mL, respectively) if mice were immunized with a formulation containing lower concentration of AIOH (Diluted conventional formulation) and even further more reduced if mice were immunized with equal amounts of allergen in the complete absence of AIOH. Hence, in contrast to the *in vitro* induced proliferation of human T cells by the conventional and alternative formulation, the magnitude of *in vivo* induced T cell stimulation depended primarily on the AIOH concentration and not to allergen concentration at least within the concentration range tested here.

Basophil activation induced by the alternatively formulated vaccine is decreased compared with the conventionally formulated vaccine

The allergenicity of the alternatively and conventionally formulated grass-pollen vaccines were compared by measuring the degree of activation of human basophils from grass allergic donors in parallel experiments following exposure to dilution series of the two vaccines. Dilutions were made proportionally to the maintenance dose of the vaccines. Fresh whole blood was incubated with the vaccines for 1 h, and the cells were subsequently stained with fluorochrome-labelled antibodies against the basophil activation marker CD63 and antibodies against CD123/HLA-DR for detection of basophils. The percentage of cells with increased expression of CD63 within the basophil population was calculated for each sample.

Dose-response curves showing mean values of CD63 expression on basophil from 6 grass allergic donors activated with conventionally or alternatively formulated Phl p vaccines, respectively, are shown in Fig. 5a. Even though the sensitivity of basophils from individual patients varied, basophils from all patients reacted more strongly to the conventionally formulated vaccine compared with the alternatively formulated vaccine when the two vaccines were diluted to the same extent compared with their respective clinical maintenance doses (Fig. 5b). Traditionally, grass-pollen SCIT immunotherapy may also be performed with a mixture of pollen extracts from different but related grass species. Consistent with our findings for the *Phleum pratense* single-species formulations, the alternatively formulated multi-species vaccine gave rise to similar results, as this grass mix formulation was less efficient in activating basophils compared with the conventionally formulated grass mix vaccine (Fig. 5c and d) indicating lower

