Recombinant Allergens in Structural Biology, Diagnosis, and Immunotherapy

Angelika Tscheppe Heimo Breiteneder

Department of Pathophysiology and Allergy Research, Medical University of Vienna, Vienna, Austria

Keywords

Recombinant allergens · Structural biology · Allergy diagnosis · Vaccine development · Allergy immunotherapy · Clinical studies

Abstract

The years 1988–1995 witnessed the beginning of allergen cloning and the generation of recombinant allergens, which opened up new avenues for the diagnosis and research of human allergic diseases. Most crystal and solution structures of allergens have been obtained using recombinant allergens. Structural information on allergens allows insights into their evolutionary biology, illustrates clinically observed cross-reactivities, and makes the design of hypoallergenic derivatives for allergy vaccines possible. Recombinant allergens are widely used in molecule-based allergy diagnosis such as protein microarrays or suspension arrays. Recombinant technologies have been used to produce well-characterized, noncontaminated vaccine components with known biological activities including a variety of allergen derivatives with reduced IgE reactivity. Such recombinant hypoallergens as well as wild-type recombinant allergens have been used successfully in several immunotherapy trials for more than a decade to treat birch and grass pollen allergy.

As a more recent application, the development of antibody repertoires directed against conformational epitopes during immunotherapy has been monitored by recombinant allergen chimeras. Although much progress has been made, the number and quality of recombinant allergens will undoubtedly increase and keep improving our knowledge in basic scientific investigations, diagnosis, and therapy of human allergic diseases.

© 2017 The Author(s)
Published by S. Karger AG, Basel

Introduction

The history of recombinant allergens started with the very first publications on allergen cloning. In January 1988, the cDNA sequence coding for the major house dust mite allergen Der p 1 was discovered in Australia and published as the first complete allergen sequence worldwide [1]. Interestingly, the publication of the identification of the λ gt11 clone expressing Der p 1 was only published later that year [2]. The sequence of a major allergen of the white-faced hornet *Dolichovespula maculata*, then named antigen 5 and now designated as Dol m 5, was cloned in February 1988 in New York City and published by Fang et al. [3]. The following year witnessed the dis-

covery of the cDNA of the very first plant allergen, the major birch pollen allergen Bet v 1, in Austria, which was published in 1989 [4]. What these 3 allergens have in common is their expression in the bacterium *Escherichia coli* following the infection by λ gt11 phages. Hence, they can justifiably be regarded as the first recombinant allergens.

So the era of recombinant allergens began in 1988. Der p 1 was identified by screening the λ gt11 cDNA expression library with a rabbit anti-Der p 1 antiserum [2]. Dol m 5 was discovered by screening a λ gt11 cDNA expression library with hornet antigen 5-specific mouse sera [3]. Bet v 1 was the first recombinant allergen that was identified by screening the expression library with IgE from sera of birch pollen-allergic patients [4]. Previously, Bet v 1 had been expressed in a cell-free wheat germ system in vitro and identified using sera from patients allergic to birch pollen [5]. Again, in 1989, the second plant allergen, Dac g 2 from cocksfoot grass pollen, was identified by screening a λ gt11 expression library with sera obtained from cocksfoot grass pollen-allergic individuals [6]. The first fungal allergen, Asp f 1 from *Aspergillus fumigatus*, was published in 1990 [7], the first animal allergen, Fel d 1 from the domestic cat, in 1991 [8], and the first plant food allergen, Ara h 1 from peanut, in 1995 [9].

Recombinant Allergens in Structural Biology

Almost all crystal and solution structures of allergens have been obtained from recombinant allergens. There are 2 reasons for this: (1) most allergens occur in the allergen source as a mixture of several isoforms that are very difficult to separate and (2) some allergens are present at very low concentrations, making it impossible to obtain a sufficient quantity of the purified allergen for crystallization. Proteomic profiling or characterization by 2-dimensional electrophoresis has shown the presence of allergen isoforms in all analyzed allergen sources including birch pollen [10], Timothy grass pollen [11], *Parietaria judaica* pollen [12], peanut [13], house dust mite [14], fire ant [15], and cow's milk [16]. The recombinant production of allergens allows the deconvolution of isoform mixtures and the crystallization of individual isoforms. This approach has yielded the crystal structures of 3 Bet v 1 isoforms, Bet v 1.0101 (former designation Bet v 1a, PDB 4A88) [17], Bet v 1.0106 (Bet v 1j, 4A8U) [17], and Bet v 1.0107 (Bet v 1l, 1FM4) [18].

The Structural Database of Allergens (SDAP, <https://fermi.utmb.edu/>) is a repository of allergenic proteins

and various computational tools that can assist structural biology studies related to allergens. SDAP provides a list of and links to 92 allergen structures that are included in the Protein Data Bank (PDB, www.rcsb.org/pdb/). SDAP also provides 458 models for allergen and isoallergen structures. In their 2014 paper, Dall'Antonia et al. [19] presented 103 structures of allergens from the PDB. Structural information on allergens is helpful in reconstructing the evolutionary history of protein architectures where amino acid sequence comparisons fail to reveal sequence similarities. Thus, a large superfamily of structurally related proteins that are all based on the Bet v 1 fold could be arranged into the Bet v 1 superfamily [20]. The presence of Bet v-like molecules in Archaea such as *Aeropyrum pernix* [21] indicates that the Bet v 1 fold originated in the very early stages of life on earth. Likewise, the origin of the cupin superfamily which harbors many allergenic seed storage proteins, e.g., Ara h 1 from peanut, can be traced back to the Archaea based on available crystal structures [22]. AllFam (<http://www.meduniwien.ac.at/allfam/>), the recently updated database of allergen families, is built around the membership of allergens to protein families [23]. AllFam groups allergens according to the classification of the protein family database Pfam (<http://pfam.xfam.org/>). The September 2016 version of AllFam contains 1,018 allergens, 939 of which can be assigned to 1 of 216 Pfam families. This number corresponds to 1.3% of the 16,306 entries contained in Pfam v30.0, illustrating the highly limited distribution of allergens into protein families.

It is important to note that the vast majority of proteins in an allergen-containing family are nonallergenic. Cross-reactivity occurs almost exclusively between allergens of the same family and spans the spectrum from high (e.g., profilins, polcalcins, and parvalbumins) to medium (e.g., PR-10 and nsLTPs) and low cross-reactivity (e.g., cupins, 2S albumins, and lipocalins).

Table 1 shows ribbon representations of selected allergens from the protein superfamilies or families that contain important allergens. Proteins with a long evolutionary past are distributed over a wide range of species, e.g., the members of the Bet v 1 family, the cupin superfamily, and the lipocalin family. Other types of allergens are more restricted in their distribution, e.g., parvalbumins are restricted to fish, polcalcins to pollen, and β -expansins to grass pollen.

The available crystal and solution structures of recombinant allergens illustrate the clinically observed cross-reactivity even between distantly related allergens, as is the case for the kiwi allergen Act d 11 and also Bet v 1

Table 1. Structures of selected allergens from prominent allergen families

Prolamin superfamily: Nonspecific lipid transfer proteins and 2S albumins

Peach Pru p 3 (2ALG) nsLTP	Hazelnut Cor a 8 (4XUW) nsLTP	Peanut Ara h 2 (3OB4) 2S albumin	Brazil nut Ber e 1 (2LVF) 2S albumin
EF-hand family*: Parvalbumins and polcalcins

Carp Cyp c 1	Cod Gad m 1 (2MBX)	Iambsquatrers pollen Che a 3 dimer (2OPO)	Timothy grass pollen Phl p 7 dimer (1K9U)
Cupin superfamily: vicilins and legumins

Peanut Ara h 1 (3SMH) trimer	Soy Gly m 5 (1IPK) trimer	Peanut Ara h 3 (3C3V) subunit of hexamer	Soy Gly m 6 (1OD5) subunit of hexamer
Bet v 1 family

Birch Bet v 1 (4A88)	Celery Api g 1 (2BK0)	Peanut Ara h 8 (4M9B)	Kiwi Act d 11 (4IHR)
Lipocalin family

Cow Bos d 2 (1BJ7)	Horse Equ c 1 (1EW3)	Dog Can f 2 (3L4R)	Cockroach Per a 4 (3EBW)
Expansin and expansin-like family

Timothy grass pollen Phl p 1 (1N10)	Timothy grass pollen Phl p 2 (1WHO), C- terminal expansin domain	Maize pollen Zea m 1 (2HCZ)	Kiwi fruit Act d 5 (4X9U), N-terminal expansin domain

Numbers in parentheses are Protein Data Bank (PDB; <http://www.rcsb.org/pdb/home/home.do>) accession numbers.

* Bound calcium ions are shown as grey spheres. The ribbon models of the allergens were created with the molecular modeling system UCSF Chimera (<http://www.cgl.ucsf.edu/chimera>).

[24]. Structural information obtained from recombinant allergens together with IgE cross-reactivity data can be combined to predict conformational epitopes in the absence of allergen-antibody complexes [25]. These predicted epitopes offer a useful starting point for introducing mutations or structural changes into known allergens in an effort to create allergen derivatives as candidate molecules for allergen-specific immunotherapy (AIT). Structural homologs of allergens can also be used as scaffolds for epitope grafting, whereby the individual surface areas of allergens are transplanted onto low or nonallergenic members of the same protein (super)family. Such chimeric molecules were produced by grafting surface areas of Bet v 1 onto either a structurally related protein from celery [26] or meadow rue [27]. The norcoclaurine synthase from meadow rue was further used as a scaffold for presenting conformational epitopes of the soybean allergen Gly m 4, in an effort to characterize its IgE epitope profile [28].

Recombinant Allergens in Diagnostic Tests

Although diagnostic assays based on purified recombinant allergens are spreading fast, extracts from natural allergen sources are still in wide use. The composition of an allergenic extract strongly affects the results of any extract-based IgE assay. Commercially available extracts may lack important allergens or may vary considerably in their allergen composition, leading to false-negative test results for certain patients. Varying allergen compositions and contents have been shown for skin prick test (SPT) solutions for the house dust mite *Dermatophagoides pteronyssinus* [29], the mould *Alternaria alternata* [30], and hazelnut [31] as well as for sublingual allergen immunotherapy products for house dust mite, storage mite, and birch pollen [32, 33]. At least for routine diagnostics, a process called spiking (i.e., the addition of recombinant allergens) has been used to improve the quality of allergenic extracts such as latex [34], hazelnut [35], and wasp venom [36]. Moreover, recombinant allergens represent an alternative to natural allergens for allergy diagnosis when the natural allergen can only be isolated in low quantities from the original source [37].

