university of groningen

University Medical Center Groningen

University of Groningen

Subcutaneous and sublingual allergen specific immunotherapy in experimental models for allergic asthma

Hesse, Laura

DOI:
10.33612/diss.158737284

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2021

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Hesse, L. (2021). *Subcutaneous and sublingual allergen specific immunotherapy in experimental models for allergic asthma.* University of Groningen. https://doi.org/10.33612/diss.158737284

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Download date: 04-10-2022

Chapter 9

General discussion and future perspectives

GENERAL DISCUSSION

Allergen immunotherapy (AIT) has the unique capacity to modify the natural course of disease in allergic disorders, by inducing a permanent state of tolerance to the causative allergen, resulting in long-term disease remission[1]. AIT is the sole treatment regime that has the capacity to modify the Th2 immune responses underlying the allergic response, by activation of Tregs and immunosuppressive cytokines. Through these immunological mechanisms, AIT is able to achieve successful suppression of clinical symptoms, such as watery itchy eyes, sneezing, congestion, and coughing upon exposure to aeroallergens[2]. Moreover, this treatment is capable of preventing new sensitizations, as well as progression to asthma in patients with allergic rhinitis[3]. AIT is usually performed by repeated administration of increasing dosages of allergens until a plateau phase is reached, after which treatment is continued for three to five years. The long-term treatment is required for sustainable success effects in patients with allergic diseases. However, the current AIT treatment is also limited by safety concerns, including the risk of severe side-effects, the long duration of treatment resulting in suboptimal adherence, and the lack of efficacy in some allergic disorders including allergic asthma. To address these challenges, optimization strategies for AIT have been the focus of clinical and translational research over the last decade. In parallel, numerous more basic studies attempting to unravel the cellular and immunological mechanism of tolerance induction have highlighted the importance of various subpopulations of immune cells derived from the innate as well as the adaptive arm of our immune system in successful immunotherapy and long-term induction of tolerance.

Clinical studies into the efficacy and mechanisms of AIT have remained largely observational. These studies are essential to evaluate safety and treatment efficacy and routinely record a range of outcome parameters associated with long-term tolerance induced by AIT, such as improved quality of life, decrease in medication use, reductions in nasal symptoms (also during natural exposure), accompanied by persistent immunological changes. However, the opportunities in clinical studies for discovering the underlying cellular and immunological mechanisms during the treatment that determine success of therapy, and the possibilities to explore optimizations of the therapy are limited due to their rigorous design and the focus on very specific primary and secondary outcome parameters needed to draw meaningful conclusions on the safety, effectivity and added value of a novel AIT therapy. To overcome these drawbacks of observational clinical studies in patient cohorts, an experimental mouse model for AIT was developed and published for the first time in 1998 by van Oosterhout *et al*.[4]. In this landmark study, a semi-rush protocol with increasing allergen doses as well as a rush high-dose protocol was evaluated. It was shown that semi-rush AIT causes a change in serum antibody isotypes and is successful in reducing airway eosinophilia and hyper responsiveness in a mouse model with allergic airway inflammation that was largely based on the OVA asthma mouse model, which was the mainstream experimental model for allergic airway disease at that time.

In the research presented in this thesis, we developed a reproducible preclinical grass pollen (GP) *Phleum pratense* (Phl p) and House dust mite (HDM) *Dermatophagoides pteronissinus* (Der p) mouse model of allergic airway disease using BALBc/ByJ mice or C57Bl6 mice. These improved experimental mouse models apply standardized treatment protocols for both SCIT and SLIT, allowing the evaluation of the contribution of several essential immune cell populations in tolerance induction, direct comparison of both application routes, and the testing of new formulations and adjuvants with the overall aim to improve the efficacy of AIT and provide a proof-of-concept for the future clinical trials. To this end, we here discuss our main findings presented in this thesis in the context of the current literature.

In our mouse model of subcutaneous immunotherapy (SCIT), the original protocol developed by van Oosterhout *et al*. [4] has been reduced to three s.c. injections containing high doses of crude extract allergens (300kSQ GP or 250µg HDM) which results in effective suppression of most parameters of allergic airway inflammation using the BALBc/ByJ mouse strain, with the exact dosage of the allergen determining the effectivity of the treatment, whereas an up-dosing regimen of seven injections (25-300kSQ GP) in total was developed for the C57Bl/6 anaphylaxis-sensitive mice (**Chapter 2 and 8**). Both of these protocols rely on sensitization by intraperitoneal (i.p.) injections of Alum-adsorbed allergen extracts as well as three intranasal (i.n.) allergen challenges to provoke the phenotypes of allergic airway inflammation, using the same crude extract of allergens. Differences in disease phenotypes, such as allergic rhinitis (upper airways) and allergic asthma (lower airways), can be modelled in mice through differences in challenge methods. Passive inhalation of an aerosolized allergen extract leads to 83% retention in the nasal mucosa[5], whereas administering intranasal (i.n.) droplets containing allergen extracts in anaesthetized mice leads to active deep inhalation and much more allergen activity in the lower airways, leading to a more asthmatic phenotype[6].

The first experimental studies on the (buccal) sublingual application and allergen uptake via the oral mucosal layer were published around the same time, in 1994[7,8], however the first sublingual immunotherapy (SLIT) protocol in a mouse model for allergic rhinitis was published in 2006 by Brimnes *et al.* In this study, the authors established a mouse model of allergic rhinitis using a crude GP allergen extract and demonstrated that SLIT using 25-125kSQ five days per week for 6-9 weeks, was able to reduce allergic symptoms in a time- and dose dependent manner[9]. To induce allergic rhinitis in their murine model, mice were challenged i.n. daily with 5µL GP (0,1 - 0,5kSQ GP) for one or two weeks and the allergen distribution was confirmed to remain in the nasal cavity, leaving the lower airways completely clean. The protocol for SLIT in a mouse model of allergic asthma used in this dissertation was based on their protocol, with minor adaptations to introduce a more asthmatic phenotype. SLIT in our mouse model also relies on sensitization by two i.p. injections with Alum adsorbed allergens and induction of allergic airway inflammation by three i.n. allergen challenges (25kSQ GP) every other day, which is exactly the same as in our SCIT protocol. The SLIT treatment phase consists of five sublingual administrations of allergen per week for a total of eight weeks, totalling up to 40 administrations using high doses of allergen extracts (300kSQ GP) directly placed under the tongue for a minimum of 30 seconds (Chapter 3). This means that the individual dose of

allergen in the optimal protocol in SCIT per application is exactly the same of that in SLIT, whereas SCIT is administered three times and SLIT is given 40 times (Chapter 3, Figure 1).

In fact, the dose of allergen used in AIT treatment as well as the total cumulative dose achieved in the total treatment period, are critical parameters for successful AIT. Based on the conclusions drawn from a double blind placebo controlled (DBPC)-study in ragweed pollen hay fever patients performed by Van Metre *et al.* during the pollen season in 1980[10], the WHO considered low-dose SCIT was inadequate in the consensus study in 1998 on AIT, with high doses achieving a therapeutic effect but likely also resulting in a higher degree of systemic reactions[11]. Moreover, the EAACI taskforce compared immunological and clinical data on treatment efficacy and safety in studies where with multiple doses were tested[12]. Herein, only 15 out of the 1379 clinical studies that were screened used multiple allergen-doses of which only nine were SCIT for respiratory allergies, four SLIT and two venom-SCIT. Out of the nine SCIT studies for respiratory allergies, almost all reported a clear dose dependent efficacy. Herein, one of the best examples included was a RDBPC SCIT study using Alutard SQ® *Phl p* (ALK) by Frew *et al.* in the UK amongst 26 centres in adult patients with allergic rhinitis[13]. Of the two doses (10kSQ-U and 100KSQ-U) used throughout the whole pollen season, both were found to be effective in improving quality of life, and reducing symptom and medication scores, although the highest dose overall performed better. In contrast, using the lower dose fever local and systemic allergic side effects and other adverse events within an hour after injection were reported[13].

Although treatment efficacy of SCIT for both rhinitis and asthma has been proven numerous times, major recent systematic studies show a broad variance in the study results can be seen[14,15]. For instance, the RDBPC phase II/III study on two dosages of AVANZ® (Phl p, ALK) did not reveal any significant effects of GP-SCIT over placebo treatment in the primary and secondary outcome parameters[16]. Herein, primary outcome parameters included daily symptoms and medication scores and reports of adverse events, and the secondary endpoints were calculated as the sum of all scores during the entire pollen season divided by the numbers of score entries. This outcome was at odds with previous studies that report a clear efficacy of GP-SCIT, using other products with higher or lower allergen content[17,18]. For instance, Zenner *et al*. reported lowered symptoms scores in allergic patients after a successful short 10week-SCIT course using rye and grass extracts in increasing doses (up to 1000 standard units, equals ~ 1-2 µg for individual grasses)[18]. Moreover, with only a few mild local responses reported at the injection site reported, this study was considered efficient and completely safe.

In clinical studies, only very rarely is an adverse effect with actual worsening of symptoms reported. One notable exception is a Phase II RDBPC study using intradermal GP injections in patients with allergic rhinitis, that led to the suppression of late phase skin reactions, while there was no difference between GP and placebo treatment in the primary endpoint and Th2 and spIgE responses were both increased[19]. Moreover, amongst the secondary endpoints, daily scored nasal symptoms worsened with 44% in the AIT treatment group, when compared to controls. The heterogeneity in safety and clinical efficacy in these study results underscore the scientific importance of a reproducible model that allows a more detailed analyses of all parameters of AIT.

In line herewith, the experimental mouse models were designed to study the immunological mechanisms of AIT in detail and help improve the treatment by finding the optimal route of administration, dose and formulation. The importance of finding the correct dose without adverse effects is evident from a study in a mouse model of OVA-induced allergic asthma, where SCIT using peptides derived from an immune-dominant epitope in the OVA protein resulted in increased AHR and eosinophilia in the lungs, indicating an exaggerated Th2 driven airway inflammation after SCIT treatment[20]. Similarly, in Chapter 2, we found that in a rush SCIT protocol using three GP injections, low dose GP-SCIT aggravated some of the allergic symptoms, resulting in elevated GP-splgE induction, reduced GP-splgG-levels, increased ear swelling responses upon intradermal GP challenge, and enhanced airway resistance and eosinophil numbers in BALF and lung after GP inhalation challenges (Figure 1). In line with a strictly dose-dependent efficacy of SCIT, we observed that an intermediate dose of GP (3-10kSQ GP) had very limited effects while higher dosages (100-300kSQ GP) were able to achieve some, or even almost complete, suppression of allergic manifestations in the experimental mouse model, indicative of successful SCIT (Chapter 3, Figure 1). These data indicate that AIT depends on a delicate balance between an aggravated immune response versus an effective treatment as function of allergen dose, which stresses the need for further optimizations by increasing the cumulative allergen dose, and by using safe application routes or adjuvantia that increase therapeutic effectivity at a specific allergen dose, thereby inducing a more dominant tolerogenic response to the allergen injections.