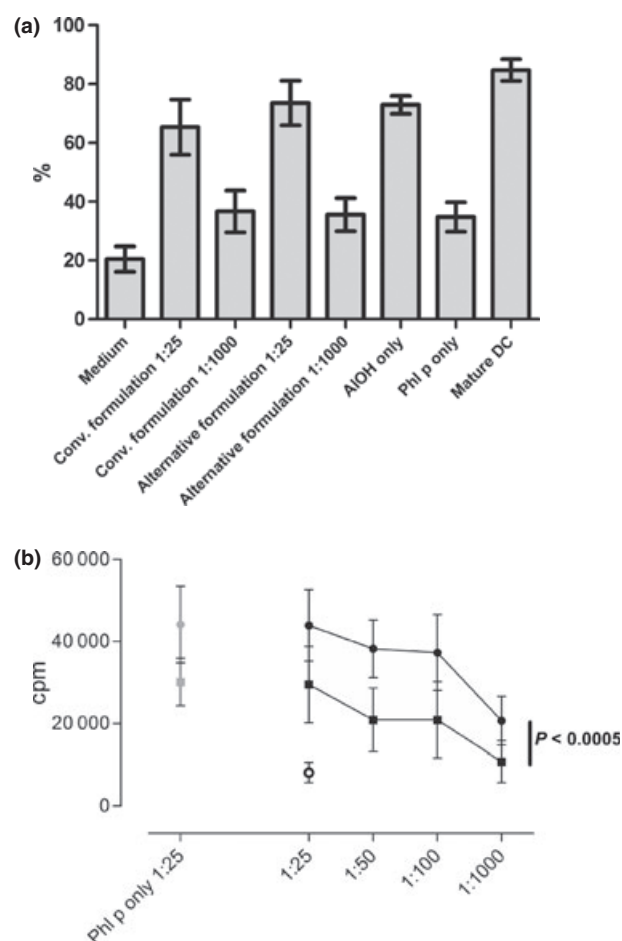


Fig. 3. *In vitro* effects of conventionally or alternatively formulated grass-pollen vaccines. Immature human monocyte-derived DCs were pulsed with the conventional or the alternative formulation of Phl p vaccines or control formulations containing either AIOH only or Phl p extract only. The final 1 : 25 dilution of the conventional and alternative formulations represents 800 SQ Phl p + 0.00912 mg AIOH and 120 SQ Phl p + 0.00456 mg AIOH, respectively per well. Further dilutions of the formulations are made both with regard to allergen extract and AIOH content. The control group stimulated with AIOH only was stimulated with an AIOH dose corresponding to the dose in the conventional formulation group. 'Phl p only' group was stimulated with allergen extract (no AIOH) in the same concentration as present in 1 : 25 dilution of the conventional formulation. Percentages of all DCs expressing CD83 are determined by flow cytometry. Group means $\pm$ SEM ($n = 4-5$) are depicted (a). Pulsed monocyte-derived DCs were co-cultured with autologous T cells and proliferation was measured after 5 days of culture; pulsing with conventional formulation ($\bullet$) and alternative formulation ($\blacksquare$), respectively, with doses as described above. In the control stimulation group with 'Phl p only', DCs were pulsed with allergen extract (no AIOH) in the same concentrations as present in 1 : 25 dilution of the conventional formulation ($\circ$) and alternative formulation ($\blacksquare$), respectively. The control group stimulated with AIOH only was stimulated with a dose corresponding to the AIOH dose in the conventional formulation group ($\circ$). Group means $\pm$ SEM ($n = 5$) are shown and statistical comparisons were performed by two-way ANOVA test with Bonferroni correction (b).

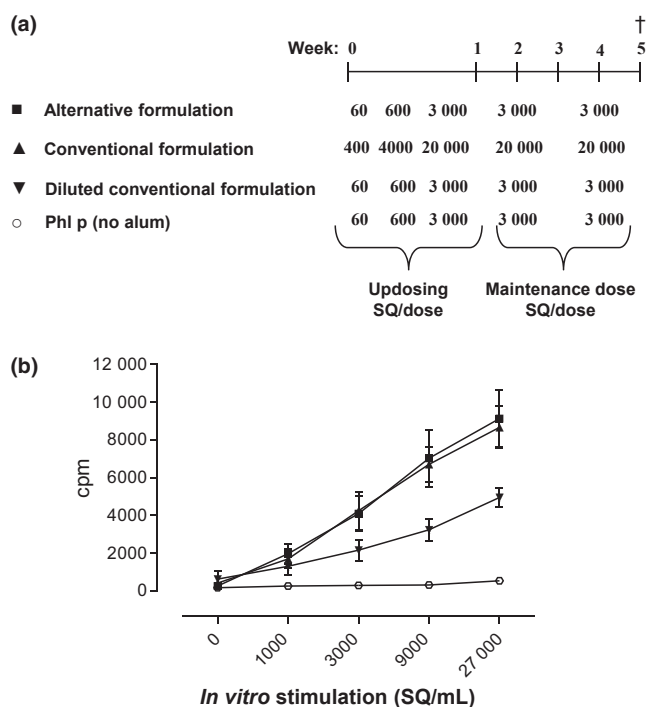


Fig. 4. *In vivo* T cell responses are dependent on AIOH concentration. Mice were i.p. immunized with 1/5th of the clinical dose of each formulation. Immunization schedule started with three updosing steps (day 1, 3 and 5) followed by two immunizations with maintenance dose every second week. Alternative and conventional formulations contain 3 kSQ + Phl p + 0.114 mg of Al³⁺ in AIOH/dose (■) and 20 kSQ Phl p + 0.228 mg of Al³⁺ in AIOH/dose (▲), respectively. 'Diluted conventional formulation' contains 3 kSQ Phl p + 0.0342 mg of Al³⁺ in AIOH/dose (▼) and Phl p (no AIOH) (○) contains 3 kSQ Phl p extract alone (a). Mice were sacrificed 1 week after the final immunization, and spleens were collected and single cells suspensions were prepared and stimulated *in vitro* with indicated concentrations of Phl p extract (SQ/mL). Proliferation after 6 days of culture was measured. Group means ± SEM are depicted (b).

degree of allergenicity of the new alternatively formulated grass mix vaccine compared with the conventional formulation.

Allergenicity of alternatively formulated grass mix and single grass vaccines are comparable

Often, the active ingredient in SCIT grass-pollen vaccines consist of extracts of pollens from more than one grass species, typically mixtures of different but related grass species. In addition to being a measure of the allergenicity, the basophil activation assay can also be used to examine differences in the IgE epitope repertoires of related allergens or allergen extracts. To investigate whether the formulation with AIOH would affect the allergenicity of the alternatively formulated vaccine, basophil activation induced by either vaccines with single grass specie or a vaccine composed of a mixture of five related temperate grasses were compared. No

significant difference in the ability of the vaccines or the unformulated extracts (data not shown) to activate basophils from grass-pollen sensitized individuals were found (Fig. 6). Together these data indicate that the reduced allergenicity observed in the alternatively formulated vaccines compared with the conventionally formulated vaccines is consistent for vaccines produced by different but related grass species.