Molecule-based allergy diagnosis uses purified natural and recombinant allergens to elucidate the sensitization patterns of a patient at the molecular level [38]. This procedure can (i) increase the diagnostic accuracy, (ii) distinguish genuine sensitization from sensitization due to cross-reactivity, (iii) help to assess the risk and type of al-

lergic reaction, and (iv) facilitate the selection of eligible patients and suitable allergens for allergen-specific immunotherapy [39].

Today, singleplex (1 assay/serum sample) and multiplex (multiple assays/serum sample) platforms for measuring sIgE antibodies to allergens are available. Singleplex assays allow testing for the presence of IgE specific to only those allergens that are indicated by the patient's clinical history. In contrast, a multiplex platform allows the definition of an individual's IgE response to the whole spectrum of allergens arrayed on a chip. Thus, the multiplex microarray approach often measures the presence of specific (s)IgE to allergens not indicated by the patient's history. This results in the generation of IgE antibody data that can be inconsistent with the patient's history and difficult to explain to the patient.

Screening large populations by allergen microarrays may also produce unexpected results. Panzner et al. [40] could detect sensitizations patterns to allergen sources which were not present in the study area. The microarrayed allergen approach which facilitated the measurement of sIgE to a large number of allergenic proteins using a small volume of serum was first introduced in 2002 by Hiller et al. [41]. VBC Genomics developed the allergen microarray technology which was then advanced into the commercial product ImmunoCAP Immuno Solid-Phase Allergen Chip (ISAC) by ThermoFisher Scientific. Further development of the ISAC was funded by the European Union (EU) project MeDALL to increase the original number of allergens from 112 to 176, 127 of which are recombinant allergens [42]. The ISAC makes it possible to semiquantitatively test for sensitization to 112 allergens, providing, as is sometimes argued, too much information. Incorvaia et al. [43] caution against basing an allergy diagnosis on ISAC data alone.

In addition to ThermoFisher Scientific's ISAC, several other companies offer systems to test for the presence of allergen-specific IgE in patients' sera. These companies include Siemens with their Immulite immunoassay system and HYCOR with their HYTEC 288 Plus system. The technological advancements for in vitro allergy diagnosis do not stop there [44]. Small-scale suspension arrays for a small number of purified recombinant and natural allergens have been developed on the Luminex xMAP® platform [45] and on the Becton Dickinson cytometric bead array system [46]. Indoor Biotechnology produces an IgE quantitative binding array for the simultaneous detection and quantitative determination of total and allergen-specific human IgE against 11 allergens. Kühne et al. [47] demonstrated, in a proof-of-concept study, that

Table 2. Mutational strategies to produce recombinant hypoallergens

Strategy	Definition	Allergen source	Molecule/s	Ref.
Fragmentation	The cDNA coding for the allergen is fragmented into ≥ 2 parts; the fragments may overlap; the fragments are expressed individually	birch pollen	Bet v 1	58, 59
		cow dander	Bos d 2	60
		storage mite <i>L. destructor</i>	Lep d 2	61
		Timothy grass pollen	Phl p 1	62
		house dust mite <i>D. pteronyssinus</i>	Der p 2	63
Oligomerization	Two or more copies of the allergen-encoding cDNA are linked by short oligonucleotide spacers with an open reading frame; the complete construct is expressed	birch pollen	Bet v 1	64
Mosaics	The allergen-encoding cDNA is fragmented into several parts and the fragments are re-joined in an order different from the original sequence; if the sequence parts originate from >1 allergen, the resulting protein is regarded as a hybrid mosaic	Timothy grass pollen	Phl p 2 Phl p 1	66 67
		birch pollen	Bet v 1	68
		cat	Fel d 1	69
Chimeras/allergen hybrids	Chimeric proteins or hybrid proteins are created by joining the genetic information of at least 2 different proteins; such constructs may contain parts of or the complete original proteins	house dust mite <i>D. pteronyssinus</i>	Der p 1, Der p 2	71, 72
		Timothy grass pollen	Phl p 2, Phl p 6 Phl p 1, Phl p 2, Phl p 5, Phl p 6	73 74
		yellowjacket, paper wasp	Ves v 5, Pol a 5	75
		honey bee	Api m 1, Api m 2 Api m 3	76
		Japanese cedar	Cry j 1, Cry j 2	77
Point mutations	One or more nucleotide triplets coding for a specific amino acid is/are altered to replace the original amino acid at its exact position by an amino acid with different physicochemical characteristics	birch pollen	Bet v 1 Bet v 4	79, 101 83
		<i>Brassica rapa</i> pollen	Bra r 1	84
		carp	Cyp c 1	82
		<i>P. judaica</i> pollen	Par j 1	85
		latex <i>H. brasiliensis</i>	Hev b 6.02	86, 98
		house dust mite <i>D. pteronyssinus</i>	Der p 2	87
		peanut	Ara h 1, Ara h 2, Ara h 3	94–96
		latex <i>H. brasiliensis</i>	Hev b 5	97
		ryegrass pollen	Lol p 5	99
		egg white	Gal d 1	100

multi-peptide microarrays of the peanut allergen Ara h 1 and all 3 subunits of the soybean allergen Gly m 5 could be used for large-scale screening of IgE epitopes of food allergens.

In response to the need of allergologists for guidance and background information on allergen molecule-based diagnostics, the European Academy of Allergy and Clinical Immunology (EAACI) has published the Molecular Allergy User's Guide, compiled by a panel of 65 ex-

perts in the field [48]. The EAACI regards the need to learn to interpret "allergomics" data as a challenge for practicing allergologists. The list of important allergenic molecules used in the microarray format is still incomplete. Recently, Becker et al. [49] demonstrated that Der p 23 was a new important house dust mite allergen which could contribute to the clarification of perennial allergic symptoms when the known major house dust mite allergens Der p 1, Der p 2, and Der p 10 test negatively. This

importance of Der p 23 was confirmed by a recent study in Thailand, where 54% of 222 house dust mite-allergic individuals displayed Der p 23-specific IgE responses [50]. The Ani s 11-like protein, Ani s 11.0201, a recently discovered new major allergen of the fish parasite *Anisakis simplex* was shown to be recognized by 78% of the patients in the study group while 13.5% only detected the Ani s 11-like allergen [51]. It is predicted that in vitro IgE assays based on allergenic extracts or purified allergens will coexist in clinical practice for quite a while. Hence, the EAACI suggests combining the results from SPTs, IgE assays using allergenic extracts, and molecular diagnosis with the clinical history of the patient, in order to provide the best possible allergy diagnosis for the patient.

Vaccine Development

The first AIT trial was reported in 1911 by Leonard Noon [52], who injected grass pollen extracts subcutaneously into grass pollen-allergic patients and thus achieved a reduction in allergic symptoms. Since then, several clinical trials have shown that AIT is able to modify allergic diseases and can produce long-lasting effects in treated patients [53–55]. However, natural allergen extracts often show great variations in allergen content, may lack important allergens, and may be contaminated with allergens from other sources [29, 56, 57]. In contrast, recombinant technologies allow the production of well-characterized, noncontaminated vaccine components of known biologic activity. Moreover, genetic engineering allows the modification of wild-type allergens to generate allergen derivatives with reduced IgE reactivity (“hypoallergens”), a reduced risk of triggering undesirable allergic reactions during the course of AIT, and a retained immunogenic activity. Modifications that are used to destroy conformation-dependent B-cell epitopes, while conserving T-cell epitopes, include the fragmentation or fusion of molecules, point mutations and deletions, and the formation of chimeras and mosaics. Table 2 gives an overview of the most commonly applied methods of producing hypoallergens.

Recombinant Allergen Fragments

The IgE antibody response to respiratory allergens is mainly directed to conformational epitopes. Hence, the disruption of the 3-dimensional structure by separately expressing fragments of the allergen can reduce or abolish the allergen’s IgE reactivity. The production of allergen-derived fragments was first described for the major birch pollen allergen Bet v 1 by Vrtala et al. [58, 59]. Two parts

of Bet v 1, representing amino acid residues 1–74 and 75–160, were expressed in *E. coli*. The 2 recombinant fragments showed almost no IgE-binding activity and exhibited random coil conformation. Both fragments induced the proliferation of human Bet v 1-specific T cell clones. Two overlapping recombinant fragments (amino acid residues 1–131 and 81–172) of the respiratory allergen Bos d 2 from cow dander showed only a low level of residual IgE reactivity but vigorously stimulated Bos d 2-specific T-cell clones [60]. Six fragments of the major allergen Lep d 2 of the storage mite *Lepidoglyphus destructor* displayed only weak IgE binding, indicating that Lep d 2 does not possess dominant linear B-cell epitopes [61]. When fragmentation of an allergen is taken to the extreme, IgE haptens are the result. An expression cDNA library was constructed from a randomly fragmented cDNA coding for the major Timothy grass pollen allergen Phl p 1 [62]. An immunodominant 15mer peptide was identified with IgE from allergic patients; it bound IgE from 30% of grass pollen-allergic patients but did not induce basophil histamine release in most patients. Two recombinant fragments of the major house dust mite allergen Der p 2 (residues 1–53 and 54–129) displayed a >10-fold reduction in allergenic activity, shown by a basophil activation assay and an SPT [63].

Recombinant Allergen Oligomers

The concept of allergen oligomerization was realized by expressing 3 copies of the Bet v 1 cDNA linked by short oligonucleotide spacers with an open reading frame in *E. coli* [64]. The recombinant (r)Bet v 1 trimer contained Bet v 1-specific IgE and IgG as well as T-cell epitopes, but it exhibited a profoundly reduced allergenic activity. This could be explained by microaggregation, steric hindrance, and/or unfavorable charge interactions that hid some of the IgE epitopes required for effective cross-linking. The rBet v 1 trimer induced IgG antibodies in mice and rabbits that blocked human IgE binding to Bet v 1, Aln g 1, and Mal d 1. It was later found that the rBet v 1 trimer formed high-molecular-weight aggregates with an altered presentation of IgE epitopes to effector cell-bound IgE [65].