While the lower limit of allergen dose in AIT is determined by the ability to achieve immune suppression, the upper limit is determined by the risk for severe side effects that increases with allergen dose applied, including large local reactions and anaphylaxis. The experimental mouse models again offer a great opportunity to study both sides of the coin when increasing the allergen dose in AIT treatment. In our Balbc/ByJ experimental mouse models of AIT, IgG1 is considered to be the main neutralizing antibody. It has been shown that the IgG produced during an antigen-specific immune response or administrated passively can suppress IgE mediated anaphylaxis through two mechanisms: by capturing the allergen before it can induce mast cell activation upon crosslinking of IgE/FcεRI complexes, or by crosslinking FcεRI to FcγRIIB[21]. Given the strongly increased specific IgG1 levels after treatment in our mouse models, and the suppression of phenotypes of allergic airway inflammation such as eosinophilia and AHR after allergen challenges in the absence of any signs of anaphylaxis in the allergen challenged mice, we feel that under these conditions these IgG1 antibodies play a protective role in suppressing allergic responses. To further support the role for splgG1 in preventing allergic phenotypes after allergen challenges in AIT treated mice, we have calculated the correlation of specific IgG1 titers to the other read-out parameters in the model and found that splgG1 levels are significantly and inversely correlated to airway eosinophilia and lung levels of IL-5 and IL-13 (Chapter 3). This indicates that the effect of the increased systemic splgG1 titers on suppressing IgE dependent allergic responses are more dominant than the potential IgG1 dependent anaphylaxis in our experimental model. Such a role for IgG1 in anaphylaxis is quite specific for the mouse, and has been shown in a number of studies that have identified two separate pathways to contribute to anaphylaxis: the classic pathway which depends on IgE mediated

crosslinking of FcεRI on mast cells and release of histamine and platelet activating factor (PAF), which is also found in humans, and the alternative pathway, which depends on IgG, FcγRIII and PAF secretion by macrophages and basophils[22]. The two pathways differ in their antigen dependence, with the IgE mediated pathway requiring far less allergen compared to the IgG mediated pathway. In the BALB/cByJ strain, we do not observe any anaphylactic responses induced by the injection of allergens in SCIT, without the need for an updosing phase. This is in contrast to the C57Bl6/J mouse, where anaphylaxis is readily induced by high dose allergen injections and a careful updosing scheme is required for successful SCIT. Similarly, Smit *et al*. tested anaphylactic responses upon peanut-allergen exposures in three mouse strains, C3H/HeOuJ, C57BL/6 and BALB/c[23]. Herein, specific immunoglobulin responses, mast cell degranulation and specific T cell responses were different in all three strains, with the BALBc mice the most responsive, followed by C57BL/6 mice, and least pronounced in C3H/HeOuJ mice. Moreover, while anaphylactic responses were absent in BALBc mice, they were notably present in both C57BL/6 mice and to an even greater extent in C3H/HeOuJ mice demonstrating that these three strains displayed a highly divergent susceptibility to anaphylaxis[23]. Next, Marco-Martín *et al*. performed an experiment using C3H/HeOuJ and BALB/c mice to study both clinical and immune responses in peanut allergen-induced systemic anaphylaxis, and found that, although BALB/c mice exhibited higher levels of Th2 cytokines, the C3H/HeOuJ strain showed more pronounced levels of spIgE, IgG1, IgG2a, and more severe clinical symptoms [24]. These data indicate that in the mouse strain used in our current studies, the BALB/cByJ strain, anaphylaxis is not a dominant response. In the C57BL/6 strain however, we found that injection of only 25kSQ of GP (as compared to 300kSQ GP in BALB/cByJ mice) already induced pronounced clinical signs of anaphylaxis as described in Chapter 2. Clearly, the BALB/cByJ model is not the optimal model to evaluate the safety of AIT. In the C57Bl/6J strain seven s.c. injections using increasing dosages of GP revealed marked increases in spIgE, spIgG1 and spIgG2a levels. However, we do not know the relative contribution of IgE and IgG1 to the anaphylactic response observed in C57Bl/6J mice, which would need experimental validation prior to employing this strain as a preclinical model for safety screening of novel AIT treatment modalities. Overall, although the C57BL/6 strain was shown to be more susceptible for anaphylaxis induction, we were able to adapt the treatment schedule of AIT in such a way that it was still successful in inducing tolerance, based on lowered numbers of eosinophilia and Th2 cytokines in lung cells. This model might be of relevance for future experimental modelling of the safety aspects of SCIT treatment. The clinical signs of anaphylaxis were only visible in the first two days of s.c. injections, which is similar to the early desensitization response observed in patients with venom allergy, where venom-specific immunotherapy induced a rapid desensitization associated with local (piecemeal) degranulation of the mast cells, requiring monitoring of the response at the lower allergen dosages in the outpatient clinic during the updosing scheme of the VIT treatment[25] (J.N.G. Oude Elberink, personal communication).

Allergen immunotherapy: SCIT versus SLIT

In DBPC-trials, both SCIT and SLIT have been shown to effectively suppress allergic manifestations upon allergen exposure, providing long term relief from symptoms in allergic disorders including allergic asthma[26,27]. Clinical studies directly comparing SCIT and SLIT report differences in kinetics

and magnitude of the immunological changes induced during treatment [28–32]. Both SCIT and SLIT require regular administration of allergens for 3-5 years with the goal of desensitizing the effector cells, and converting the adaptive immune response to a state of long-term tolerance. In general, maintenance dosage in SCIT treatment involves ~20 µg of allergen that can be applied using various treatment regimens (applied as a single allergen or a mixture of allergens and with a variety of frequency of injections). SLIT is available in daily tablet (GP or 5-grass, HDM, or ragweed pollen) or in aqueous droplets usually in higher concentrations (ORALVAC® 7,680 Therapeutic Units (TU)/ mL) but easier to use and therefore allowing self-administration[33]. According to international guidelines, both treatment routes have been considered effective, received FDA approval for general use as AIT and achieve substantial improvement in symptom scores, most notably in adults[34]. Although SCIT is traditionally considered to be more effective than SLIT, and adherence to treatment can be better monitored, the superior safety profile and the home use are important advantages of SLIT, while efficacy is not necessarily lower than SCIT as shown in the recent large-scale clinical trials[35]. The prescribing practitioner has to consider the risks, advantages, costs and patient motivation when deciding on the use of either SCIT or SLIT.

While SCIT and SLIT have been proven effective for both rhinitis and asthma, as shown in recent major systematic studies, a broad variance in the study results as well as a substantial variation in the meta-analyses was described[14,15]. Comparing studies of different treatment routes (or different allergens) is even more challenging, when taking variable methods, results, and outcomes into account. Based on indirect evidence from systematic reviews and DBRPC trials, Durham and Penagos published results on a comparison of SCIT versus SLIT for allergic rhinitis and concluded both administration routes leave the patient and the doctor in complete equality and personal preference[32].

The number of clinical studies that directly compare sublingual versus subcutaneous application in the same patient population and using similarly sourced allergen preparations is very limited. One hallmark study was performed in which rhinitis patients were randomly assigned to either SCIT, SLIT or placebo treatment groups and were treated with GP for 15-24 months[29,36]. Although both SCIT and SLIT prevented seasonal induction of specific IgE, SCIT was observed to induce a more rapid and robust change in sp-IgG4, inhibition of facilitated antigen presentation and basophil activation test[29]. After 15 months, however, SLIT and SCIT resulted in similar levels of inhibition of facilitated antigen presentation and symptom scores. After 24 months, both SCIT and SLIT resulted in a temporal increase in IL-10 producing T cells, while the numbers of IL-5 producing T cells were significantly more decreased in the SCIT-treated patients when compared to the SLIT treated group[36]. In this study, SCIT involved the use of Alum as an adjuvant, in contrast to the adjuvant-free SLIT, which might have potentiated the neutralizing immunoglobulin responses in SCIT. Notwithstanding this limitation and the relatively small patient numbers, it is clear that SCIT treatment induced immunological changes with faster kinetics and greater magnitude compared to SLIT treatment. However, on typical clinical endpoints for large clinical studies such as symptom scores and use of other medication, the two treatments performed equal upon successful finishing the treatment regimen[32].

Experimental studies dissecting the mechanisms of AIT or attempting to enhance its efficacy have often focused on a single route of application, precluding direct comparison of the efficacy of sublingual versus subcutaneous administration of allergen extracts for induction of neutralizing antibodies and suppression of allergic inflammation or lung function parameters. In **Chapter 3**, we presented the first study directly comparing SCIT and SLIT treatments in a mouse model for allergic airways disease using clinically relevant allergens. We performed a direct comparison of GP-SCIT and GP-SLIT treatments in the experimental mouse model using a standardized protocol and on the basis of immunological and translational outcome parameters (Chapter 3). We uniquely treat the mice with either SCIT or SLIT after parenteral immunization with the allergen, which means in the presence of an immunological memory population. Altogether, both administrative routes rendered suppression of certain phenotypes of the experimental mouse model of GP-driven allergic asthma. To allow qualitative evaluation of the relative efficacy of either administration route of GP-AIT on the various parameters of the allergic asthma mouse model, we provide an overview of all parameters and their dose-dependence (Figure 1). GP-SCIT treatment regimens using 3, 10 and 30kSQ GP, were unable to reduce symptoms of allergic airway disease, but in fact resulted in a more inflammatory profile, as outlined by strong GP-spIgE induction, low GP-spIgG-levels, increased swelling responses and airway resistance, and enhanced/exaggerated eosinophil numbers in BALF and lung. Both SCIT and SLIT using 100kSQ GP resulted in suppression of a subset of the parameters of allergic airway inflammation only, and this dose was considered suboptimal in later experiments. At the highest dose of 300kSQ, the immunoglobulin responses showed marked differences between SCIT and SLIT. The total and specific IgE responses measured after GP challenges were in the same range in SCIT and SLIT. The induction of IgE after GP challenges, however, was far more effectively suppressed by GP-SLIT. The blocking immunoglobulins differ based on isotype: GP-spIgG1 is superior in the (faster) SCIT protocol, while the IgG2a levels are higher after SLIT treatment. Consequently, the neutralizing capacity by IgG1 was much better in SCIT than in SLIT, while the opposite is true for IgG2a (Figure 1). When comparing airway hyperresponsiveness to methacholine after GP challenges, GP-SLIT achieved a stronger inhibition, while GP-SCIT merely achieved a trend towards suppression compared to Sham-treated controls. Moreover, only SLIT treatment was able to increase lung compliance. In contrast, marked suppression of eosinophilic inflammation in BALF and lung was achieved in the SCIT protocol, while in SLIT these findings were less profound and only significant in the lung cells. Interestingly, SCIT was able to suppress IL-13 production by lung cells, while SLIT failed to do so, while both treatments resulted in reduced levels of IL-5 in lung tissue homogenates. Apparently, suppression of Th2 cell activity was slightly more effective in GP-SCIT.