Discussion

The benefits of SIT compared to symptomatic treatment of grass pollen induced allergic rhinoconjunctivitis have been demonstrated in a number of clinical trials and includes sustained clinical effects after completion of treatment, and decreased risk of developing more severe allergic asthma [24–28,45].

Sensitized individuals experience the typical immediate allergic reactions such as sneezing, itchy eyes and running noses upon exposure to airborne allergens due to the interaction of allergen-specific IgE with individual allergens. The primary cell types responsible for the immediate allergic reaction are mast cells and basophils, and both cell types express the high-affinity IgE-receptor on their surface. The encounter of cell-surface bound IgE and allergen (airborne or contained in e.g. insect venom) will lead to cross-linking of two or more IgE molecules which bind simultaneously to different epitopes of a particular allergen. A consequence of the extracellular IgE/allergen complex formation is the concomitant ligation of the intracellular domains of the high-affinity IgE receptors which in turn will initiate a cascade of intracellular events leading to cellular degranulation and release of histamine, cytokines and other pro-inflammatory and symptom-inducing substances [46]. The immunological mechanism of the therapeutic effect of SIT has not yet been described in full, but it may at least partly be based on treatment-induced non-IgE antibodies (e.g. allergen-specific IgG1 and IgG4) which will bind to the surface of allergens and thereby prevent IgE binding and subsequent effector-cell degranulation [31–35].

Protective vaccines against infectious diseases are often composed of complex mixtures of proteins from individual pathogens which lead to a broad immune response towards a large number of individual antigens from that particular pathogen and long-term protection against subsequent infections. To increase the safety of such vaccines, the pathogens may be killed or their infectiousness attenuated by different means, leading to a favourable safety/efficacy profile. Likewise, most vaccines currently in use for SCIT are composed of complex extracts of natural allergens such as grass pollen, house dust mites, insect venoms and animal danders. Inherent to SIT with intact allergens is of course the