Recombinant Allergen Mosaics

Mosaic proteins created by genetic engineering are defined as artificial recombinant proteins designed from a set of peptides that are found in the reference protein(s). If the peptides originate from ≥ 2 proteins, then the resulting protein is regarded as a hybrid mosaic (see below). Mosaic allergens are constructed by reassembling the peptides derived from the sequence of the wild-type al-

Table 3. Recombinant allergens and recombinant allergen derivatives used in AIT studies

Allergen source	Vaccine component	Molecule	Intervention	Clinical study type	Patients, <i>n</i>	Year	Ref.
Birch pollen	recombinant WT protein, fragments, dimer, and trimer	rBet v 1, 2 rBet v 1 fragments, (rBet v1) ₂ , (rBet v1) ₃	SPT, intradermal injections	Phase 0	29/30	2000	107
Birch pollen	recombinant fragments and trimer	2 rBet v 1 fragments, (rBet v1) ₃	subcutaneous injection	Phase I safety and tolerability	124 in 3 groups	2004	108
Birch pollen	recombinant WT protein	rBet v 1, nBet v 1, birch pollen extract	subcutaneous injection	Phase II safety and efficacy	134 in 4 groups	2008	111
Birch pollen	recombinant WT protein	rBet v 1	sublingual tablet	Phase II safety and efficacy	485 in 4 groups	2015	112
Birch pollen	recombinant WT protein folding variant	rBet v 1-FV	subcutaneous injection	Phase I safety	37 in 4 groups	2013	114
Birch pollen	recombinant WT protein folding variant	rBet v 1-FV, birch pollen extract	subcutaneous injection	Phase II	51 in 2 groups	2015	115
Timothy grass pollen	recombinant WT protein	mixture of rPhl p 1, rPhl p 2, rPhl p 5a, rPhl p 5b, rPhl p 6	subcutaneous injection	Phase I	62	2005	116
Timothy grass pollen	recombinant WT protein	mixture of rPhl p 1, rPhl p 2, rPhl p 5a, rPhl p 5b, rPhl p 6	subcutaneous injection	Phase II	50 in 5 groups	2012	117
Timothy grass pollen	recombinant fusion proteins of HBV surface protein preS domain and allergen-derived B-cell epitope peptides	peptides derived from Phl p 1, Phl 2, Phl p 5, Phl p 6	subcutaneous injection	Phase II	71 in 4 groups	2016	118
Cat	recombinant fusion protein	rMAT-Fel d 1	intralymphatic injection	Phase I	20 in 2 groups	2012	121
Peanut	recombinant modified proteins	modified rAra h 1, 2, and 3 in <i>E. coli</i> cells	rectal administration	Phase 0	10	2013	122
Carp	recombinant hypoallergen	modified rCyp c 1	subcutaneous injection	Phase I/IIa	16	2016	124

WT, wild-type; FV, folding variant; r, recombinant; n, natural.

lergen to produce molecules that lack IgE binding due to changes in their 3-dimensional structure. The major allergen Phl p 2 from Timothy grass pollen was converted into a mosaic by reassembling its peptides in a changed order [66]. The recombinant mosaic protein had lost its IgE reactivity but induced high levels of IgG in mice and rabbits that inhibited IgE binding from allergic patients to group 2 allergens from Timothy grass and other grass species. Similarly, a rearranged mosaic of Phl p 1 displayed a reduction in IgE reactivity >85% and induced inhibitory IgG in immunized rabbits [67]. Three recombinant mosaic proteins were derived from Bet v 1 and displayed a strongly reduced IgE reactivity and allergenic activity [68]. The immunological properties of the de-

signed mosaic proteins cannot always be predicted. Only 3 out of 7 mosaic proteins derived from the major cat allergen Fel d 1 possessed the desired hypoallergenic characteristics [69].

Recombinant Allergen Chimeras/Allergen Hybrids

Chimeric proteins or hybrid proteins are created by joining the genetic information of at least 2 different proteins. Such constructs may contain parts of or the complete original proteins. Hybrid molecules are ideal for producing vaccine candidates for allergen sources that contain several important allergens, such as house dust mite, grass pollen, or insect venoms. Hybrid molecules increase the immunogenicity and reduce the number of

molecules that need to be produced and quality-checked [70]. Asturias et al. [71] produced a hybrid molecule derived from the house dust mite allergens Der p 1 and Der p 2 which had a significantly lower potency to induce reactions in SPTs than the individual allergens, and even induced higher T cell proliferation responses than an equimolar mixture of Der p 1 and Der p 2. Chen et al. [72] engineered hybrids from Der p 1 and Der p 2 and also replaced the cysteines with serines, showing that these molecules could be used safely for both tolerance and vaccination approaches. Linhart et al. [73] produced hybrid mosaic hypoallergens derived from the Timothy grass pollen allergens Phl p 2 and 6, and then, in another study [74], from Phl p 1, 2, 5, and 6. In both cases, the hybrid molecules displayed an increased immunogenicity by inducing higher allergen-specific IgG titers in immunized animals than a mixture of the wild-type allergens. The homologous venom allergens Ves v 5 from the yellow-jacket and Pol a 5 from the paper wasp have only low degrees of cross-reactivity. Hybrids containing different segments of these allergens can therefore be used to map discontinuous B-cell epitopes but have also been shown to be useful for immunotherapy due to their reduction in allergenicity [75]. Karamloo et al. [76] could show that a hybrid consisting of fragmented sequences of the 3 bee venom major allergens Api m 1, 2, and 3 had preserved T-cell epitopes, had lost the B cell epitopes of all 3 allergens, and possessed a highly reduced IgE cross-linking ability on human mast cells and basophils as well as a strongly reduced skin test reactivity. The 2 major allergens of Japanese cedar (*Cryptomeria japonica*) pollen, Cry j 1 and Cry j 2, were expressed as a fusion protein and then conjugated to polyethylene glycol to improve their solubility and to create a safer vaccine [77]. Treatment of mice and monkeys with this vaccine resulted in a significant increase of Cry j 1-specific IgG. In general, allergen fusions allow the production of multiallergen vaccine components as a single molecule, thus facilitating their manufacture, purification, and characterization [78].

Recombinant Allergens Modified by Mutations

Genetic engineering of genes coding for allergens requires the knowledge of their B- and T-cell epitopes and, ideally, also their 3-dimensional structure. Thus, mutations of an allergen sequence can be used to alter its functions and immunological properties.

Ferreira et al. [79] compared amino acid substitutions in several Bet v 1 isoallergens and homologs by an algorithm developed to predict residues important for IgE binding. A Bet v 1 6-point mutant, developed by site-

directed mutagenesis, exhibited extremely low reactivity with serum IgE from birch pollen-allergic patients. Its ability to induce reactions in SPTs was significantly lower than for wild-type Bet v 1. In addition, the mutations had not influenced T cell recognition.

The IgE recognition of allergenic members of the EF-hand superfamily of calcium-binding proteins is influenced by the presence of bound calcium, as has been shown for polcalcins [80] and the fish allergen parvalbumin [81, 82]. The EF-hand calcium-binding domains were disrupted resulting in recombinant hypoallergens for the birch pollen allergen Bet v 4 [83], the *Brassica* pollen allergen Bra r 1 [84], and the carp allergen Cyp c 1 [82]. All 3 hypoallergens displayed reduced IgE-binding activities. Cyp c 1 was analyzed in an SPT and induced significantly fewer skin reactions than wild-type Cyp c 1. Mouse IgG raised by immunization with mutated Cyp c 1 cross-reacted with parvalbumins from various fish species and inhibited the binding of the IgE in fish-allergic patients to the wild-type allergen.

Disulfide bonds stabilize the structure of several important allergen families and have therefore been a target of site-directed mutagenesis experiments. Hypoallergens displaying a decreased IgE-binding activity and a conserved T cell reactivity were generated for the nonspecific lipid transfer protein Par j 1 from *P. judaica* pollen [85] and the major latex allergen Hev b 6.02 [86]. Disulfide bonds present in the group 2 allergens of mites were also destroyed by site-directed mutagenesis to destabilize the antigenic structure and ablate IgE binding of Der p 2 [87], Der f 2 [88], and Lep d 2 [89, 90].

The introduction of point mutations in several peanut allergens was based on detailed studies of their IgE epitopes. For Ara h 1, 4 linear immunodominant IgE epitopes were identified by screening overlapping synthetic decapeptides [91]. Likewise, 10 linear IgE epitopes were identified in the Ara h 2 sequence [92], and 4 in the Ara h 3 sequence [93]. Amino acids critical to each IgE epitope of these 3 peanut allergens were changed by site-directed mutagenesis [94–96]. The resulting recombinant proteins had a lower capacity to bind patients' IgE but retained the ability to stimulate T cell proliferation. Beezheld et al. [97] produced a recombinant latex hypoallergen Hev b 5 by simultaneously replacing the critical amino acid residues in 8 IgE epitopes to achieve a significantly reduced IgE-binding activity. Karisola et al. [98] produced 29 Hev b 6.02 mutants to identify 6 IgE-interacting residues in the sequence of this latex allergen. SPT reactivity of the 6-residue mutant was completely abolished for all patients examined in the study.

Based on B- and T-cell epitope mapping studies and sequence comparison of group 5 allergens from different grasses, point mutations were introduced by site-directed mutagenesis in the highly conserved sequence domains of Lol p 5, the group 5 allergen from ryegrass [99]. The authors succeeded in producing rLol p 5 mutants with low IgE-binding capacity and reduced allergenic activity, determined by basophil histamine release and SPTs in allergic patients. Two amino acid positions in the egg-white allergen Gal d 1 have been identified as crucial for IgE binding as they contribute substantially to the structural integrity of the protein [100]. Sequence and fold analysis of members of the Bet v 1 family identified a short sequence stretch in Bet v 1, susceptible for mutations to induce an altered fold of the entire molecule [101]. The replacement of 7 consecutive amino acids of Bet v 1 by the homologous Mal d 1 sequence resulted in the loss of the Bet v 1-like fold and a drastic reduction in binding IgE in birch pollen-allergic individuals.

Recombinant Allergens in Clinical Studies

AIT is the only specific and disease-modifying treatment of allergy. The difficulty in preparing safe and effective vaccines from natural allergen extracts represents the main limitation of AIT [102]. Vaccines based on recombinant wild-type allergens or recombinant hypoallergenic allergen derivatives offer the solution to this problem and have therefore entered into clinical testing more than a decade ago [103]. Today, the structures of most of the important allergens are known and a wide array of recombinant hypoallergenic derivatives has been engineered [104]. As shown in Table 3, a number of these hypoallergenic molecules have already been tested in clinical trials [105]. However, there is still a strong need for further research in the area of recombinant allergens and allergen derivatives to increase the efficacy and safety of AIT [106].