Although there are no experimental studies available that provide results on a similar comparison of both treatment routes in a mouse model for allergic asthma, SCIT treatment with allergen extracts of birch pollen (BP) or recombinant phospholipase A2 (PLA2) in mice was shown to induce strong neutralizing antibody responses[37]. Interestingly, in the BP-SCIT mouse model, a higher number of allergen injections (eight s.c. injections) was needed to achieve suppression of AHR than to induce a neutralizing antibody response, which might be relevant for our GP-SCIT mouse model as well. Experimental mouse models of Japanese cedar (JC)-SLIT treatment have rendered variable

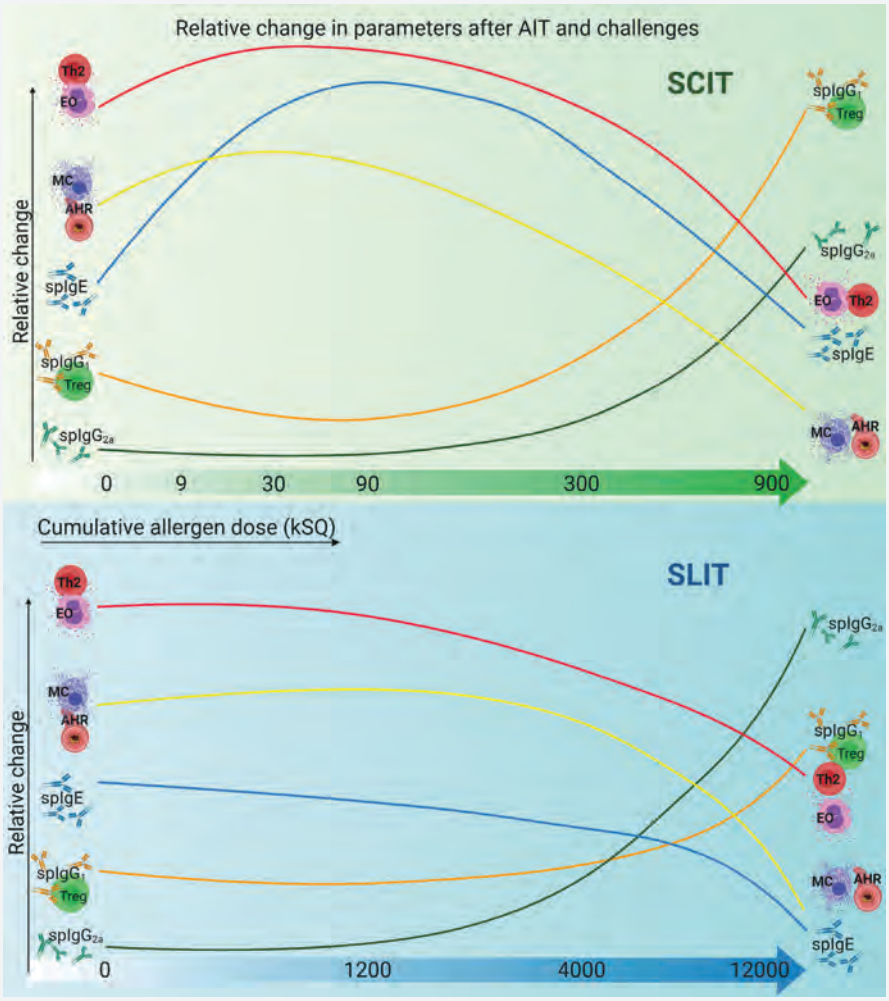

Figure 1. *Overview of parameters of inflammation and tolerance induction after SCIT (upper half) and SLIT (lower half) using increasing doses of GP to allow a qualitative comparison of both administrative routes as well as allergen dose used per administration (kSQ). The doses in SCIT include 3, 10, 30, 100, 300kSQ whereas the SLIT doses include only 30, 100, 300kSQ. Please note that SCIT is administered three times whereas SLIT is given 40 times. Th2, T helper 2 lymphocytes; EO, Eosinophils; MC, Mast cells (representative for the EST); AHR, Airway hyperresponsiveness (representative for the Resistance, ED3, and compliance levels); splgE, specific immunoglobulin E; splgG1, specific immunoglobulin G1; Treg, T regulatory cells; splgG2a, specific Immunoglobulin G2a. Figure was created using BioRender.com.*

results regarding the efficiency of suppressing the different parameters of allergic airway disease, but generally show limited induction of neutralizing antibody responses, in line with our own results[38]. Furthermore, in one experimental study using *Dermatophagoides farinae* (Der f) extracts, SLIT treatment had a more pronounced effect on AHR than on Th2-driven, eosinophilic airway inflammation[39]. In mouse models of GP-SLIT, induction of a neutralizing antibody response by GP-

SLIT treatment was also limited, although daily application of lower amounts of GP extract were able to induce a modest spIgG1 and IgG2a response in comparison to less frequent application of higher doses of GP extract[9,40].

In contrast, in clinical studies SLIT treatment was reported to be able to induce a significant antibody response. For instance, in a sub-study of the GRAZAX® asthma prevention study, a 3-year course of GRAZAX® SLIT in children with allergic rhinitis revealed increased levels of specific IgA in saliva accompanied by significantly increased serum spIgG4 measured after 3 years of treatment as well as two year after cessation thereof[41]. The increased levels of spIgG4 are considered to be immunomodulatory, as it competes with other Ig isotypes for binding allergen and does not activate complement[42]. In our findings, GP-SLIT only significantly induced GP-spIgG1 levels directly during and after SLIT, whereas GP challenges were unable to increase these blocking antibodies any further. Of note, we were only able to detect serum IgGs directly after challenges (48hrs) and did not measure the long-term immunoglobulin responses after SLIT or SCIT at later time point after these or repeated allergen challenges. In the GRAZAX® asthma prevention study, a significant reduction of the ratio of IL-5/IL-13 over IFN-γ was found to be indicative of lowered Th2 cell responses and increased levels of Th1 chemokines, CXCL10 and CXCL11. In, our mouse model of GP-SLIT we only observed modestly decreased levels of Th2 cytokines, and we were unable to detect any increase of Th1 cytokines. The effect size of SCIT on suppression of Th2 cytokines and eosinophilic airway inflammation was stronger than that of SLIT, indicating that a higher allergen dose in SLIT, or a modified delivery method, might be warranted for full efficacy.

Overall, the results in other models for AIT seem to confirm our observations described in Chapter 3, with regard to the modulation of adaptive immune responses by SCIT, as evidenced by induction of neutralizing antibody responses and suppression of Th2 driven eosinophilia, whilst SLIT has more of an effect on lung function parameters, but not so much on specific antibodies or Th2 cells. Moreover, the wide variety of allergen extracts used, the differences in treatment schemes and the resulting variable outcomes of SCIT or SLIT treatment on the clinically relevant parameters of airway inflammation and lung function underscore the added value of our approach in combining the two application routes in a single mouse model using the identical allergen extract for efficient comparison on immunological and translational parameters.

Use of adjuvants: successful preclinical validation of VitD3

Strategies to improve AIT regimens include alternative administration routes to achieve optimal antigen presentation at low levels of applied allergens[43,44], use of purified or recombinant allergens and or peptides[45,46], or addition of an adjuvant to enhance tolerance induction[47]. Use of adjuvantia in AIT aims to increase allergen delivery to and uptake by antigen presenting dendritic cells (DCs), and to enhance their tolerogenic capacity. One such candidate adjuvant for AIT, 1,25-dihydroxy-vitamin D3 (VitD3), binds to its nuclear hormone VitD3 receptor (VDR), and provides immune regulatory properties through induction of tolerogenic DCs [48]. VitD3 prevents DC-maturation leading to down-regulation of costimulatory molecules (CD40, CD80, CD86) and enhanced IL-10 production [49],

facilitating the generation of adaptive Treg cells[50].

Vitamin D insufficiency is widespread, and is thought to contribute to asthma susceptibility[51]. Childhood VitD deficiency is associated with increased risk for asthma, through increased susceptibility for allergic sensitization[52]. In some cases, supplementation of VitD3 in clinical studies has resulted in reduced risk for allergic disease or improved outcomes of treatment[53]. For instance, VitD3 supplementation during pregnancy reduced the risk of recurrent wheeze and acute respiratory tract infections in early life[51,54]. Moreover, VitD3 supplementation in asthma patients has been shown to reduce the rate of asthma exacerbations requiring treatment with systemic corticosteroids[55]. The mechanism of action of VitD3 is thought to include both steering of the immune system towards a more tolerogenic response, as well as reinforcing the barrier and antiviral properties of the bronchial epithelium[51,56,57].
In allergic rhinitis patients, clinical efficacy of SCIT was evaluated based on their VitD3 status throughout treatment, and when VitD serum levels were sufficient effects were more pronounced[58]. However, conflicting data have been obtained in clinical studies on a role for VitD3 in allergen-based SCIT and SLIT treatment protocols[59,60]. These recent studies report that VitD3 supplementation had limited positive effects on HDM-SCIT treatment, with asthma symptom score as the only improvement compared to control HDM-SCIT treatment[59]. In contrast, VitD3 supplementation of GP-SLIT was reported to suppress nasal and asthmatic symptoms in comparison to the control GP-SLIT treated group[60]. The discrepancy between these studies might be due to differences in allergen used (HDM versus GP), duration of treatment (12 versus 5 months) or the route of application of the allergen.

Based on the tolerogenic properties of VitD3, we previously showed that injection of VitD3 enhanced the therapeutic effects of SCIT in the OVA-driven mouse model for allergic airway inflammation[61]. To resolve whether VitD3 supplementation has the potential to enhance efficacy of both SCIT and SLIT, we aimed to perform a side-by-side comparison of VitD3 supplementation in SCIT versus SLIT using the same allergen extract in a mouse model of GP-driven allergic airway inflammation in **Chapter 4**. We found that the use of VitD3 supplementation augments induction of neutralizing antibody responses, and leads to enhanced suppression of eosinophilic inflammation and production of IL-10 in lung tissue in both SCIT and SLIT treatments, while an additional effect on AHR was observed in SLIT treatment only (Figure 2).