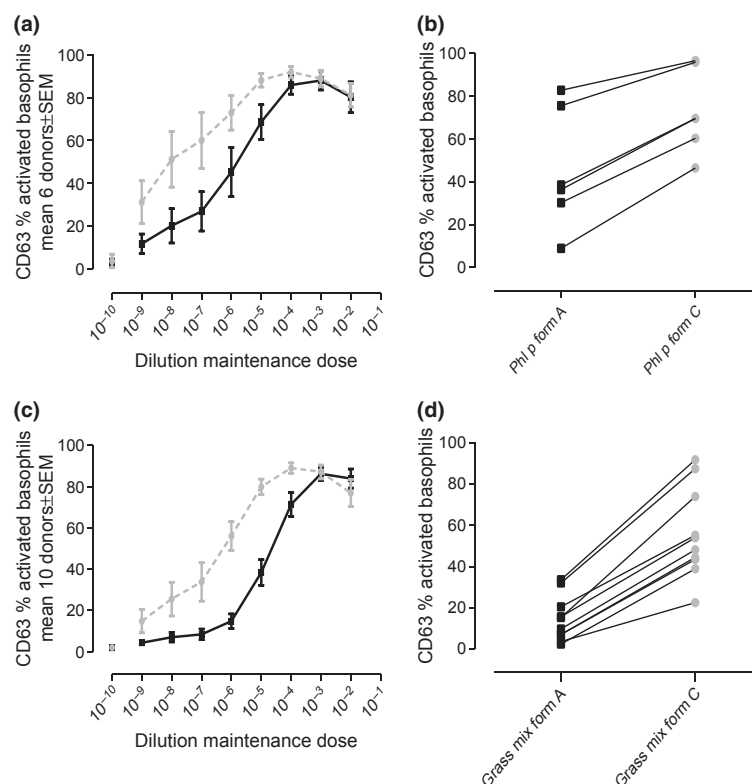


Fig. 5. Basophil activation induced by alternatively formulated *Phleum pratense* and grass mix vaccine was decreased compared to the conventionally formulated vaccines. Human blood basophils were incubated with increasing doses of the alternatively formulated and the conventionally formulated *Phleum pratense* vaccines (a and b) or the same formulations with grass mix vaccines (c and d). Percentages of basophils with increased CD63 expression are shown. Figure a and c: Graphs show CD63 expression induced by vaccines with stated dilutions of recommended maximal maintenance dose. Solid lines represent CD63 induced by alternatively formulated vaccines and dotted lines by conventionally formulated vaccines. Mean values $\pm$ SEM from experiments performed with basophils from 6 (a) or 10 (b) allergic donors. Statistical comparisons of alternative versus conventional formulation were performed by Wilcoxon signed two-tailed rank test, matched pairs based on maintenance dose, giving $P < 0.005$ and $P < 0.05$ for comparisons in figure a and c, respectively. Figure b and d: Graphs show CD63 expression induced by alternatively formulated (Phl p Form A and Grass mix Form A in b and d, respectively) and conventionally formulated (Phl p Form C and Grass mix Form C in b and d, respectively) vaccines at the dilution of 10^{-6} of clinically relevant maintenance dose. Statistical comparisons of alternative versus conventional formulation were performed by Wilcoxon signed two-tailed rank test, matched pairs based on donor, giving $P < 0.05$ and $P < 0.005$ for comparisons in figure b and d, respectively.

risk of eliciting local or systemic allergic reactions, and an appealing approach would be to denature the allergens by e.g. heat or chemical means in order to eliminate or reduce the number of intact IgE epitopes in a vaccine. Such approaches are used in some current day vaccines [47–55], and denaturation of allergens may indeed reduce unwanted IgE binding but may also reduce or eliminate the possibility of raising protective non-IgE antibodies, which are robust marker associated with successful SIT and have been suggested to be able to compete with IgE for binding to intact allergens [31–35]. It has previously been demonstrated that chemically modified allergen extracts may be reduced with regard to their allergenicity, but that they are also concomitantly reduced with regard to their immunogenicity [42, 56, 57].

One strategy to reduce IgE-mediated side-effects of SCIT has been to use allergen-derived peptides which

include T cell epitopes, but lack the conformational IgE-epitopes [58–63]. Another attempt has been to use recombinant, genetically modified allergen variants, so-called recombinant hypoallergenic allergen derivatives which to various degrees exhibit reduced IgE-reactivity [64–66]. However, there are currently no registered products of these categories on the market, and the clinical efficacy of these experimental treatment concepts are so far not well documented. An alternative to allergen modification for achieving a more favourable safety/efficacy ratio is to formulate a vaccine with a reduced content of structurally intact allergens. The lower allergen content of such a vaccine would provide a better safety profile, and the use of unmodified allergens would be optimal for raising non-IgE antibodies that compete with IgE for allergen binding. The target profile for an improved vaccine for SCIT would include improved safety, maintained or increased

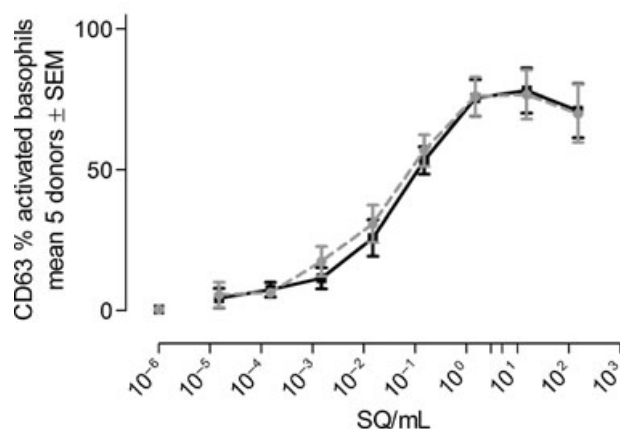


Fig. 6. No difference in basophil activation induced by one grass (Phl p) compared with a mixture of grasses. Human blood basophils were incubated with increasing dose of alternatively or conventionally formulated *Phleum pratense* or grass mix vaccines. Percentages of basophils with increased CD63 expression induced by the alternatively formulated single grass vaccine, *Phleum pratense* (dotted line) or the alternatively formulated grass mix vaccine (5 grasses) (solid line) with stated concentrations of allergen (SQ/mL) are determined. Means $\pm$ SEM from experiments performed with basophils from five allergic donors are shown. No significant difference between the two vaccine formulations, $P = 0.3$ (Wilcoxon two-tailed signed rank test, matched pairs based on maintenance dose).