Birch Pollen Allergy, Bet v 1

In 2000, Pauli et al. [107] compared the allergenic activity of rBet v 1 with rBet v 1 fragments, an rBet v 1 dimer, and an rBet v 1 trimer. In SPTs on 29 patients and in intradermal injections of 30 patients, the hypoallergenic derivatives of Bet v 1 showed a reduced capacity to induce immediate-type skin reactions. The results of the first double-blind, placebo-controlled AIT study in 124 birch pollen-allergic patients were published by Niederberger et al. [108] in 2004. One preseasonal treatment course

with the 2 rBet v 1 fragments or the rBet v 1 trimer induced protective allergen-specific IgG antibodies and resulted in the reduction of cutaneous sensitivity as well as an improvement of symptoms in actively treated patients. A significant reduction in the rise of allergen-specific IgE induced by seasonal pollen exposure was also observed. Analysis of the nasal secretions of 23/124 patients showed the induction of Bet v 1-specific IgG1, IgG2, and IgG4, and low IgA levels [109]. The observed reduced nasal sensitivity to natural Bet v 1 was significantly associated with the induced antibody levels. However, the increase in Bet v 1-specific IgG levels resulted in the improvement of birch pollen-associated OAS symptoms only in 7 of 25 patients assessed after the vaccination with either the Bet v 1 fragments or Bet v 1 trimer [110].

In 2008, Pauli et al. [111] published the comparison of a recombinant wild-type Bet v 1 vaccine, a standard birch pollen extract and natural purified Bet v 1 in a randomized, double-blind, placebo-controlled trial of 134 patients. All groups demonstrated significant and equal improvements in symptoms, medication use, and skin test reactivity in 2 pollen seasons. Clinical improvements were accompanied by marked increases in Bet v 1-specific IgG1, IgG2, and IgG4 levels, which were highest in the rBet v 1-treated group. It was clearly shown that rBet v 1 could replace the birch pollen extract in the AIT treatment of patients. Interestingly, the extract-based treatment led to de novo induction of IgE against Bet v 2, the birch pollen profilin, in 3 patients. The safety and efficacy of rBet v 1 formulated as a sublingual tablet were assessed in a multicenter, double-blind, placebo-controlled study conducted in 482 birch pollen-allergic patients [112]. The average adjusted symptom scores were significantly decreased in comparison to placebo in individuals receiving rBet v 1 tablets once daily for 5 months.

A stable monomeric and hypoallergenic folding variant of Bet v 1 (rBet v 1-FV) was produced by changing the buffers of the chromatographic steps during the purification of rBet v 1 expressed in *E. coli* [113]. Thirty adult birch pollen-allergic individuals were treated with rBet v 1-FV for 10 weeks with weekly injections of the hypoallergen [114]. Upon exposure to birch pollen in an environmental exposure chamber, a significant decrease in the total symptom score was observed. No serious adverse events occurred. A preseasonal, randomized, controlled phase II study compared the effects of subcutaneous AIT with rBet v 1-FV or an established natural birch pollen extract [115]. No statistical difference between rBet v 1-FV and the pollen extract was observed regarding the beneficial effects of the AIT. rBet v 1-FV could even be

administered in higher doses than the native protein with no increase in adverse effects.

Grass Pollen Allergy, Phl p 1, 2, 5a, 5b, and 6

A study published in 2005 by Jutel et al. [116] determined the effectiveness of a mixture of 5 recombinant wild-type Timothy grass pollen allergens, rPhl p 1, rPhl p 2, rPhl p 5a, rPhl p 5b, and rPhl p 6, in reducing symptoms and the need for symptomatic medications. This randomized, double-blind, placebo-controlled study of subcutaneous-injection immunotherapy showed a significant improvement in the symptom medication score in subjects receiving the recombinant allergens compared to the placebo group. Conjunctival provocation showed a clear trend in favor of the active treatment which was based on the pronounced development of allergen-specific IgG1 and IgG4. A randomized, double-blind, placebo-controlled, dose-ranging study was performed in 50 patients with a mixture of 5 recombinant *Phleum* pollen allergens [117]. Eight patients experienced systemic reactions grade 1 or 2, but none experienced grade 3 or 4. Hence, this first, dose-ranging safety study with Timothy grass pollen allergens showed no major side effects, even in the very high dose range.

Very recently, a randomized, double-blind, placebo-controlled AIT study of grass pollen allergy using a recombinant B-cell epitope-based vaccine was published [118]. The vaccine, called BM32, contained recombinant fusion proteins that consisted of the hepatitis B virus (HBV) surface protein preS domain as an immunogenic carrier and peptides derived from the Timothy grass pollen allergens Phl p 1, 2, 5, and 6. Sixty-eight patients completed the trial. The total nasal symptom score decreased significantly, the total ocular symptom score and SPT reactions showed a dose-dependent decrease, and only a few grade 1 systemic reactions occurred. BM32 induced highly significant allergen-specific IgG but no IgE responses. As a side effect of this grass pollen AIT, but of high interest, Cornelius et al. [119] reported, in all of the 7 recipients of the BM32 vaccine tested so far, the induction of HBV-neutralizing antibodies. Although the number of vaccine recipients evaluated was small and no titers were determined, this study showed that the preS domain alone could induce HBV-neutralizing antibodies [120].

Cat Allergy, Fel d 1

The recombinant major cat dander allergen Fel d 1 was linked to a cell membrane translocation sequence and to a truncated invariant chain for targeting the MHC class II pathway, creating the modular antigen transporter

(MAT)-Fel d 1 fusion protein [121]. In a randomized, double-blind, placebo-controlled trial, intralymphatic immunotherapy with rMAT-Fel d 1 was performed on 20 patients. After only 3 intralymphatic injections of rMAT-Fel d 1 within 2 months, nasal tolerance increased up to 74-fold and cat-dander-specific IgG4 was increased 5.7-fold. This first-in-human clinical study of intralymphatic immunotherapy was regarded as safe and efficient in inducing allergen tolerance.

Peanut Allergy, Ara h 1, 2, and 3

cDNAs coding for 3 recombinant peanut allergens (Ara h 1, Ara h 2, and Ara h 3) were modified to disrupt common IgE-binding sites [94, 95]. The modified recombinant allergens were separately expressed in *E. coli*, and remained inside the cells when these were subsequently killed using heat and phenol [122]. The 3 resulting cell suspensions were combined to form the rectally administered vaccine EMP-123 with the goal of inducing tolerance to the dominant peanut allergens. Ten peanut-allergic individuals received escalating weekly doses over 10 weeks, followed by 3 biweekly maximal doses. The rectal administration of EMP-123 resulted in mild rectal symptoms in 1 patient and adverse reactions in 5 patients, including 2 anaphylactic reactions. Due to the unexpected frequency and intensity of these adverse reactions, to date, no further development of the vaccine has been reported by the company Allertein.

Peach Allergy, Pru p 3

The EU-funded FAST (Food Allergy Specific Immunotherapy) project was aimed at developing a safe and effective subcutaneous immunotherapy for persistent and severe allergy to fish and peach [123]. For peach, FAST focused on its major allergen, the nonspecific lipid-transfer protein Pru p 3. Two rPru p 3 mutants (substitutions of surface-exposed amino acids important for IgE binding and the destruction of disulfide bridges), 2 chemically modified rPru p 3 molecules (reduction/alkylation and glutaraldehyde treatment) and a naturally occurring hypoallergen, rFra a 3 (from strawberry) were prepared and extensively tested. Of these 5 candidate molecules, 2 failed on hypoallergenicity, 2 on immunogenicity and stability, and 1 on solubility [124]. Subsequently, 2 trimeric molecules were designed with all 8 cysteines replaced by serines, 1 heat-treated wild-type rPru p 3 was prepared, and 2 mutants were produced with 4 (i.e., 8 in all) surface-exposed amino acids residues exchanged [124]. Hypoallergenicity could be achieved at the cost of immunogenicity and stability. From these results, the au-

thors concluded that Pru p 3 is a molecule that loses its immunogenicity when its structure is altered, and they decided to abandon its further clinical development.

Fish Allergy, Cyp c 1

For the development of immunotherapy for fish allergy, the FAST project focused on the major allergen from carp, the highly cross-reactive parvalbumin Cyp c 1 [123]. Hypoallergenic mutants of Cyp c 1 had been previously produced by mutating the 2 functional calcium-binding sites which are responsible for most of the IgE binding of the molecule [81, 82]. The FAST project selected the mutant Cyp c 1, in which both calcium-binding sites had been destroyed for the development of a hypoallergenic vaccine for fish AIT. Purified mutated rCyp c 1 behaved as a stable and folded molecule that had retained its immunogenicity and displayed, on average, a 1,000-fold reduction of IgE binding when tested with sera from 26 fish-allergic patients [125]. Mutated rCyp c 1 was tested in Denmark in 2013/2014 in a first-in-human, randomized, double-blind, placebo-controlled clinical study involving 16 fish-allergic subjects [124]. Due to the low level of side effects and the positive immunological response, a multicenter clinical trial in 9 clinical centers in 6 countries was initiated in October 2015.

Recombinant Chimeric Allergens for Monitoring Clinical Studies

The determination of conformational antibody epitopes on allergens is still a challenge, yet of high interest for monitoring the development of allergen-specific antibody repertoires during AIT. Epitope grafting from allergens onto scaffolds of low-allergenic homologs is an elegant approach to a solution for this problem. Four distinct areas of Bet v 1, representing in total around 80% of its surface, were separately grafted onto the scaffold of its celery homolog Api g 1 to yield the chimeras Api-Bet-1–4 [26]. Sera from 64 birch pollen-allergic patients were tested for the presence of chimera-specific IgE. The highly patient-specific IgE recognition profiles revealed that the IgE response to Bet v 1 is polyclonal and that the epitopes are spread across the entire Bet v 1 surface. The 4 Api-Bet chimeras were then applied to monitor the development of the Bet v 1-specific IgE, IgG1, and IgG4 repertoires in narrow time intervals during 3 years of AIT in 11 subjects [126]. In the majority of patients, Bet v 1-specific IgE levels increased during the early phase of treatment, followed by a gradual decrease. The overall IgE epitope di-

versity during AIT remained constant. IgE also showed the highest epitope diversity among the immunoglobulin classes investigated. All patients had Bet v 1-specific IgG4, and 7 patients had Bet v 1-specific IgG1. IgE, IgG1, and IgG4 recognized different epitope profiles. Notably, the epitopes represented by the chimera Api-Bet-3 (the C-terminus of Bet v 1 plus surrounding residues) played a prominent role in the recognition of the allergen by all studied immunoglobulin classes.