To our knowledge, Chapter 4 describes the first study comparing the adjuvant effects of VitD3 supplementation in GP-SCIT and GP-SLIT treatments in an experimental model for allergic airway disease. Strikingly, and in contrast to our previous results using unsupplemented AIT[62], we report a prominent Treg cytokine profile in lung tissue after VitD3 supplemented GP-SCIT or GP-SLIT, as demonstrated by the increased levels of IL-10 and in SLIT also of TGF-β1 (Figure 2). These results are in line with the previously reported biological effects of VitD3 on DCs, which was shown to result in enhanced generation of adaptive Treg cells and increased IL-10 and TGF-β1 production[63,64]. Subsequently, in **Chapter 5**, we aimed to find the optimal VitD3 dose in our GP-SCIT model and

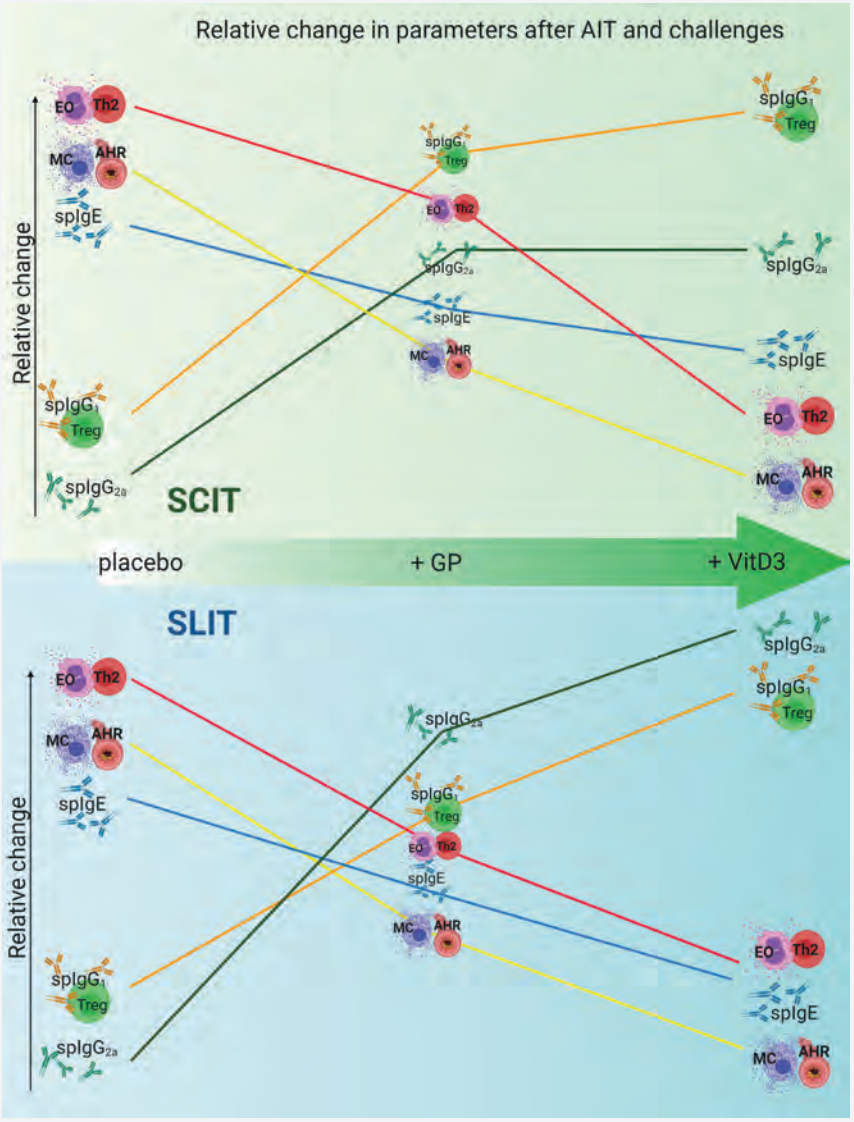

Figure 2. *Overview of parameters of inflammation and tolerance induction after SCIT (upper half) and SLIT (lower half) using GP to allow a qualitative comparison of both administrative routes as well as VitD3 adjuvanted GP-SCIT and GP-SLIT (on the right half). Th2, T helper 2 lymphocytes; EO, Eosinophils; MC, Mast cells; AHR, Airway hyperresponsiveness; splgE, specific immunoglobulin E; splgG1, specific immunoglobulin G1; Treg, T regulatory cells; splgG2a, specific Immunoglobulin G2a. Figure was created using BioRender.com.*

report a clear dose-dependent effect of VitD3 in GP-SCIT with remarkable effects of high-dose VitD3 when compared to the unsupplemented GP-SCIT group. The difference in the dose of VitD3 between the OVA and GP models (10ng vs. 300ng VitD3 per injection) most likely stems from the use

of a purified protein (OVA), which upon repeated administrations by inhalation even has tolerogenic properties[65] versus a GP extract, which contains multiple allergens and is immune-stimulatory. We feel that the latter model is a better translational model for the clinical practice where treatment depends on the use of allergen extracts or modifications thereof[62]. Our results strongly suggest that AIT efficacy can be improved by monitoring and supplementing VitD3 levels, or by combining VitD3 in the allergen formulation for AIT.

Experimental mouse models of allergic airway disease have been used to study the effect of VitD3 levels on parameters of the disease [57]. Perinatal VitD deficiency in mice has immunomodulatory effects, such as increased Th2 skewing and reduced IL-10^{+} Tregs, which was exaggerated upon challenges[66]. More importantly, when VitD-depleted neonates were placed on a diet containing high dose VitD, recovering their VitD status during weaning, their disease severity was less profound, as detected by lower AHR and eosinophilia, as well as reduced IgE levels. In line herewith, our results indicated that only three injection containing a high dose VitD3 were sufficient to enhance suppression of airway eosinophilia after challenges by GP AIT treatment (Chapter 5). Moreover, Heine *et al.* showed that VitD deficiency in mice promotes sensitization, and co-administration of 25(OH)D in OVA-SCIT resulted in lower airway inflammation, Th2 cytokines, and AHR after challenges[67]. Moreover, VitD supplemented Der p2-allergoid SCIT resulted in reduced Th2 cytokines and eosinophilia and increased Treg numbers[68].

Interestingly, in addition to suppression of Th2 inflammation by VitD3 supplementation of GP-SCIT, we also observed suppression of a number of innate cytokines and chemokines in lung. With the subcutaneous VitD3 injections, we aimed for modification of the local APCs during GP-SCIT, leading to Treg induction and Th2 effector cell suppression. The suppression of the innate responses in the lung-resident cells might indicate a strongly augmented Treg activity, or systemic effects of the locally applied VitD3[51]. These data support further clinical studies on VitD3 supplementation in allergen-specific immunotherapy treatment for patients with allergic airway diseases.

Other successful formulations and adjuvants

In addition to the use of adjuvants such as VitD3, the formulation of the allergen extract used in SCIT or SLIT also offers opportunities to improve the delivery of the allergens in a tolerogenic fashion. An example includes the use of virus-like particles (VLPs), nanoparticles of about 20-200 nm in size that are biodegradable, and can be engineered or produced to carry allergens on their surface. The use of VLPs to improve treatment efficacy has been demonstrated in many studies, as reviewed by Anzaghe *et al.*[69]. In another approach, few studies reported on the beneficial use of liposomes in new AIT formulations[70]. Lipid bilayers encapsulating allergens form liposomes, like nanoparticles, and act as delivery vehicle, as a depot or even function as an adjuvant. One RDBPC trial in patients with allergic asthma revealed that liposome-encapsulated HDM-extract resulted in blocking IgG responses and reduced eosinophil numbers, but no safety data were reported in this study[71]. In line herewith, a murine model of HDM allergy was used to test intranasal application of liposome-adhered major allergens (*Dermatophagoides pteronyssinus*, Der p) Der p1 and Der p2, and found to be equally effective in lowering Th2 responses while being superior in increasing Treg cytokines, like

IL-10 and TGF-β, when compared to the crude extract alone[72]. Due to the lipophilic nature of VitD3, simultaneous administration of the poorly water-soluble VitD3 and a freeze-dried highly water-soluble GP-extract in an AIT mixture is not optimal. Notwithstanding, simultaneous delivery of VitD3 and the allergen extract in a single injection is desirable in order to tolerize the DCs presenting the allergens, without otherwise influencing the host immune response. The use of liposomes to deliver both the hydrophilic allergen extract and the lipophilic VitD3 might enhance therapeutic efficacy. SAINT-18 (1-methyl-4-(cis-9-dioleyl)methyl-pyridinium-chlorid, SAINT) is a synthetic biocompatible lipid which can form liposomal structures, and might be able to act as a carrier for VitD3 while encapsulating the GP extract[73]. In **Chapter 5**, we tested whether use of the synthetic lipid SAINT to establish a more stable mixture of GP-SCIT extracts and VitD3 could enhance suppression of parameters of allergic inflammation in our mouse model of GP-SCIT. Addition of SAINT, however, did not further enhance the VitD3-dependent suppression of allergic manifestations, even at suboptimal GP-dosages.

Similar to previous findings using carrier formulations like chitosan and Poly-lactic-co-glycolic acid (PLGA)-nano particles, SAINT-18 is thought to capture allergens, and might induce an enhanced allergen uptake and presentation. Endmann *et al*. showed the successful capture of SAINT-18 with MIDGE-Th1 DNA vectors, resulting in stable, well-tolerated formulations with high immune responses *in vivo* induced by the formulated lipoplexes[73]. Although through a different formulation strategy and working mechanism, Liu *et al*. reported beneficial effects of using chitosan as a chitosan-Der f nano-vaccine in a mouse model of intranasal AIT[74]. Moreover, Saint-Lu *et al*. showed in a murine model that chitosan formulations had mucoadhesive properties, induced enhanced uptake of OVA when applied sublingually and enhanced tolerance induction via lowered AHR, eosinophils, as well as specific Th2 responses[75]. More recently, PLGA-nano particles, were used to enhance efficacy of SLIT in a murine model of allergic rhinitis [76]. These PLGA-nano particles are biodegradable polymeric nanoparticles, excellent delivery vehicles and considered nontoxic, completely biocompatible, and improve macromolecule penetration across the mucosal layer. In the SLIT model, PLGA-formulated rChe a3 (recombinant chenopodium album Protein/ polcalcin) SLIT reduced Th2 inflammation, eosinophilia and increased numbers of Tregs when compared to control treated mice[76].

The crucial determinant in the treatment efficacy might be the particle size of the new formulations. The smaller the particles the better the antigen uptake and presentation, classic liposomal formulations generally range between 50-250nm in size, and are unstable below 50nm due to the high curvature this requires for the lipids[77,78]. Moreover, smaller sized particles could possess adjuvant activity towards activation and maturation of DCs in a inducer of humoral and cellular immunity[79]. Our SAINT/GP formulations were prepared freshly every time, resulting in a stable formulation with acceptable particle sizes. In pilot stability experiments however, we did find that the higher the dose of VitD3 used in the mixtures, the more emulsified the mixture became and particle sizes would decrease to 78-80nm, possibly leading to more efficient phagocytosis by the APCs. However, while we show that high dose VitD3 improves GP-SCIT, this effect was not enhanced by use of SAINT-18 as a carrier.

Overall, the use of liposomal carriers as a strategy to improve formulation for SCIT and SLIT has demonstrated positive immune-modulating results and does hold the potential to improve the efficacy of AIT. However, the use of SAINT, as such, was unsuccessful in this model and improved techniques and formulations are required to gain more knowledge on the stability and mechanism of immunomodulatory properties of these liposomal formulations.