clinical efficacy, and increased patient convenience. Clinical efficacy and safety can only be determined in a double-blind placebo-controlled clinical trial but as far as providing proof of concept of an improved vaccine design the critical *in vitro* and non-clinical *in vivo* parameters are i) maintained IgG-inducing capacity, ii) maintained T cell responses and iii) reduced IgE-mediated activation of effector cells. The data presented in this paper demonstrate that a SCIT vaccine consisting of AIOH-formulated intact allergens with optimized allergen- and AIOH-concentrations complies with all three criteria: In the presence of a strong humoral immunity-inducing adjuvants like AIOH, a wide range of allergen extract concentrations gives rise to the same level of immunogenicity, measured as the treatment-induced appearance of non-IgE antibodies after i.p. administration in mice. Furthermore, the experimental design of the mouse immunization protocol reflects the recommended human dosing regimen as both vaccine concentration and volume are proportionally maintained. The current study indicates that it is possible to reduce the allergen concentration and to concomitantly reduce the absolute amount of AIOH per dose without detectable loss of immunogenicity. The concentration of the AIOH adjuvant for *in vivo* immunogenicity, however, is critical. *In vitro*, however, there was no adjuvant effect on T cell proliferation even though alum-dependent up-regulation of the maturation marker CD83 on DCs [67–69] was observed. At this stage, it

is difficult to know if the conflicting results are due to the different sources of the cells used (human versus mouse) or to the fact that the two experiments evaluate either the *in vitro* or the *in vivo* effect of alum. *In vitro* and *in vivo* discrepancies by alum or other particulate adjuvants have been described by others showing that *in vitro* activation of the inflammasome, but not the *in vivo* activation, requires a TLR-agonist as a second stimulus [70]. It has been proposed that induction of tissue damage and release of endogenous signals by dying cells following *in vivo* injection of particulate adjuvants could be effective in activating immune responses and that this could act as the second signal [70–72]. Indeed, it has been shown that in addition to IL-1 β , other pro-inflammatory mediators such as CCL2, CCL11, histamine and IL-5 are induced within hours after *in vivo* injection of alum. In addition, inflammatory cells such as neutrophils and eosinophils are recruited to the site of injection within one day post-injection [70, 72, 73]. The fact that the allergen extract as well as the alum used in this study contain very low amounts of LPS could potentially explain the absence of an alum-dependency on the *in vitro* response. The mechanism behind the adjuvant effect of alum is under debate. Likewise, the depot effect of AIOH has been challenged during the past 20 years by several studies reported very similar stabilities of antigen formulated in the presence/absence of alum [15, 74–77]. However, the discovery by Li et al. [9] that alum induces maturation of IL-1 β and IL-18 in an NLRP3-dependent way indeed revived the research field of alum adjuvant mechanism, but these findings have also been challenged. The crucial role of the inflammasome for the secretion of IL-1 β and IL-18 cytokines is undoubted. However, results are contradictory when it comes to the question of whether NLRP3 plays a role in alum-induced specific immunity, e.g. allergen-specific T cell and IgG antibody responses [9, 10, 12–14]. Recently, a novel adjuvant mechanism was introduced by Kuroda et al. who showed that alum stimulates macrophages to produce PGE₂, a mechanism being crucial for induction of specific Th2 immunity by an NALP3 independent pathway [78]. Despite lack of consensus for adjuvant mechanism of alum, it has successfully been used as adjuvants in SCIT for decades [1, 2], and no novel adjuvants have been able to outcompete alum regarding efficacy/safety profile, even though a substantial number of candidates, such as TLR-agonists and various particles, have been tested pre-clinically and to some extent also clinically [79–84].

Regardless of the actual mechanism of AIOH as an adjuvant, our experiments show that the formulation of natural unmodified grass-pollen allergens with AIOH increases the grass-pollen-specific immunological effect of the vaccine in an AIOH dependent manner, so the

magnitude of the immunological effect of the vaccine clearly depends on the presence of the adjuvant. In contrast, using basophil activation as a measure of allergenicity, it is equally clear that the potential to elicit IgE-mediated adverse events is linked to the allergen concentration. Recently, a clinical randomized controlled trial confirming tolerability and immunogenicity of the alternatively formulated SCIT product in 400 patients with grass-pollen-induced allergic rhinoconjunctivitis was published. In this trial, there was no comparison between the alternative and the conventional formulation. Instead, two different up-dosing intervals, 3–4 days or 1 week, were compared in two parallel groups. Post-treatment allergen-specific IgG4, IgE and IgE-blocking factor were significantly increased in both groups compared to baseline levels. No statistical difference in immunogenicity could be observed between the up-dosing regimens, but the 1-week interval was considered to be slightly better tolerated [85].

In conclusion, we have demonstrated that the adjuvant effect of alum allows for optimization of a grass-pollen vaccine for SCIT leading to a formulation with reduced allergenicity and maintained immunogenicity compared with the original formulation. This optimized formulation with a reduced and consequently more convenient and patient-friendly up dosing schedule is currently being tested for efficacy in a double-blind placebo-controlled clinical trial.

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