Conclusions

Recombinant allergens have considerably advanced our knowledge of the immune mechanisms of allergic diseases as well as vastly enriched our toolbox for diagnosis and therapy. While recombinant allergens have contributed significantly to allergen standardization, diagnosis, structural biology, and epitope mapping, it is still an open issue whether and when recombinant AIT will replace extract-based immunotherapy. There are 2 main aspects to consider, the regulatory aspects of AIT and the quality of the recombinant allergens. The European Community has not yet produced regulations for AIT with recombinant allergens that would be compelling for all member states. Bonini [127] states in his 2012 article that the guidelines produced by the European Medicines Agency, such as that on the production and quality issues of allergen products (EMA/CHMP/BWP/304831/2007), are only for orientation. Likewise, position papers published by expert panels or scientific societies, such as the EAACI, are of no regulatory value. In addition, recombinant allergens and hypoallergens will remain unavailable in many countries due to the strict regulatory criteria that need to be met. Hence, it is unlikely that novel allergenic molecules will be available in the near future for use as in vivo clinical diagnostic and immunotherapy reagents.

On the other hand, the recombinant production of certain allergens still needs to overcome technological hurdles. The EU-funded CREATE project, which aimed to evaluate the use of recombinant allergens as reference material, showed that some recombinant allergens only possess a mean of 50–80% of the IgE-binding capacity of their natural counterparts [128]. The absence of post-translational modifications could result in a decrease of the IgE-binding capacity, as was shown for the peanut allergen Ara h 2 [129]. A recent study on the expression of Ara h 1 and Ara h 2 in the chloroplasts of the unicellular eukaryotic green alga *Chlamydomonas reinhardtii* found that even the algal-produced recombinant allergens had

a reduced affinity for IgE compared to the native proteins [130]. Hypoallergenic variants of Ara h 1, 2, and 3 still produced frequent adverse side effects when administered to patients, resulting in the termination of any further development of a therapy for peanut allergy based on recombinant allergens [122]. Two companies have turned to natural peanut proteins in their peanut allergy drug formulations, and they have tested an oral peanut protein formulation and an epicutaneous patch that delivered peanut proteins via the skin [131]. For both drugs, desensitization was lost within weeks if patients terminated the treatment. Nevertheless, as the technology of the production of recombinant proteins is refined, they will become an increasingly attractive alternative to native allergens for allergy diagnostics and immunotherapy.

Acknowledgements

The authors acknowledge the support of the Austrian Science Fund Doctoral Program MCCA W1248-B30.

Disclosure Statement

The authors declare that they have no conflicts of interest with respect to the information presented in this article.

References

- Chua KY, Stewart GA, Thomas WR, Simpson RJ, Dilworth RJ, Plozza TM, Turner KJ: Sequence analysis of cDNA coding for a major house dust mite allergen, Der p 1. Homology with cysteine proteases. *J Exp Med* 1988;167: 175–182.
- Thomas WR, Stewart GA, Simpson RJ, Chua KY, Plozza TM, Dilworth RJ, Nisbet A, Turner KJ: Cloning and expression of DNA coding for the major house dust mite allergen Der p 1 in *Escherichia coli*. *Int Arch Allergy Appl Immunol* 1988;85:127–129.
- Fang KS, Vitale M, Fehner P, King TP: cDNA cloning and primary structure of a white-face hornet venom allergen, antigen 5. *Proc Natl Acad Sci USA* 1988;85:895–899.
- Breiteneder H, Pettenburger K, Bito A, Valenta R, Kraft D, Rumpold H, Scheiner O, Breitenbach M: The gene coding for the major birch pollen allergen Bet v 1, is highly homologous to a pea disease resistance response gene. *EMBO J* 1989;8:1935–1938.
- Breiteneder H, Hassfeld W, Pettenburger K, Jarolim E, Breitenbach M, Rumpold H, Kraft D, Scheiner O: Isolation and characterization of messenger RNA from male inflorescences and pollen of the white birch (*Betula verrucosa*). *Int Arch Allergy Appl Immunol* 1988; 87:19–24.
- Walsh DJ, Matthews JA, Denmeade R, Walker MR: Cloning of cDNA coding for an allergen of Cocksfoot grass (*Dactylis glomerata*) pollen. *Int Arch Allergy Appl Immunol* 1989; 90:78–83.
- Arruda LK, Platts-Mills TA, Fox JW, Chapman MD: *Aspergillus fumigatus* allergen 1, a major IgE-binding protein, is a member of the mitogillin family of cytotoxins. *J Exp Med* 1990;172:1529–1532.
- Morgenstern JP, Griffith IJ, Brauer AW, Rogers BL, Bond JF, Chapman MD, Kuo MC: Amino acid sequence of Fel d 1, the major allergen of the domestic cat: protein sequence analysis and cDNA cloning. *Proc Natl Acad Sci USA* 1991;88:9690–9694.
- Burks AW, Cockrell G, Stanley JS, Helm RM, Bannon GA: Recombinant peanut allergen Ara h 1 expression and IgE binding in patients with peanut hypersensitivity. *J Clin Invest* 1995;96:1715–1721.
- Erlar A, Hawranek T, Krückemeier L, Asam C, Egger M, Ferreira F, Briza P: Proteomic profiling of birch (*Betula verrucosa*) pollen extracts from different origins. *Proteomics* 2011;11:1486–1498.
- Schmidt H, Gelhaus C, Nebendahl M, Janssen O, Petersen A: Characterization of *Phleum pratense* pollen extracts by 2-D DIGE and allergen immunoreactivity. *Proteomics* 2010; 10:4352–4362.
- Barranca M, Fontana S, Taverna S, Duro G, Zanella-Cleon I, Becchi M, De Leo G, Alessandro R: Proteomic analysis of *Parietaria judaica* pollen and allergen profiling by an immunoproteomic approach. *Biotechnol Lett* 2010;32:565–570.
- Chassaigne H, Trégoat V, Nørgaard JV, Maleki SJ, van Hengel AJ: Resolution and identification of major peanut allergens using a combination of fluorescence two-dimensional differential gel electrophoresis, Western blotting and Q-TOF mass spectrometry. *J Proteomics* 2009;72:511–526.
- Weghofer M, Thomas WR, Pittner G, Horak F, Valenta R, Vrtala S: Comparison of purified *Dermatophagoides pteronyssinus* allergens and extract by two-dimensional immunoblotting and quantitative immunoglobulin E inhibitions. *Clin Exp Allergy* 2005;35:1384–1391.
- dos Santos Pinto JR, Fox EG, Saidenberg DM, Santos LD, da Silva Menegasso AR, Costa-Manso E, Machado EA, Bueno OC, Palma MS: Proteomic view of the venom from the fire ant *Solenopsis invicta* Buren. *J Proteome Res* 2012;11:4643–4653.
- Natale M, Bisson C, Monti G, Peltran A, Garoffo LP, Valentini S, Fabris C, Bertino E, Coscia A, Conti A: Cow's milk allergens identification by two-dimensional immunoblotting and mass spectrometry. *Mol Nutr Food Res* 2004;48:363–369.
- Kofler S, Asam C, Eckhard U, Wallner M, Ferreira F, Brandstetter H: Crystallographically mapped ligand binding differs in high and low IgE binding isoforms of birch pollen allergen Bet v 1. *J Mol Biol* 2012;422:109–123.
- Markovic-Housley Z, Degano M, Lamba D, von Roepenack-Lahaye E, Clemens S, Susani M, Ferreira F, Scheiner O, Breiteneder H: Crystal structure of a hypoallergenic isoform of the major birch pollen allergen Bet v 1 and its likely biological function as a plant steroid carrier. *J Mol Biol* 2003;325:123–133.
- Dall'Antonia F, Pavkov-Keller T, Zangger K, Keller W: Structure of allergens and structure based epitope predictions. *Methods* 2014;66: 3–21.
- Radauer C, Lackner P, Breiteneder H: The Bet v 1 fold: an ancient, versatile scaffold for binding of large, hydrophobic ligands. *BMC Evol Biol* 2008;8:286.
- Sako Y, Nomura N, Uchida A, Ishida Y, Morii H, Koga Y, Hoaki T, Maruyama T: *Aeropyrum pernix* gen. nov., sp. nov., a novel aerobic hyperthermophilic archaeon growing at temperatures up to 100 degrees C. *Int J Syst Bacteriol* 1996;46:1070–1077.
- Dunwell JM, Purvis A, Khuri S: Cupins: the most functionally diverse protein superfamily? *Phytochemistry* 2004;65:7–17.