From crude extracts to short peptides

Traditionally, allergologists around the world are familiar with treatment regimens based on application of crude allergen extracts, which are easy to produce and prepare. Crude extracts contain multiple major allergens from a specific source and are easy to combine when patients are poly-sensitized. However, the safety of this treatment has always been a concern and standardizing natural allergen extracts remains a major challenge. Use of crude allergen extracts has several disadvantages: production of the crude extracts has high batch-to-batch variation; extracts may contain contaminations from other sources; and crude extracts always contain a mixture of allergenic and non-allergenic substances. In SCIT, the majority of the adverse events are the result of IgE-dependent basophil and mast cell activation upon injection or administration of crude allergen extracts[80], that retain their allergenicity.

Numerous attempts have been made to reduce the allergenicity of AIT while retaining the potential to suppress Th2 activity and restore tolerance to the allergen (Figure 3). As an alternative to crude extracts, the use of purified proteins might be less immunogenic, since full extracts contain impurities and a number of non-protein constituents, which might activate antigen presenting cells through pattern recognition receptors. Consequently, use of purified proteins might increase the treatment efficacy (Figure 3). Preclinical mouse studies already established that proteins purified from crude extract HDM can be used in AIT [45]. Native proteins retain their 3D conformation and therefore can crosslink IgE and induce side-effects upon injection, especially in highly purified form. However, synthetic peptides that represent the dominant T-cell epitopes of a major allergen but lack the tertiary structure required to bind to and crosslink IgE, may have a better safety profile than the native protein (Figure 3)[81].

Recently, the use of peptides was successfully implemented in murine models using the major epitopes from Fel d1 [82]. Furthermore, Kettner *et al*. presented a successful phase IIb AIT-trial based on contiguous overlapping peptides from birch pollen Bet v1, reporting significant and persistent clinical improvement extending to at least 2-seasons post-treatment in birch pollen allergic subjects[83]. The use of purified proteins or selected peptides instead of a full allergen extract, however, might limit the efficacy of the treatment due to incomplete coverage of the range of potential allergens that patients can be sensitized to. Moreover, the use of peptides is limited by differences in MHC usage between individual patients, which impacts on the identity of the T-cell epitopes to which each individual will respond. Furthermore, peptides may have a shorter half-life after administration, and need to be taken up by DCs in order to be presented to T-cells and exert a putative tolerogenic activity.

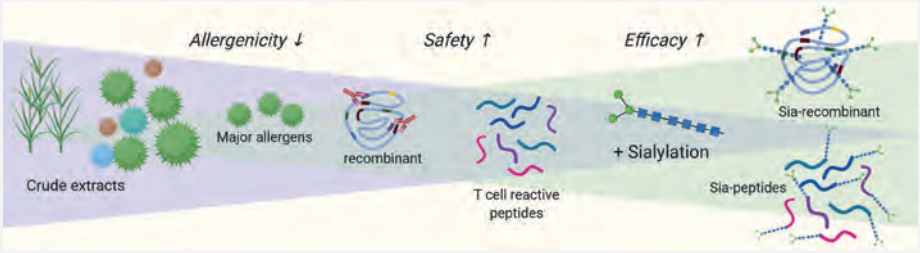

Figure 3. *Overview of improvement strategies to obtain reduction in allergenicity and to increase safety and efficacy of AIT. From Left to right: Crude allergen extracts, like pollens, house dust, animal dander, spores of mold, cosmetics, feathers etc. can be identified as highly capable of inducing an allergic reaction upon sensitization. Crude extracts contain major allergens that can be naturally purified or its isolated mRNA can be used to produce recombinant allergens with reduced allergenicity. Usually, both purified and recombinant allergens maintain T cell recognition sites as well as IgE binding sites. Shorter peptide sequences containing T cell recognition can be identified and isolated using T cell epitope mapping from allergen specific T cell clones. Addition of polysaccharide chains, sialylation (Sia-), is an attractive strategy to improve treatment efficacy. Figure was created using BioRender.com.*

Besides suppressing Th2 cells, strategies to improve AIT using purified or modified major allergens or peptides might include ways to promote or actively induce a tolerogenic state of the DCs. DCs express sialic acid-binding Ig-like lectins (Siglecs), which bind sialic acids - monosaccharides on glycan chains on proteins, peptides or lipids. Siglecs function as endocytic receptors, enhancing phagocytosis by the DCs[84]. In mice, sialylation of antigens has been shown to instruct an antigen-specific tolerogenic state onto DCs via binding to Siglec-E (human Siglec 7 and 9 homologue), enhancing generation and propagation of Treg cells while reducing the generation and function of inflammatory T-cells[85]. Hence, sialylation of allergen-derived peptides might enhance the induction of allergen-specific Treg-cells, and consequently increase the efficacy of peptide AIT. In **Chapter 6**, we asked whether glycan modification of peptides derived from Phl p5a could improve the efficacy of peptide AIT in suppressing symptoms of allergic airway inflammation. We showed that sialylation of Phl p5a peptides resulted in increased proliferation, FoxP3 expression and TGF-β1 release by $CD4^+$ T-cells isolated from GP-sensitized mice. Thereafter, we compared SCIT using sialylated and unmodified Phl p5a peptides to the use of a GP-extract (GP-SCIT). Our *in vivo* findings indicate that GP-SCIT with the full extract was successful in suppressing asthmatic manifestations, whereas the use of a mix of two Phl p5a-derived peptides was not as effective, and failed to induce neutralizing antibody or spIgE responses. A direct comparison between unmodified and sialylated peptides revealed a significantly increased induction of $FoxP3^+$ T-cells, and decreased numbers of $GATA3^+$ T-cells associated with an enhanced suppression of eosinophilia in both BALF and lung tissue by the sialylated peptides. Herein, we concluded that the use of sialylated allergen-derived peptides encoding T-cell epitopes is a promising approach towards efficient AIT that lacks the risk of adverse effects associated with IgE-cross linking or inflammatory cell activation.

In line with our findings, studies unravelling the role of the Siglec-sialic acid pathway in several

cells residing in inflamed tissues, highlighted the essential role of its activation in the induction of tolerance as well as resolution of inflammation[86,87]. For example, Orgel *et al*. used the peanut allergen Ara h2 in a nanoparticle formulation completely covered with a high-affinity Siglec-2 ligand (CD22) to obtain B cell receptor and Siglec-2 binding simultaneously. These so-called, Siglec-engaging tolerance-inducing antigenic liposomes (STALs), were able to prevent peanut allergy in mice[88]. This antigen-specific approach in improved formulations of AIT hold great potential for future therapeutic interventions in food allergies as well as allergic asthma. However, this approach requires a more patient-tailored diagnosis and a specific panel of antigens the patient is sensitized to.

An easier approach to increase treatment efficacy, while avoiding use of crude extracts, would be to include naturally purified major allergens. In contrast to crude extracts, use of purified proteins allows for treatment with specific allergens in the absence of numerous non-protein constituents such as chitins, β-glycans and endotoxins, all of which can act on innate immune cells in a pro-inflammatory fashion, which might interfere with the tolerance-inducing capacity[89]. This is expected to enhance efficacy by more efficiently inducing a tolerogenic response due to the absence of TLR agonists during allergen administration, harnessing an immunoregulatory phenotype of the allergen-presenting cell upon SCIT[90]. Moreover, use of purified allergens in combination with a component-resolved diagnosis of sensitization patterns holds the promise of personalized intervention strategies[89]. HDM is the most prominent source of indoor exposure to allergens, and is a cause for allergic rhinitis and asthma[91]. The HDM species Der p has at least 23 major allergens that are thought to contribute to allergic sensitization through their proteolytic activity, activating cells of the innate immune system and priming an adaptive type-2 immune response[91,92]. In the MAS prospective birth cohort, sensitization patterns for HDM-allergens were studied into detail by component-resolved analysis[93]. Herein, spIgE for Der p1, 2 and 23 can be detected before sensitization to any of the other major allergens. Treatment options for HDM-allergy include allergen avoidance and AIT[94]. However, HDM extracts are variable in content of[95,96] and stability is limited[97]. Given the fact that sensitization to Der p1 and 2 identifies more than 95% of HDM-allergic individuals[91,96] and their causal role in early sensitization[93], SCIT with purified Der p1 and 2 might be a more attractive therapeutic approach compared to the use of HDM extracts.

In **Chapter 7**, we provide evidence that Der p1 and 2 can be used as a pharmacologically well-defined AIT in the HDM-sensitized host to suppress allergic responses, with superior activity compared to HDM-extract with regard to Th2 cell cytokines as well as the chemokines and alarmins released by the lung structural cells upon HDM exposure. HDM extract and purified allergens were equally effective in suppressing eosinophilic airway inflammation and AHR. We observed a reduction in HDM-induced CCL20 levels and a trend towards reduction in eotaxin, TARC and IL-33 levels in DerP1/2-SCIT mice, which might reflect a reduced activation status of the innate immune system. In agreement herewith, it has been reported that full extract-challenges in allergic asthma patients induced a stronger late allergic response compared to challenges with Der p1 and 2, while early responses were identical, which was attributed to non-protein constituents of the HDM extract [98].

Notwithstanding, using biochemically and pharmacologically defined products for AIT will increase quality of the treatment. Hence, we postulate that naturally purified antigen-formulations are at least as effective in suppressing the HDM-induced adaptive and innate response as whole body extracts, warranting translational studies to evaluate whether a similar approach is also efficacious in patients. These data warrant clinical studies to explore the safety and efficacy of the use of these purified natural allergens as a novel vaccine for HDM induced allergic disease, including rhinitis and allergic asthma.

CONCLUSIONS

Most clinical studied are observational by nature and the cellular and immunological mechanisms of AIT have often been investigated using preclinical murine models of allergic diseases. Animal models of allergic asthma should resemble to the human pathophysiology of allergic airway disease as close as possible, so that mechanistic insights obtained in the experimental models can be easily translated to the human situation. The aim of the research described in this thesis was to optimize and validate the use of a standardized animal model for allergic airway inflammation and therein test improved treatment regimens for AIT. This thesis describes findings based on a scientifically well-defined animal model with high translational value. Not only are we able to correctly interpret the readout parameters of inflammation, airway resistance and serological responses using crude allergen extracts in AIT treatment regimens, but also adjust formulations in two mouse models for allergic asthma (chapter 2). Moreover, we can directly compare the administration routes (chapter 3), test the use an adjuvant in both SCIT and SLIT (chapter 4 and 5), make new formulations (chapter 5), optimize peptide-AIT (chapter 6), and make the model completely allergen-independent to test naturally purified major allergens or any other AIT composition (chapter 7). Moreover, the book chapter on the experimental methods used (chapter 8) also gives other researchers the opportunity to apply this model for their specific research purposes. Despite the new insights described in this thesis, further research is needed to increase the treatment efficiency of AIT for allergic diseases.