- 23 Radauer C, Bublin M, Wagner S, Mari A, Breiteneder H: Allergens are distributed into few protein families and possess a restricted number of biochemical functions. *J Allergy Clin Immunol* 2008;121:847–852.
- 24 Chruszcz M, Ciardiello MA, Osinski T, Majorek KA, Giangrieco I, Font J, Breiteneder H, Thalassinou K, Minor W: Structural and bioinformatic analysis of the kiwifruit allergen Act d 11, a member of the family of ripening-related proteins. *Mol Immunol* 2013;56:794–803.
- 25 Dall'Antonia F, Gieras A, Devanaboyina SC, Valenta R, Keller W: Prediction of IgE-binding epitopes by means of allergen surface comparison and correlation to cross-reactivity. *J Allergy Clin Immunol* 2011;128:872–879.
- 26 Gepp B, Lengger N, Bublin M, Hemmer W, Breiteneder H, Radauer C: Chimeras of Bet v 1 and Api g 1 reveal heterogeneous IgE responses in patients with birch pollen allergy. *J Allergy Clin Immunol* 2014;134:188–194.
- 27 Berkner H, Seutter von Loetzen C, Hartl M, Randow S, Gubesch M, Vogel L, Husslik F, Reuter A, Lidholm J, Ballmer-Weber B, Vieths S, Rösch P, Schiller D: Enlarging the toolbox for allergen epitope definition with an allergen-type model protein. *PLoS One* 2014;9:e111691.
- 28 Husslik F, Nürnberg J, Seutter von Loetzen C, Mews T, Ballmer-Weber BK, Kleine-Tebbe J, Treudler R, Simon JC, Randow S, Völker E, Reuter A, Rösch P, Vieths S, Holzhauser T, Schiller D: The conformational IgE epitope profile of soybean allergen Gly m 4. *Clin Exp Allergy* 2016;46:1484–1497.
- 29 Casset A, Mari A, Purohit A, Resch Y, Weghofer M, Ferrara R, Thomas WR, Alessandri C, Chen KW, de Blay F, Valenta R, Vrtala S: Varying allergen composition and content affects the in vivo allergenic activity of commercial *Dermatophagoides pteronyssinus* extracts. *Int Arch Allergy Immunol* 2012;159:253–262.
- 30 Kespohl S, Maryska S, Zahradnik E, Sander I, Brüning T, Raulf-Heimsoth M: Biochemical and immunological analysis of mould skin prick test solution: current status of standardization. *Clin Exp Allergy* 2013;43:1286–1296.
- 31 Akkerdaas JH, Wensing M, Knulst AC, Aalberse RC, Hefle SL, van Ree R: In vitro and in vivo characterization of hazelnut skin prick test extracts. *Arb Paul Ehrlich Inst Bundesamt Sera Impfstoffe Frankf A M* 2003;87–95.
- 32 van Ree R: Indoor allergens: relevance of major allergen measurements and standardization. *J Allergy Clin Immunol* 2007;119:270–277.
- 33 Moreno Benítez F, Espinazo Romeu M, Le-trán Camacho A, Mas S, García-Cózar FJ, Tabar AI: Variation in allergen content in sublingual allergen immunotherapy with house dust mites. *Allergy* 2015;70:1413–1420.
- 34 Lundberg M, Chen Z, Rihs HP, Wrangsjö K: Recombinant spiked allergen extract. *Allergy* 2001;56:794–795.
- 35 Andersson K, Ballmer-Weber BK, Cistero-Bahima A, Ostling J, Lauer I, Vieths S, Lidholm J: Enhancement of hazelnut extract for IgE testing by recombinant allergen spiking. *Allergy* 2007;62:897–904.
- 36 Yoshida N, Hirata H, Watanabe M, Sugiyama K, Arima M, Fukushima Y, Ishii Y: Improved sensitivity to venom specific-immunoglobulin E by spiking with the allergen component in Japanese patients suspected of Hymenoptera venom allergy. *Allergol Int* 2015;64:248–252.
- 37 Mas S, Boissy P, Monsalve RI, Cuesta-Herranz J, Díaz-Perales A, Fernández J, Colás C, Rodríguez R, Barderas R, Villalba M: A recombinant Sal k 1 isoform as an alternative to the polymorphic allergen from *Salsola kali* pollen for allergy diagnosis. *Int Arch Allergy Immunol* 2015;167:83–93.
- 38 Asero R: Looking for sensitization profiles in different populations by recombinant allergens. *Int Arch Allergy Immunol* 2014;164:106–108.
- 39 Canonica GW, Ansotegui IJ, Pawankar R, Schmid-Grendelmeier P, van Hage M, Baena-Cagnani CE, Melioli G, Nunes C, Passalacqua G, Rosenwasser L, Sampson H, Sastre J, Bousquet J, Zuberbier T: A WAO-ARIA-GA²LEN consensus document on molecular-based allergy diagnostics. *World Allergy Organ J* 2013;6:17.
- 40 Panzner P, Vachová M, Vitovcová P, Brodská P, Vlas T: A comprehensive analysis of middle-European molecular sensitization profiles to pollen allergens. *Int Arch Allergy Immunol* 2014;164:74–82.
- 41 Hiller R, Laffer S, Harwanegg C, Huber M, Schmidt WM: Microarrayed allergen molecules: diagnostic gatekeepers for allergy treatment. *FASEB J* 2002;16:414–416.
- 42 Lupinek C, Wollmann E, Baar A, Banerjee S, Breiteneder H, Broecker BM, Bublin M, Curin M, Flicker S, Garmatiuk T, Hochwallner H, Mittermann I, Pahr S, Resch Y, Roux KH, Srinivasan B, Stentzel S, Vrtala S, Willison LN, Wickman M, Lødrup-Carlson KC, Antó JM, Bousquet J, Bachert C, Ebner D, Schleiderer T, Harwanegg C, Valenta R: Advances in allergen-microarray technology for diagnosis and monitoring of allergy: the MeDALL allergen-chip. *Methods* 2014;66:106–119.
- 43 Incorvaia C, Mauro M, Ridolo E, Makri E, Montagni M, Ciprandi G: A pitfall to avoid when using an allergen microarray: the incidental detection of IgE to unexpected allergens. *J Allergy Clin Immunol Pract* 2015;3:879–882.
- 44 Chapman MD, Wuenschmann S, King E, Pomés A: Technological innovations for high-throughput approaches to in vitro allergy diagnosis. *Curr Allergy Asthma Rep* 2015;15:36.
- 45 King EM, Vailes LD, Tsay A, Satinover SM, Chapman MD: Simultaneous detection of total and allergen-specific IgE by using purified allergens in a fluorescent multiplex array. *J Allergy Clin Immunol* 2007;120:1126–1131.
- 46 Pomponi D, Bernardi ML, Liso M, Palazzo P, Tuppo L, Rafaiani C, Santoro M, Labrada A, Ciardiello MA, Mari A, Scala E: Allergen micro-bead array for IgE detection: a feasibility study using allergenic molecules tested on a flexible multiplex flow cytometric immunoassay. *PLoS One* 2012;7:e35697.
- 47 Kühne Y, Reese G, Ballmer-Weber BK, Niggemann B, Hanschmann KM, Vieths S, Holzhauser T: A novel multiplexed microarray for the specific and sensitive mapping of linear IgE-binding epitopes of food allergens. *Int Arch Allergy Immunol* 2015;166:213–224.
- 48 Matricardi PM, Kleine-Tebbe J, Hoffmann HJ, Valenta R, Hilger C: EAACI Molecular Allergy User's Guide. *Pediatr Allergy Immunol* 2016;27(suppl 23):1–250.
- 49 Becker S, Schleiderer T, Kramer MF, Haack M, Vrtala S, Resch Y, Lupinek C, Valenta R, Gröger M: Real-life study for the diagnosis of house dust mite allergy – the value of recombinant allergen-based IgE serology. *Int Arch Allergy Immunol* 2016;170:132–137.
- 50 Soh WT, Le Mignon M, Surattanon N, Satitsuksanoa P, Chatchatee P, Wongpiyaboron J, Vangveravong M, Rerkpattanapipat T, Sangasapaviliya A, Nony E, Piboonpocanun S, Ruxrungham K, Jacquet A: The house dust mite major allergen Der p 23 displays O-glycan-independent IgE reactivities but no chitin-binding activity. *Int Arch Allergy Immunol* 2015;168:150–160.
- 51 Carballeda-Sangiao N, Rodríguez-Mahillo AI, Careche M, Navas A, Caballero T, Dominguez-Ortega J, Jurado-Palomo J, González-Muñoz M: Ani s 11-like protein is a pepsin- and heat-resistant major allergen of *Anisakis* spp. and a valuable tool for *Anisakis* allergy component-resolved diagnosis. *Int Arch Allergy Immunol* 2016;169:108–112.
- 52 Noon L: Prophylactic inoculation against hay fever. *Lancet* 1911;1:1572–1573.
- 53 Bousquet J, Lockey R, Malling HJ: Allergen immunotherapy: therapeutic vaccines for allergic diseases. A WHO position paper. *J Allergy Clin Immunol* 1998;102:558–562.
- 54 Durham SR, Walker SM, Varga EM, Jacobson MR, O'Brien F, Noble W, Till SJ, Hamid QA, Nouri-Aria KT: Long-term clinical efficacy of grass-pollen immunotherapy. *N Engl J Med* 1999;341:468–475.
- 55 Möller C, Dreborg S, Ferdousi HA, Halken S, Høst A, Jacobsen L, Koivikko A, Koller DY, Niggemann B, Norberg LA, Urbanek R, Valovirta E, Wahn U: Pollen immunotherapy reduces the development of asthma in children with seasonal rhinoconjunctivitis (the PAT-study). *J Allergy Clin Immunol* 2002;109:251–256.
- 56 Focke M, Marth K, Flicker S, Valenta R: Heterogeneity of commercial Timothy grass pollen extracts. *Clin Exp Allergy* 2008;38:1400–1408.
- 57 Casset A, Valenta R, Vrtala S: Allergen content and in vivo allergenic activity of house dust mite extracts. *Int Arch Allergy Immunol* 2013;161:287–288.

- 58 Vrtala S, Hirtenlehner K, Vangelista L, Pastore A, Eichler HG, Sperr WR, Valent P, Ebner C, Kraft D, Valenta R: Division of the major birch pollen allergen, Bet v 1, into two non-anaphylactic fragments. *Int Arch Allergy Immunol* 1997;113:246–248.
- 59 Vrtala S, Hirtenlehner K, Vangelista L, Pastore A, Eichler HG, Sperr WR, Valent P, Ebner C, Kraft D, Valenta R: Conversion of the major birch pollen allergen, Bet v 1, into two nonanaphylactic T cell epitope-containing fragments: candidates for a novel form of specific immunotherapy. *J Clin Invest* 1997;99:1673–1681.
- 60 Zeiler T, Taivainen A, Rytönen M, Rautiainen J, Karjalainen H, Mäntytjärvi R, Tuomisto L, Virtanen T: Recombinant allergen fragments as candidate preparations for allergen immunotherapy. *J Allergy Clin Immunol* 1997;100:721–727.
- 61 Elfman LH, Whitley P, Schmidt M, van Hage-Hamsten M: IgE binding capacity of synthetic and recombinant peptides of the major storage mite (*Lepidoglyphus destructor*) allergen, Lep d 2. *Int Arch Allergy Immunol* 1998;117:167–173.
- 62 Ball T, Vrtala S, Sperr WR, Valent P, Susani M, Kraft D, Valenta R: Isolation of an immunodominant IgE hapten from an epitope expression cDNA library. Dissection of the allergic effector reaction. *J Biol Chem* 1994;269:28323–28328.
- 63 Chen KW, Fuchs G, Sonneck K, Gieras A, Swoboda I, Douladiris N, Linhart B, Jankovic M, Pavkov T, Keller W, Papadopoulos NG, Valent P, Valenta R, Vrtala S: Reduction of the in vivo allergenicity of Der p 2, the major house-dust mite allergen, by genetic engineering. *Mol Immunol* 2008;45:2486–2498.
- 64 Vrtala S, Hirtenlehner K, Susani M, Akdis M, Kusébi F, Akdis CA, Blaser K, Hufnagl P, Binder BR, Politou A, Pastore A, Vangelista L, Sperr WR, Sempfer H, Valent P, Ebner C, Kraft D, Valenta R: Genetic engineering of a hypoallergenic trimer of the major birch pollen allergen Bet v 1. *FASEB J* 2001;15:2045–2047.
- 65 Campana R, Vrtala S, Maderegger B, Dall'Antonia Y, Zafred D, Blatt K, Herrmann H, Focke-Tejkl M, Swoboda I, Scheibhofer S, Gieras A, Neubauer A, Keller W, Valent P, Thalhamer J, Spitzauer S, Valenta R: Altered IgE epitope presentation: A model for hypoallergenic activity revealed for Bet v 1 trimer. *Mol Immunol* 2011;48:431–441.
- 66 Mothes-Luksch N, Stumvoll S, Linhart B, Focke M, Krauth MT, Hauswirth A, Valent P, Verdino P, Pavkov T, Keller W, Grote M, Valenta R: Disruption of allergenic activity of the major grass pollen allergen Phl p 2 by reassembly as a mosaic protein. *J Immunol* 2008;181:4864–4873.
- 67 Ball T, Linhart B, Sonneck K, Blatt K, Herrmann H, Valent P, Stoecklinger A, Lupinek C, Thalhamer J, Fedorov AA, Almo SC, Valenta R: Reducing allergenicity by altering allergen fold: a mosaic protein of Phl p 1 for allergy vaccination. *Allergy* 2009;64:569–580.
- 68 Campana R, Vrtala S, Maderegger B, Jertschin P, Stegellner G, Swoboda I, Focke-Tejkl M, Blatt K, Gieras A, Zafred D, Neubauer A, Valent P, Keller W, Spitzauer S, Valenta R: Hypoallergenic derivatives of the major birch pollen allergen Bet v 1 obtained by rational sequence reassembly. *J Allergy Clin Immunol* 2010;126:1024–1031.
- 69 Curin M, Weber M, Thalhamer T, Swoboda I, Focke-Tejkl M, Blatt K, Valent P, Marth K, Garmatiuk T, Grönlund H, Thalhamer J, Spitzauer S, Valenta R: Hypoallergenic derivatives of Fel d 1 obtained by rational reassembly for allergy vaccination and tolerance induction. *Clin Exp Allergy* 2014;44:882–894.
- 70 Linhart B, Valenta R: Vaccine engineering improved by hybrid technology. *Int Arch Allergy Immunol* 2004;134:324–331.
- 71 Asturias JA, Ibarrola I, Arilla MC, Vidal C, Ferrer A, Gamboa PM, Viñuela JE, Sanz ML, Andreu C, Martínez A: Engineering of major house dust mite allergens Der p 1 and Der p 2 for allergen-specific immunotherapy. *Clin Exp Allergy* 2009;39:1088–1098.
- 72 Chen KW, Blatt K, Thomas WR, Swoboda I, Valent P, Valenta R, Vrtala S: Hypoallergenic Der p 1/Der p 2 combination vaccines for immunotherapy of house dust mite allergy. *J Allergy Clin Immunol* 2012;130:435–443.e4.
- 73 Linhart B, Mothes-Luksch N, Vrtala S, Kneidinger M, Valent P, Valenta R: A hypoallergenic hybrid molecule with increased immunogenicity consisting of derivatives of the major grass pollen allergens, Phl p 2 and Phl p 6. *Biol Chem* 2008;389:925–933.
- 74 Linhart B, Focke-Tejkl M, Weber M, Narayanan M, Neubauer A, Mayrhofer H, Blatt K, Lupinek C, Valent P, Valenta R: Molecular evolution of hypoallergenic hybrid proteins for vaccination against grass pollen allergy. *J Immunol* 2015;194:4008–4018.
- 75 King TP, Jim SY, Monsalve RI, Kagey-Sobotka A, Lichtenstein LM, Spangfort MD: Recombinant allergens with reduced allergenicity but retaining immunogenicity of the natural allergens: hybrids of yellow jacket and paper wasp venom allergen antigen 5s. *J Immunol* 2001;166:6057–6065.
- 76 Karamloo F, Schmid-Grendelmeier P, Kusébi F, Akdis M, Salagianni M, von Beust BR, Reimers A, Zumkehr J, Soldatova L, Housley-Markovic Z, Müller U, Kündig T, Kemeny DM, Spangfort MD, Blaser K, Akdis CA: Prevention of allergy by a recombinant multi-allergen vaccine with reduced IgE binding and preserved T cell epitopes. *Eur J Immunol* 2005;35:3268–3276.
- 77 Fujimura T, Fujinami K, Ishikawa R, Tateno M, Tahara Y, Okumura Y, Ohta H, Miyazaki H, Taniguchi M: Recombinant fusion allergens, Cry j 1 and Cry j 2 from Japanese cedar pollen, conjugated with polyethylene glycol potentiate the attenuation of Cry j 1-specific IgE production in Cry j 1-sensitized mice and Japanese cedar pollen allergen-sensitized monkeys. *Int Arch Allergy Immunol* 2015;168:32–43.
- 78 Moingeon P: Will allergen fusion bring new energy to immunotherapy? *Int Arch Allergy Immunol* 2015;168:107–109.
- 79 Ferreira F, Ebner C, Kramer B, Casari G, Briza P, Kungl AJ, Grimm R, Jahn-Schmid B, Breiteneder H, Kraft D, Breitenbach M, Rheinberger HJ, Scheiner O: Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy. *FASEB J* 1998;12:231–242.
- 80 Valenta R, Hayek B, Seiberler S, Bugajska-Schretter A, Niederberger V, Twardosz A, Natter S, Vangelista L, Pastore A, Spitzauer S, Kraft D: Calcium-binding allergens: from plants to man. *Int Arch Allergy Immunol* 1998;117:160–166.
- 81 Swoboda I, Bugajska-Schretter A, Verdino P, Keller W, Sperr WR, Valent P, Valenta R, Spitzauer S: Recombinant carp parvalbumin, the major cross-reactive fish allergen: a tool for diagnosis and therapy of fish allergy. *J Immunol* 2002;168:4576–4584.
- 82 Swoboda I, Bugajska-Schretter A, Linhart B, Verdino P, Keller W, Schulmeister U, Sperr WR, Valent P, Peltre G, Quirce S, Douladiris N, Papadopoulos NG, Valenta R, Spitzauer S: A recombinant hypoallergenic parvalbumin mutant for immunotherapy of IgE-mediated fish allergy. *J Immunol* 2007;178:6290–6296.
- 83 Engel E, Richter K, Obermeyer G, Briza P, Kungl AJ, Simon B, Auer M, Ebner C, Rheinberger HJ, Breitenbach M, Ferreira F: Immunological and biological properties of Bet v 4, a novel birch pollen allergen with two EF-hand calcium-binding domains. *J Biol Chem* 1997;272:28630–28637.
- 84 Okada T, Swoboda I, Bhalla PL, Toriyama K, Singh MB: Engineering of hypoallergenic mutants of the *Brassica* pollen allergen, Bra r 1, for immunotherapy. *FEBS Lett* 1998;434:255–260.
- 85 Bonura A, Amoroso S, Locorotondo G, Di Felice G, Tinghino R, Geraci D, Colombo P: Hypoallergenic variants of the *Parietaria judaica* major allergen Par j 1: a member of the non-specific lipid transfer protein plant family. *Int Arch Allergy Immunol* 2001;126:32–40.
- 86 Drew AC, Eusebius NP, Kenins L, de Silva HD, Suphioglu C, Rolland JM, O'Hehir R E: Hypoallergenic variants of the major latex allergen Hev b 6.01 retaining human T lymphocyte reactivity. *J Immunol* 2004;173:5872–5879.
- 87 Smith AM, Chapman MD: Reduction of IgE antibody binding to rDer p 2 variants generated by site-directed mutagenesis. *Adv Exp Med Biol* 1996;409:391–394.
- 88 Takai T, Yokota T, Yasue M, Nishiyama C, Yuuki T, Mori A, Okudaira H, Okumura Y: Engineering of the major house dust mite allergen Der f 2 for allergen-specific immunotherapy. *Nat Biotechnol* 1997;15:754–758.