FUTURE PERSPECTIVES

Although AIT has been used successfully for more than a century, patients with allergic airway diseases still have a lot to gain from improvements in AIT treatment, not only through a better quality of life and cheaper treatments with increased efficacy or safety and shorter treatment duration, but also from an earlier diagnosis and start of treatment. Current strategies to improve the treatment of allergic disorders using AIT are focused on several aspects including: (i) **prevention strategies**, allergen vaccination strategies to prevent progression from rhinitis to asthma or from mono-sensitization to multi-sensitization; (ii) strategies **for selection of patients** that will benefit most from AIT through the (identification of the) right **biomarkers**; (v) strategies to increase the **safety profile** and thereby the tolerability of the treatment regimens, that can be increased via purification, use of recombinant allergens and or allergen-derived peptides; (v) to improve treatment **efficacy** that can be enhanced via the use of adjuvantia, like CTLA-4 and VitD3; or via **antigen-targeting**, like

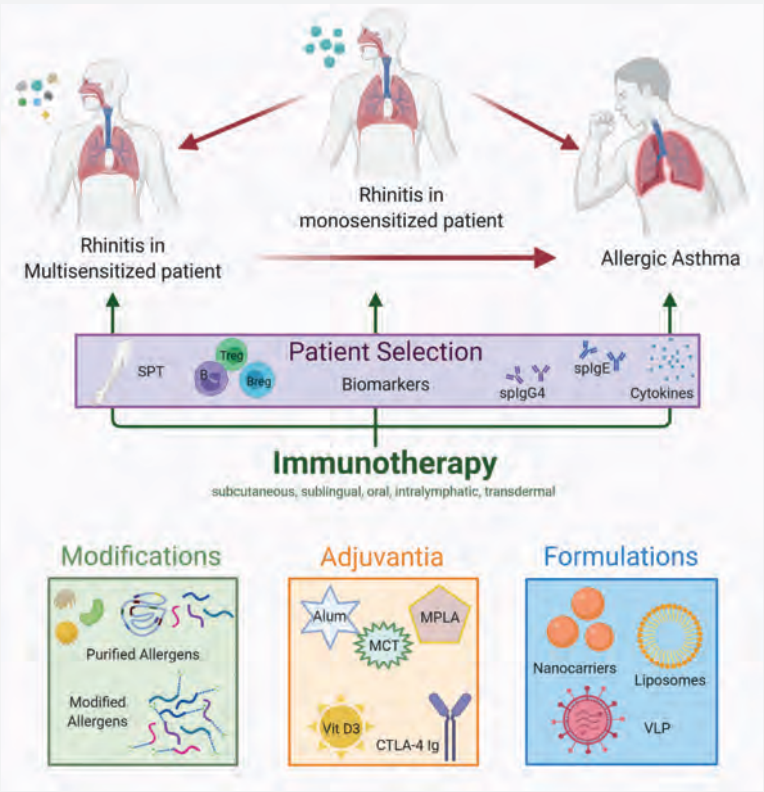

Figure 4. *Perspectives on precise diagnostics and inclusion, possibilities and strategies in the broad field of allergen immunotherapy. Abbreviations: SPT: Skin prick test; B: Basophil, Treg: T regulatory cell; Breg: B regulatory cell; sIgE: Specific Immunoglobulin E; sIgG4: specific Immunoglobulin 4; Alum: Aluminium hydroxide; MCT: microcrystalline tyrosine; MPLA: monophosphoryl lipid A; Vit D3: Vitamin D3; CTLA-4 Ig: Cytotoxic T lymphocyte-associated antigen-4-Ig; VLP: Virus like particles. Figure was created using BioRender.com.*

the use of glycated products; or via improved treatment formulation by using carriers, like liposomal formulated depots, that encapsulate allergens, whether or not supplemented with an adjuvants (Figure 4).

Before starting AIT, allergen avoidance is the most commonly used preventive approach that may help to prevent progression from rhinitis to asthma and multi-sensitization in mono-sensitized patients, and that can improve asthma control in patients with allergic asthma, including sending children to holiday homes and admission to a hospital. Although this strategy will allow relief of symptoms temporarily, studies showed that complete avoidance of allergenic triggers was impractical and unsuccessful in maintaining asthma control [99]. Besides, the main pollination period for the different tree and grass species spans over 6 months, from spring to autumn in Europe,

making allergen avoidance not a realistic approach to prevent progression to asthma or to multi-sensitization. An improved AIT treatment should therefore offer a realistic alternative to the 'allergen avoidance strategy'.

An improved AIT would be safe and efficacious. While technological advanced such as use of adjuvantia, formulations and targeted delivery can improve the efficacy of AIT, another approach to optimization of AIT treatment is patient selection and development of a precision AIT, with multiple possible treatment regimens that depend on the individual patient profile. Treatment of mono-sensitized patients suffering from upper airway symptoms versus multi-sensitized patients and/or those that have involvement of the lower airways likely requires different AIT treatment protocols (Figure 4). One approach would be to combine component-resolved diagnostics with a 'toolbox' of AIT peptides with decreased allergenicity and improved immunogenicity. In addition, other biomarkers than allergen-specific IgE are urgently needed that predict treatment response to AIT, further allowing an improved patient selection for AIT treatment.

Other approaches to develop novel, safe therapeutic allergy vaccines for use in AIT for HDM, GP, or any other allergen include the generation of **recombinant hypoallergenic combination vaccines**, which were shown to have limited IgE reactivity, whilst retaining its T cell epitopes and the ability to induce neutralizing antibody response in experimental models that could block IgE[94,100]. The use of these hypoallergenic recombinant vaccines holds the promise of inducing fewer side effects during therapy. For example, Der p1 peptides have also been delivered on virus-like particles, inducing IgG responses within 4 weeks after a single injection in healthy subjects[101]. These approaches involve the use of recombinant hypoallergenic proteins or peptides, while the use of purified natural proteins, with high purity and pharmacologically well defined, retains the capacity to crosslink IgE. Although hypoallergenic proteins are considered to have a better safety profile during treatment, is currently unknown whether hypoallergenic vaccines have a comparable therapeutic efficacy compared to IgE-activating allergens. Initial studies show that hypoallergenic proteins can induce neutralizing antibodies that inhibit allergen-mediated crosslinking of IgE[94,102]. IgE crosslinking vaccines might have some additional therapeutic efficacy due to so-called piecemeal degranulation of mast cells and basophils, which is thought to contribute to the immediate desensitization and protection against allergic responses especially during the early phase of treatment due to inactivation or exhaustion of these effector cells[102]. Further research will need to establish whether purified natural allergens, that can be produced in relatively high quantities under strictly controlled conditions at relatively low costs and address both effector cell responses, B and T cell activity, or recombinant hypoallergenic or peptide vaccines that require far higher productions costs and mainly address the T cell response, will be the most optimal treatment for AIT.

Prioritize on preventive strategies

The global increased prevalence of allergic airway diseases is, at least in part, explained by changes in our environment and lifestyle. Environmental changes that contribute to the increasing prevalence of allergic airway diseases may include greater international travel (air pollution) and

climate change[103]. Moreover in the last years, new adaptations to the *hygiene hypothesis* have placed emphasis on the potential depletion or reduction of our microbiome diversity (lung, gut and skin), which causes susceptibility to chronic inflammatory disease[104]. Such effects are thought to be enhanced by our 'Western' way of living, characterized by a decline in physical exercise, increased numbers of caesarian sections, increased use of antibiotics and structural changes in diet. Restoring the natural balance within our environment and actively combating climate change might, at least in part, actively curtail the allergic epidemic to aerosolized allergens. In line herewith, lifestyle changes could be tailored much more towards a more natural microbial exposure, including pre- and probiotic dietary supplements, like Vitamin D, antioxidants, folate as well as polyunsaturated fatty acids[105].

Especially in pediatrics, early onset allergic reactions, to for example cow milk and foods, can be the start of a natural progression of sensitizations and an important indicator for allergic disease progression in later life stages[106–110]. Identification of this susceptible group at an early stage of the so-called atopic march, might be an interesting strategy to select the right patient population suitable for prophylactic allergen vaccinations.

Despite all the optimization strategies currently in place for AIT treatment regimens, prevention of sensitization as well as progression of the disease remains the best strategy in the long term to reduce the ever increasing burden of the allergy epidemic. In theory, if prevention strategies worked, the remaining group with a specific sensitization pattern would be the ideal starting point for the development of well-designed, prospective, longitudinal studies with the ultimate goal of precision medicine for every unique patient.

References

1. Brompton, R., National, H., Service, H., Laboratories, M. C. & Uni-, M. Long-term clinical efficacy of grass-pollen immunotherapy. (1999).
2. Pfaar, O. *et al.* Perspectives in allergen immunotherapy: 2019 and beyond. *Allergy Eur. J. Allergy Clin. Immunol.* **74**, 3–25 (2019).
3. Jacobsen, L. *et al.* Specific immunotherapy has long-term preventive effect of seasonal and perennial asthma: 10-year follow-up on the PAT study. *Allergy* **62**, 943–8 (2007).
4. Van Oosterhout, A. J. M. *et al.* Allergen immunotherapy inhibits airway eosinophilia and hyperresponsiveness associated with decreased IL-4 production by lymphocytes in a Murine model of allergic asthma. *Am. J. Respir. Cell Mol. Biol.* **19**, 622–628 (1998).
5. Hellings, P. W. & Ceuppens, J. L. Mouse models of global airway allergy: What have we learned and what should we do next? *Allergy Eur. J. Allergy Clin. Immunol.* **59**, 914–919 (2004).
6. Herz, U., Renz, H. & Wiedermann, U. Animal models of type I allergy using recombinant allergens. *Methods* **32**, 271–280 (2004).
7. Mistrello, G., Roncarolo, D., Gentili, M., Zanoni, D. & Falagiani, P. Modified par j I allergen from P. judaica pollen and its rate of absorption in rats. *Immunol. Lett.* **40**, 31–36 (1994).
8. van Wilsem E, van Hoogstraten I, Brevé J, Zaman Y, K. G. In vivo antigen presentation capacity of dendritic cells from oral mucosa and skin draining lymph nodes. *Adv Exp Med Biol.* **355**, 63–67 (1994).
9. Brimnes, J., Kildsgaard, J., Jacobi, H. & Lund, K. Sublingual immunotherapy reduces allergic symptoms in a mouse model of rhinitis. *Clin. Exp. Allergy* **37**, 488–497 (2007).
10. Van Metre, T. E. *et al.* A controlled study of the effectiveness of the Rinkel method of immunotherapy for ragweed pollen hay fever. *J. Allergy Clin. Immunol.* **65**, 288–297 (1980).
11. Bousquet, J. *et al.* Allergen immunotherapy: Therapeutic vaccines for allergic diseases - A WHO position paper. *J. Allergy Clin. Immunol.* (1998). doi:10.1016/s0091-6749(98)70271-4
12. Demoly, P. & Calderon, M. A. Dosing and efficacy in specific immunotherapy. *Allergy Eur. J. Allergy Clin. Immunol.* **66**, 38–40 (2011).
13. Frew, A. J., Powell, R. J., Corrigan, C. J. & Durham, S. R. Efficacy and safety of specific immunotherapy with SQ allergen extract in treatment-resistant seasonal allergic rhinoconjunctivitis. *J. Allergy Clin. Immunol.* **117**, 319–325 (2006).
14. Dhami, S. *et al.* Allergen immunotherapy for allergic asthma: A systematic review and meta-analysis. *Allergy Eur. J. Allergy Clin. Immunol.* **72**, 1825–1848 (2017).
15. Dhami, S. *et al.* Allergen immunotherapy for allergic rhinoconjunctivitis: A systematic review and meta-analysis. *Allergy Eur. J. Allergy Clin. Immunol.* **72**, 1597–1631 (2017).
16. Kleine-Tebbe, J. *et al.* Negative clinical results from a randomised, double-blind, placebo-controlled trial evaluating the efficacy of two doses of immunologically enhanced, grass subcutaneous immunotherapy despite dose-dependent immunological response. *Clin. Drug Investig.* **34**, 577–586 (2014).
17. Klimek, L. *et al.* The effects of short-term immunotherapy using molecular standardized grass and rye allergens compared with symptomatic drug treatment on rhinoconjunctivitis symptoms, skin sensitivity, and specific nasal reactivity. *Otolaryngol. - Head Neck Surg.* **133**, 538–543 (2005).
18. Zenner, H. P. *et al.* Short-term immunotherapy: A prospective, randomized, double-blind, placebo-controlled multicenter study of molecular standardized grass and rye allergens in patients with grass pollen-induced allergic rhinitis. *J. Allergy Clin. Immunol.* **100**, 23–29 (1997).
19. Slovick, A. *et al.* Intradermal grass pollen immunotherapy increases TH2 and IgE responses and worsens respiratory allergic symptoms. *J. Allergy Clin. Immunol.* (2016). doi:10.1016/j.jaci.2016.09.024
20. Janssen, E. M. *et al.* Opposite effects of immunotherapy with ovalbumin and the immunodominant T-cell epitope on airway eosinophilia and hyperresponsiveness in a murine model of allergic asthma. *Am. J. Respir. Cell Mol. Biol.* **21**, 21–29 (1999).
21. Strait, R. T., Morris, S. C. & Finkelman, F. D. IgG-blocking antibodies inhibit IgE mediated anaphylaxis in vivo through both antigen interception and Fcgamma RIIb cross-linking. *J. Clin. Invest.* **116**, 833–841 (2006).
22. Finkelman, F. D. Anaphylaxis: Lessons from mouse models. *J. Allergy Clin. Immunol.* **120**, 506–515 (2007).

23. Smit, J. J. *et al.* Contribution of classic and alternative effector pathways in peanut-induced anaphylactic responses. *PLoS One* **6**, 1–12 (2011).
24. Marco-Martín, G. *et al.* Differences in the Anaphylactic Response between C3H/HeOuJ and BALB/c Mice. *Int. Arch. Allergy Immunol.* **173**, 204–212 (2017).
25. Novak, N. *et al.* Early suppression of basophil activation during allergen-specific immunotherapy by histamine receptor 2. *J. Allergy Clin. Immunol.* **130**, 2–9 (2012).
26. Klimek, L., Fox, G. C. & Thum-Oltmer, S. SCIT with a high-dose house dust mite allergoid is well tolerated: safety data from pooled clinical trials and more than 10 years of daily practice analyzed in different subgroups. *Allergo J. Int.* **27**, 131–139 (2018).
27. Tao, L., Shi, B., Shi, G. & Wan, H. Efficacy of sublingual immunotherapy for allergic asthma: Retrospective meta-analysis of randomized, double-blind and placebo-controlled trials. *Clin. Respir. J.* **8**, 192–205 (2014).
28. Pokladnikova, J., Krcmova, I. & Vlcek, J. Economic evaluation of sublingual vs subcutaneous allergen immunotherapy. *Ann. Allergy. Asthma Immunol.* **100**, 482–9 (2008).
29. Aasbjerg, K. *et al.* Immunological comparison of allergen immunotherapy tablet treatment and subcutaneous immunotherapy against grass allergy. *Clin. Exp. Allergy* **44**, 417–428 (2014).
30. Nelson, H. S. Subcutaneous immunotherapy versus sublingual immunotherapy: Which is more effective? *J. Allergy Clin. Immunol. Pract.* **2**, 144–149 (2014).
31. Dretzke, J. *et al.* Subcutaneous and sublingual immunotherapy for seasonal allergic rhinitis: a systematic review and indirect comparison. *J. Allergy Clin. Immunol.* **131**, 1361–6 (2013).
32. Durham, S. R. & Penagos, M. Sublingual or subcutaneous immunotherapy for allergic rhinitis? *J. Allergy Clin. Immunol.* **137**, 339–349 (2016).
33. Mösges, R. *et al.* Shortened up-dosing with sublingual immunotherapy drops containing tree allergens is well tolerated and elicits dose-dependent clinical effects during the first pollen season. *World Allergy Organ. J.* **12**, (2019).
34. Agache, I. *et al.* <scp>EAACI</scp> Guidelines on Allergen Immunotherapy: House dust mite- driven allergic asthma. *Allergy* **74**, 855–873 (2019).
35. Passalacqua, G., Bagnasco, D. & Canonica, G. W. 30 Years Of Sublingual Immunotherapy. *Allergy* 1–14 (2019). doi:10.1111/all.14113
36. Schulten, V. *et al.* Distinct modulation of allergic T cell responses by subcutaneous vs. sublingual allergen-specific immunotherapy. *Clin. Exp. Allergy* **46**, 439–448 (2016).
37. Van Rijt, L. S. *et al.* Birch pollen immunotherapy in mice: Inhibition of Th2 inflammation is not sufficient to decrease airway hyper-reactivity. *Int. Arch. Allergy Immunol.* **165**, 128–139 (2014).
38. Tofukuji, S. *et al.* Allergen-specific sublingual immunotherapy is dose and duration dependent in a murine allergic rhinitis model. *J. Allergy Clin. Immunol.* **142**, 1977-1979.e9 (2018).
39. Shima, K. *et al.* Effects of sublingual immunotherapy in a murine asthma model sensitized by intranasal administration of house dust mite extracts. *Allergol. Int.* **66**, 89–96 (2017).
40. Kildsgaard, J., Brimnes, J., Jacobi, H. & Lund, K. Sublingual immunotherapy in sensitized mice. *Ann. Allergy. Asthma Immunol.* **98**, 366–72 (2007).
41. Huoman, J. *et al.* Sublingual immunotherapy alters salivary IgA and systemic immune mediators in timothy allergic children. *Pediatr. Allergy Immunol.* **30**, 522–530 (2019).
42. van de Veen, W. & Akdis, M. Role of IgG4 in IgE-mediated allergic responses. *J. Allergy Clin. Immunol.* **138**, 1434–1435 (2016).
43. Senti, G. *et al.* Intralymphatic Immunotherapy: Update and Unmet Needs. *Int. Arch. Allergy Immunol.* **178**, 141–149 (2019).
44. Bach, D. *et al.* Transcutaneous immunotherapy via laser-generated micropores efficiently alleviates allergic asthma in Phl p 5-sensitized mice. *Allergy Eur. J. Allergy Clin. Immunol.* **67**, 1365–1374 (2012).
45. Hesse, L. *et al.* Subcutaneous immunotherapy with purified Der p1 and 2 suppresses type 2 immunity in a murine asthma model. *Allergy* **73**, 862–874 (2018).
46. Hesse L, Feenstra R, Ambrosini M, de Jager WA, Petersen AH, Vietor H, Unger WWJ, van Kooyk Y, N. M. Subcutaneous immunotherapy using modified Phl p5a - derived peptides efficiently alleviates allergic asthma in mice. *Allergy* **74**, 2495–2498 (2019).

47. Hesse L, Petersen AH, Oude Elberink JNG, van Oosterhout AJM, Nawijn MC. 1,25(OH)2VitD3 supplementation in subcutaneous and sublingual immunotherapy in a grass pollen driven mouse model of asthma augments beneficial effects. *Sci. Rep.* (2020).
48. Adorini, L., Giarratana, N. & Penna, G. Pharmacological induction of tolerogenic dendritic cells and regulatory T cells. *Semin. Immunol.* **16**, 127–34 (2004).
49. Vanherwegen, A. S. *et al.* Vitamin D controls the capacity of human dendritic cells to induce functional regulatory T cells by regulation of glucose metabolism. *J. Steroid Biochem. Mol. Biol.* **187**, 134–145 (2019).
50. Griffin, M. D. *et al.* Dendritic cell modulation by 1 ,25 dihydroxyvitamin D3 and its analogs: A vitamin D receptor-dependent pathway that promotes a persistent state of immaturity in vitro and in vivo. *Proc. Natl. Acad. Sci.* **98**, 6800–6805 (2002).
51. Pfeffer, P. E. & Hawrylowicz, C. M. Vitamin D in Asthma: Mechanisms of Action and Considerations for Clinical Trials. *Chest* **153**, 1229–1239 (2018).
52. Hollams, E. M. *et al.* Vitamin D over the first decade and susceptibility to childhood allergy and asthma. *J. Allergy Clin. Immunol.* **139**, 472–481 (2017).
53. Martineau, A. R. *et al.* Vitamin D supplementation to prevent acute respiratory tract infections: Systematic review and meta-analysis of individual participant data. *BMJ* **356**, (2017).
54. Wolsk, H. M. *et al.* Vitamin D supplementation in pregnancy, prenatal 25(OH)D levels, race, and subsequent asthma or recurrent wheeze in offspring: Secondary analyses from the Vitamin D Antenatal Asthma Reduction Trial. *J. Allergy Clin. Immunol.* **140**, 1423-1429.e5 (2017).
55. Jolliffe, D. A., Greenberg, L. & Hooper, R. L. Europe PMC Funders Group Vitamin D supplementation to prevent asthma exacerbations : a systematic review and meta-analysis of individual participant data. **5**, 881–890 (2018).
56. Colotta, F., Jansson, B. & Bonelli, F. Modulation of inflammatory and immune responses by vitamin D. *J. Autoimmun.* **85**, 78–97 (2017).
57. Hufnagl, K. & Jensen-Jarolim, E. Vitamin A and D in allergy: From experimental animal models and cellular studies to human disease. *Allergo J. Int.* **27**, 72–78 (2018).
58. Joudi, M. *et al.* Effects of Serum Vitamin D and Efficacy of Subcutaneous Immunotherapy in Adult Patients With Allergic Rhinitis. *Allergy Asthma Immunol Res.* **11**, 885–893 (2019).
59. Baris, S. *et al.* Vitamin D as an adjunct to subcutaneous allergen immunotherapy in asthmatic children sensitized to house dust mite. *Allergy* **69**, 246–53 (2014).
60. Jerzynska J, Stelmach W, Rychlik B, Lechańska J, Podlecka D, S. I. The clinical effect of vitamin D supplementation combined with grassspecific sublingual immunotherapy in children with allergic rhinitis. *Allergy Asthma Proc.* **37**, 105–114 (2016).
61. Taher, Y. A., van Esch, B. C. A. M., Hofman, G. A., Henricks, P. A. J. & van Oosterhout, A. J. M. 1 ,25-Dihydroxyvitamin D3 Potentiates the Beneficial Effects of Allergen Immunotherapy in a Mouse Model of Allergic Asthma: Role for IL-10 and TGF- . *J. Immunol.* **180**, 5211–5221 (2008).
62. Hesse, L. *et al.* Subcutaneous immunotherapy suppresses Th2 inflammation and induces neutralizing antibodies, but sublingual immunotherapy suppresses airway hyperresponsiveness in grass pollen mouse models for allergic asthma. *Clin. Exp. Allergy* **48**, 1035–1049 (2018).
63. Akbari, O., DeKruyff, R. H. & Umetsu, D. T. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nat. Immunol.* **2**, 725–731 (2001).
64. Weiner, H. L. The mucosal milieu creates tolerogenic dendritic cells and TR l and TH3 regulatory cells. *Nat. Immunol.* **2**, 671–672 (2001).
65. McMillan, S. J. & Lloyd, C. M. Prolonged allergen challenge in mice leads to persistent airway remodelling. *Clin. Exp. Allergy* **34**, 497–507 (2004).
66. Vasiliou, J. E. *et al.* Vitamin D deficiency induces Th2 skewing and eosinophilia in neonatal allergic airways disease. *Allergy Eur. J. Allergy Clin. Immunol.* **69**, 1380–1389 (2014).
67. Heine, G. *et al.* 25-Hydroxvitamin D3 Promotes the Long-Term Effect of Specific Immunotherapy in a Murine Allergy Model. *J. Immunol.* **193**, 1017–1023 (2014).
68. Petrarca, C. *et al.* Vitamin D3 improves the effects of low dose Der p 2 allergoid treatment in Der p 2 sensitized BALB/c mice. *Clin. Mol. Allergy* **14**, 1–10 (2016).