- 89 Eriksson TL, Gafvelin G, Elfman LH, Johansson C, Van Hage-Hamsten M, Olsson S: T cell responses to recombinant isoforms, synthetic peptides and a mutant variant of Lep d 2, a major allergen from the dust mite *Lepidoglyphus destructor*. Clin Exp Allergy 2001;31:1881–1890.
- 90 Kronqvist M, Johansson E, Whitley P, Olsson S, Gafvelin G, Scheynius A, van Hage-Hamsten M: A hypoallergenic derivative of the major allergen of the dust mite *Lepidoglyphus destructor*, Lep d 2.6Cys, induces less IgE reactivity and cellular response in the skin than recombinant Lep d 2. Int Arch Allergy Immunol 2001;126:41–49.
- 91 Burks AW, Shin D, Cockrell G, Stanley JS, Helm RM, Bannon GA: Mapping and mutational analysis of the IgE-binding epitopes on Ara h 1, a legume vicilin protein and a major allergen in peanut hypersensitivity. Eur J Biochem 1997;245:334–339.
- 92 Stanley JS, King N, Burks AW, Huang SK, Sampson H, Cockrell G, Helm RM, West CM, Bannon GA: Identification and mutational analysis of the immunodominant IgE binding epitopes of the major peanut allergen Ara h 2. Arch Biochem Biophys 1997;342:244–253.
- 93 Rabjohn P, Helm EM, Stanley JS, West CM, Sampson HA, Burks AW, Bannon GA: Molecular cloning and epitope analysis of the peanut allergen Ara h 3. J Clin Invest 1999;103:535–542.
- 94 Bannon GA, Cockrell G, Connaughton C, West CM, Helm R, Stanley JS, King N, Rabjohn P, Sampson HA, Burks AW: Engineering, characterization and in vitro efficacy of the major peanut allergens for use in immunotherapy. Int Arch Allergy Immunol 2001;124:70–72.
- 95 King N, Helm R, Stanley JS, Vieths S, Lüttkopf D, Hatahet L, Sampson H, Pons L, Burks W, Bannon GA: Allergenic characteristics of a modified peanut allergen. Mol Nutr Food Res 2005;49:963–971.
- 96 Rabjohn P, West CM, Connaughton C, Sampson HA, Helm RM, Burks AW, Bannon GA: Modification of peanut allergen Ara h 3: effects on IgE binding and T cell stimulation. Int Arch Allergy Immunol 2002;128:15–23.
- 97 Beezhold DH, Hickey VL, Sussman GL: Mutational analysis of the IgE epitopes in the latex allergen Hev b 5. J Allergy Clin Immunol 2001;107:1069–1076.
- 98 Karisola P, Mikkola J, Kalkkinen N, Airenne KJ, Laitinen OH, Repo S, Pentikäinen OT, Reunala T, Turjanmaa K, Johnson MS, Palosuo T, Kulomaa MS, Alenius H: Construction of hevein (Hev b 6.02) with reduced allergenicity for immunotherapy of latex allergy by comutation of six amino acid residues on the conformational IgE epitopes. J Immunol 2004;172:2621–2628.
- 99 Swoboda I, De Weerd N, Bhalla PL, Niederberger V, Sperr WR, Valent P, Kahlert H, Fiebig H, Verdino P, Keller W, Ebner C, Spitzauer S, Valenta R, Singh MB: Mutants of the major ryegrass pollen allergen, Lol p 5, with reduced IgE-binding capacity: candidates for grass pollen-specific immunotherapy. Eur J Immunol 2002;32:270–280.
- 100 Mine Y, Sasaki E, Zhang JW: Reduction of antigenicity and allergenicity of genetically modified egg white allergen, ovomucoid third domain. Biochem Biophys Res Commun 2003;302:133–137.
- 101 Wallner M, Hauser M, Himly M, Zaborsky N, Mutschlechner S, Harrer A, Asam C, Pichler U, van Ree R, Briza P, Thalhamer J, Bohle B, Achatz G, Ferreira F: Reshaping the Bet v 1 fold modulates T_H polarization. J Allergy Clin Immunol 2011;127:1571–1578.
- 102 Focke M, Swoboda I, Marth K, Valenta R: Developments in allergen-specific immunotherapy: from allergen extracts to allergy vaccines bypassing allergen-specific immunoglobulin E and T cell reactivity. Clin Exp Allergy 2010;40:385–397.
- 103 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 2016;137:351–357.
- 104 Linhart B, Valenta R: Mechanisms underlying allergy vaccination with recombinant hypoallergenic allergen derivatives. Vaccine 2012;30:4328–4335.
- 105 Ferreira F, Wolf M, Wallner M: Molecular approach to allergy diagnosis and therapy. Yonsei Med J 2014;55:839–852.
- 106 Jutel M, Kosowska A, Smolinska S: Allergen immunotherapy: past, present, and future. Allergy Asthma Immunol Res 2016;8:191–197.
- 107 Pauli G, Purohit A, Oster JP, de Blay F, Vrtala S, Niederberger V, Kraft D, Valenta R: Comparison of genetically engineered hypoallergenic rBet v 1 derivatives with rBet v 1 wild-type by skin prick and intradermal testing: results obtained in a French population. Clin Exp Allergy 2000;30:1076–1084.
- 108 Niederberger V, Horak F, Vrtala S, Spitzauer S, Krauth MT, Valent P, Reisinger J, Pelzmann M, Hayek B, Kronqvist M, Gafvelin G, Grönlund H, Purohit A, Suck R, Fiebig H, Cromwell O, Pauli G, van Hage-Hamsten M, Valenta R: Vaccination with genetically engineered allergens prevents progression of allergic disease. Proc Natl Acad Sci USA 2004;101(suppl 2):14677–14682.
- 109 Reisinger J, Horak F, Pauli G, van Hage M, Cromwell O, König F, Valenta R, Niederberger V: Allergen-specific nasal IgG antibodies induced by vaccination with genetically modified allergens are associated with reduced nasal allergen sensitivity. J Allergy Clin Immunol 2005;116:347–354.
- 110 Niederberger V, Reisinger J, Valent P, Krauth MT, Pauli G, van Hage M, Cromwell O, Horak F, Valenta R: Vaccination with genetically modified birch pollen allergens: immune and clinical effects on oral allergy syndrome. J Allergy Clin Immunol 2007;119:1013–1016.
- 111 Pauli G, Larsen TH, Rak S, Horak F, Pastorello E, Valenta R, Purohit A, Arvidsson M, Kavina A, Schroeder JW, Mothes N, Spitzauer S, Montagut A, Galvain S, Melac M, André C, Poulsen LK, Malling HJ: Efficacy of recombinant birch pollen vaccine for the treatment of birch-allergic rhinoconjunctivitis. J Allergy Clin Immunol 2008;122:951–960.
- 112 Nony E, Bouley J, Le Mignon M, Lemoine P, Jain K, Horiot S, Mascarelli L, Pallardy M, Vincentelli R, Leone P, Roussel A, Batard T, Abiteboul K, Robin B, de Beaumont O, Arvidsson M, Rak S, Moingeon P: Development and evaluation of a sublingual tablet based on recombinant Bet v 1 in birch pollen-allergic patients. Allergy 2015;70:795–804.
- 113 Kahlert H, Suck R, Weber B, Nandy A, Wald M, Keller W, Cromwell O, Fiebig H: Characterization of a hypoallergenic recombinant Bet v 1 variant as a candidate for allergen-specific immunotherapy. Int Arch Allergy Immunol 2008;145:193–206.
- 114 Meyer W, Narkus A, Salapatek AM, Hafner D: Double-blind, placebo-controlled, dose-ranging study of new recombinant hypoallergenic Bet v 1 in an environmental exposure chamber. Allergy 2013;68:724–731.
- 115 Klimek L, Bachert C, Lukat KF, Pfaar O, Meyer H, Narkus A: Allergy immunotherapy with a hypoallergenic recombinant birch pollen allergen rBet v 1-FV in a randomized controlled trial. Clin Transl Allergy 2015;5:28.
- 116 Jutel M, Jaeger L, Suck R, Meyer H, Fiebig H, Cromwell O: Allergen-specific immunotherapy with recombinant grass pollen allergens. J Allergy Clin Immunol 2005;116:608–613.
- 117 Klimek L, Schendzielorz P, Pinol R, Pfaar O: Specific subcutaneous immunotherapy with recombinant grass pollen allergens: first randomized dose-ranging safety study. Clin Exp Allergy 2012;42:936–945.
- 118 Ziegler P, Focke-Tejkl M, Schmutz R, Lemell P, Ziegler P, Weber M, Kiss R, Blatt K, Valent P, Stolz F, Huber H, Neubauer A, Knoll A, Horak F, Henning R, Valenta R: Mechanisms, safety and efficacy of a B cell epitope-based vaccine for immunotherapy of grass pollen allergy. EBioMedicine 2016;11:43–57.
- 119 Cornelius C, Schöneweis K, Georgi F, Weber M, Niederberger V, Ziegler P, Niespodziana K, Trauner M, Hofer H, Urban S, Valenta R: Immunotherapy with the preS-based grass pollen allergy vaccine BM32 induces antibody responses protecting against hepatitis B infection. EBioMedicine 2016;11:58–67.

- 120 Gerlich WH, Glebe D: Development of an allergy immunotherapy leads to a new type of hepatitis B vaccine. *EBioMedicine* 2016; 11:5–6.
- 121 Senti G, Cramer R, Kuster D, Johansen P, Martinez-Gomez JM, Graf N, Steiner M, Hothorn LA, Grönlund H, Tivig C, Zaleska A, Soyer O, van Hage M, Akdis CA, Akdis M, Rose H, Kündig TM: Intralymphatic immunotherapy for cat allergy induces tolerance after only 3 injections. *J Allergy Clin Immunol* 2012;129:1290–1296.
- 122 Wood RA, Sicherer SH, Burks AW, Grishin A, Henning AK, Lindblad R, Stablein D, Sampson HA: A phase 1 study of heat/phenol-killed, *E. coli*-encapsulated, recombinant modified peanut proteins Ara h 1, Ara h 2, and Ara h 3 (EMP-123) for the treatment of peanut allergy. *Allergy* 2013;68:803–808.
- 123 Zuidmeer-Jongejan L, Fernández-Rivas M, Poulsen LK, Neubauer A, Asturias J, et al: FAST: towards safe and effective subcutaneous immunotherapy of persistent life-threatening food allergies. *Clin Transl Allergy* 2012;2:5.
- 124 Jongejan L, van Ree R, Poulsen LK: Hypoallergenic molecules for subcutaneous immunotherapy. *Expert Rev Clin Immunol* 2016; 12:5–7.
- 125 Zuidmeer-Jongejan L, Huber H, Swoboda I, Rigby N, Versteeg SA: Development of a hypoallergenic recombinant parvalbumin for first-in-man subcutaneous immunotherapy of fish allergy. *Int Arch Allergy Immunol* 2015;166:41–51.
- 126 Gepp B, Lengger N, Möbs C, Pfützner W, Radauer C, Bohle B, Breiteneder H: Monitoring the epitope recognition profiles of IgE, IgG1, and IgG4 during birch pollen immunotherapy. *J Allergy Clin Immunol* 2016; 137:1600–1603.
- 127 Bonini S: Regulatory aspects of allergen-specific immunotherapy: Europe sets the scene for a global approach. *World Allergy Organ J* 2012;5:120–123.
- 128 van Ree R, Chapman MD, Ferreira F, Vieths S, Bryan D: The CREATE project: development of certified reference materials for allergenic products and validation of methods for their quantification. *Allergy* 2008;63: 310–326.
- 129 Bernard H, Guillon B, Drumare MF, Paty E, Dreskin SC, Wal JM, Adel-Patient K, Hazebrouck S: Allergenicity of peanut component Ara h 2: contribution of conformational versus linear hydroxyproline-containing epitopes. *J Allergy Clin Immunol* 2015;135: 1267–1274.e1–e8.
- 130 Gregory JA, Shepley-McTaggart A, Umpierrez M, Hurlburt BK, Maleki SJ, Sampson HA, Mayfield SP, Berin MC: Immunotherapy using algal-produced Ara h 1 core domain suppresses peanut allergy in mice. *Plant Biotechnol J* 2016;14:1541–1550.
- 131 Kingwell K: Food allergy drugs step up to phase III. *Nat Rev Drug Discov* 2016;15: 149–150.