69. Anzaghe, M., Schülke, S. & Scheurer, S. Virus-Like Particles as Carrier Systems to Enhance Immunomodulation in Allergen Immunotherapy. *Curr. Allergy Asthma Rep.* **18**, 1–12 (2018).
70. Zubeldia, J. M., Ferrer, M., Dávila, I. & Justicia, J. L. Adjuvants in allergen-specific immunotherapy: Modulating and enhancing the immune response. *J. Investig. Allergol. Clin. Immunol.* **29**, 103–111 (2019).
71. Basomba, A. *et al.* Allergen vaccination with a liposome-encapsulated extract of Dermatophagoides pteronyssinus: A randomized, double-blind, placebo-controlled trial in asthmatic patients. *J. Allergy Clin. Immunol.* **109**, 943–948 (2002).
72. Chaisri, U. *et al.* Immunotherapeutic efficacy of liposome-encapsulated refined allergen vaccines against Dermatophagoides pteronyssinus allergy. *PLoS One* **12**, 1–20 (2017).
73. Endmann, A. *et al.* Combination of MIDGE-Th1 DNA vaccines with the cationic lipid SAINT-18: Studies on formulation, biodistribution and vector clearance. *Vaccine* **32**, 3460–3467 (2014).
74. Liu, Z. *et al.* Local nasal immunotherapy: Efficacy of dermatophagoides farinae-chitosan vaccine in murine asthma. *Int. Arch. Allergy Immunol.* **150**, 221–228 (2009).
75. Saint-Lu, N. *et al.* Targeting the allergen to oral dendritic cells with mucoadhesive chitosan particles enhances tolerance induction. *Allergy Eur. J. Allergy Clin. Immunol.* **64**, 1003–1013 (2009).
76. Hajavi, J., Hashemi, M. & Sankian, M. Evaluation of size and dose effects of rChe a 3 allergen loaded PLGA nanoparticles on modulation of Th2 immune responses by sublingual immunotherapy in mouse model of rhinitis allergic. *Int. J. Pharm.* **563**, 282–292 (2019).
77. Fifis, T. *et al.* Size-Dependent Immunogenicity: Therapeutic and Protective Properties of Nano-Vaccines against Tumors. *J. Immunol.* **173**, 3148–3154 (2004).
78. Cruz, L. J. *et al. Targeting nanoparticles to dendritic cells for immunotherapy*. *Methods in Enzymology* **509**, (Elsevier Inc., 2012).
79. Xiang, S. D. *et al.* Pathogen recognition and development of particulate vaccines: Does size matter? *Methods* **40**, 1–9 (2006).
80. Valenta, R., Campana, R., Focke-Tejkl, M. & Niederberger, V. Vaccine development for allergen-specific immunotherapy based on recombinant allergens and synthetic allergen peptides: Lessons from the past and novel mechanisms of action for the future. *J. Allergy Clin. Immunol.* **137**, 351–357 (2016).
81. Larché, M. Update on the current status of peptide immunotherapy. *J. Allergy Clin. Immunol.* **119**, 906–909 (2007).
82. Moldaver, D. M. *et al.* Induction of bystander tolerance and immune deviation after Fel d 1 peptide immunotherapy. *J. Allergy Clin. Immunol.* (2018). doi:10.1016/j.jaci.2018.03.023
83. Kettner, A. *et al.* Benefit of Bet v 1 contiguous overlapping peptide immunotherapy persists during first follow-up season. *J. Allergy Clin. Immunol.* **142**, 678-680.e7 (2018).
84. Mösges, R. *et al.* Lolium perenne peptide immunotherapy is well tolerated and elicits a protective B-cell response in seasonal allergic rhinitis patients. *Allergy Eur. J. Allergy Clin. Immunol.* **73**, 1254–1262 (2018).
85. Perdicchio, M. *et al.* Sialic acid-modified antigens impose tolerance via inhibition of T-cell proliferation and de novo induction of regulatory T cells. *Proc. Natl. Acad. Sci.* **113**, 3329–3334 (2016).
86. Bork, K., Horstkorte, R. & Weidemann, W. Increasing the sialylation of therapeutic glycoproteins: The potential of the sialic acid biosynthetic pathway. *J. Pharm. Sci.* **98**, 3499–3508 (2009).
87. Lübbers, J., Rodríguez, E. & Kooyk, Y. Van. Modulation of Immune Tolerance via Siglec-Sialic Acid Interactions. **9**, 1–13 (2018).
88. Orgel, K. *et al.* Exploiting CD22 on Antigen-Specific B-Cells to Prevent Allergy to the Major Peanut Allergen Ara h 2. *J Allergy Clin Immunol.* **139**, 366–369 (2017).
89. Marth, K., Focke-Tejkl, M., Lupinek, C., Valenta, R. & Niederberger, V. Allergen Peptides, Recombinant Allergens and Hypoallergens for Allergen-Specific Immunotherapy. *Curr. Treat. options allergy* **1**, 91–106 (2014).
90. Hoffmann, H. J. *et al.* Novel Approaches and Perspectives in Allergen Immunotherapy. *Allergy* **72**, 1022–1034 (2017).
91. Tourdot, S. *et al.* Evaluation of therapeutic sublingual vaccines in a murine model of chronic house dust mite allergic airway inflammation. *Clin. Exp. Allergy* 1784–1792 (2011). doi:10.1111/j.1365-2222.2011.03865.x
92. Hammad, H. & Lambrecht, B. N. Barrier Epithelial Cells and the Control of Type 2 Immunity. *Immunity* **43**,

29–40 (2015).
93. Posa, D. *et al.* Evolution and predictive value of IgE responses toward a comprehensive panel of house dust mite allergens during the first 2 decades of life. *J. Allergy Clin. Immunol.* **139**, 541-549.e8 (2016).
94. Chen, K. W. *et al.* Hypoallergenic der p 1/Der p 2 combination vaccines for immunotherapy of house dust mite allergy. *J. Allergy Clin. Immunol.* **130**, (2012).
95. Valenta, R. *et al.* From Allergen Genes to Allergy Vaccines. (2010). doi:10.1146/annurev-immunol-030409-101218
96. Pittner, G. *et al.* Component-resolved diagnosis of house-dust mite allergy with purified natural and recombinant mite allergens. *Clin. Exp. Allergy* **34**, 597–603 (2004).
97. Vidal-Quist, J. C., Ortego, F., Castañera, P. & Hernández-Crespo, P. Quality control of house dust mite extracts by broad-spectrum profiling of allergen-related enzymatic activities. *Allergy Eur. J. Allergy Clin. Immunol.* **72**, 425–434 (2016).
98. Van Der Veen, M. J., Jansen, H. M., Aalberse, R. C. & Van Der Zee, J. S. Der p 1 and Der p 2 induce less severe late asthmatic responses than native Dermatophagoides pteronyssinus extract after a similar early asthmatic response. *Clin. Exp. Allergy* **31**, 705–714 (2001).
99. De Blay, F., Barnig, C. & Ott, M. House dust mite control measures for asthma. *Allergy Eur. J. Allergy Clin. Immunol.* **64**, 1404 (2009).
100. Asturias, J. A. *et al.* Engineering of major house dust mite allergens der p 1 and der p 2 for allergen-specific immunotherapy. *Clin. Exp. Allergy* **39**, 1088–1098 (2009).
101. Kundig, T. M. *et al.* Der p 1 peptide on virus-like particles is safe and highly immunogenic in healthy adults. *J. Allergy Clin. Immunol.* **117**, 1470–1476 (2006).
102. Akdis, C. A. & Akdis, M. Mechanisms of allergen-specific immunotherapy. *J. Allergy Clin. Immunol.* **127**, 18–27 (2011).
103. D'Amato, G. *et al.* Climate change, allergy and asthma, and the role of tropical forests. *World Allergy Organ. J.* **10**, 1–8 (2017).
104. Lambrecht, B. N. & Hammad, H. The immunology of the allergy epidemic and the hygiene hypothesis. *Nat. Immunol.* **18**, 1076–1083 (2017).
105. Prescott, S. & Nowak-Wgrzyn, A. Strategies to prevent or reduce allergic disease. *Ann. Nutr. Metab.* **59**, 28–42 (2011).
106. Crevel, R. W. R. & Pickup, J. Too clean , or not too clean : the Hygiene Hypothesis and home hygiene Clinical and Experimental Allergy. *Clin. Exp. Allergy* **36**, 402–425 (2006).
107. Kim, B. J., Lee, S. Y., Kim, H. Bin, Lee, E. & Hong, S. J. Environmental changes, microbiota, and allergic diseases. *Allergy, Asthma Immunol. Res.* **6**, 389–400 (2014).
108. Risk, R. *et al.* Specific Patterns of Allergic Sensitization in Early Childhood and Asthma & Rhinitis Risk. *Clin Exp Allergy.* **43**, 233–241 (2013).
109. Maciag, M. & Phipatanakul, W. Preventing the development of asthma stopping the allergic march. *Curr Opin Allergy Clin Immunol.* **19**, 161–168 (2019).
110. Pfaar, O. *et al.* Perspectives in allergen immunotherapy: 2019 and beyond. *Allergy Eur. J. Allergy Clin. Immunol.* **74**, 3–25 (2019).