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REVIEW



Allergen-specific immunotherapy of Hymenoptera venom allergy – also a matter of diagnosis

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ABSTRACT

Stings of hymenoptera can induce IgE-mediated hypersensitivity reactions in venom-allergic patients, ranging from local up to severe systemic reactions and even fatal anaphylaxis. Allergic patients' quality of life can be mainly improved by altering their immune response to tolerate the venoms by injecting increasing venom doses over years. This venom-specific immunotherapy is highly effective and well tolerated. However, component-resolved information about the venoms has increased in the last years. This knowledge is not only able to improve diagnostics as basis for an accurate therapy, but was additionally used to create tools which enable the analysis of therapeutic venom extracts on a molecular level. Therefore, during the last decade the detailed knowledge of the allergen composition of hymenoptera venoms has substantially improved diagnosis and therapy of venom allergy. This review focuses on state of the art diagnostic and therapeutic options as well as on novel directions trying to improve therapy.

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Hymenoptera venom hypersensitivity

There are 24 described orders of insects out of which hymenoptera are the main inducers of severe allergies in humans. Hymenoptera belonging to the genus bee (*Apis*), bumblebee (*Bombus*), wasp (Family: *Vespidae*, Genus: *Dolichovespula*, *Polistes*, *Vespula*), hornet (*Vespa*) and stinging ant (Family: *Formicidae*, Genus: *Myrmecia*, *Solenopsis*), sting humans with high frequency, and thus are the most studied in terms of allergy and allergen-specific immunotherapy¹ (Fig. 1A). Honeybees (*Apis mellifera*) and yellow jackets (*Vespula vulgaris*, in Europe also called wasp) are observed as the most frequent inducers of allergic reactions to hymenoptera venoms in humans, and therefore this review will mainly focus on specific immunotherapy for allergy to stings of these species (also in comparison to *Polistes* species (paper wasps), relevant in the US and Mediterranean areas of Europe). For the review of allergy to other insects, the reader is referred to recent publications in the field.^{2,3}

The frequency of stings, and thus of subsequent allergic reactions, is dependent on geographic, environmental and ecological factors.¹ These factors can change very fast, which is reflected by the fact that species like *Polistes dominula*, known to be domestic in southern Europe, are invading the US (1970s) from the north-east area to the west coast (1990s),⁴ South Africa (2008)⁵ and also central Europe (1956),⁶ which is most probably due to climate change. Therefore, allergy to

Polistes will gain importance in these areas within the next years.

Hymenoptera venoms are complex mixtures of various substances including numerous relevant allergens. The amount of venom that is injected during a sting is species specific. Honeybees inject up to 140 μg of venom,⁷ with a protein content of around 59 μg .⁸ In comparison, wasps inject venom with a protein content ranging from 1.7 to 3.1 μg (yellow jacket), up to 17 μg (*Polistes* species).⁷ Nevertheless, 70.6% of anaphylactic reactions reported in Europe are caused by stings of wasps and only 23.4% and 4.1% are caused by bees and hornets, respectively.⁹

Clinical manifestations of hymenoptera venom hypersensitivity

In contrast to airborne allergens that have to cross mucosal barriers, venom allergens are injected into the skin and reach the blood easy and fast. Transient pain, itching and swelling are part of the normal response to stings of hymenoptera due to irritative and toxic venom components. In contrast, large local reactions (LLRs) that peak at one to 2 d after the sting and resolve 3 to 10 d later are thought to be part of an allergic reaction to the venom. LLRs are defined by edema, erythema and pruritus and have diameters greater than 10 cm.¹⁰ LLRs are supposed to be

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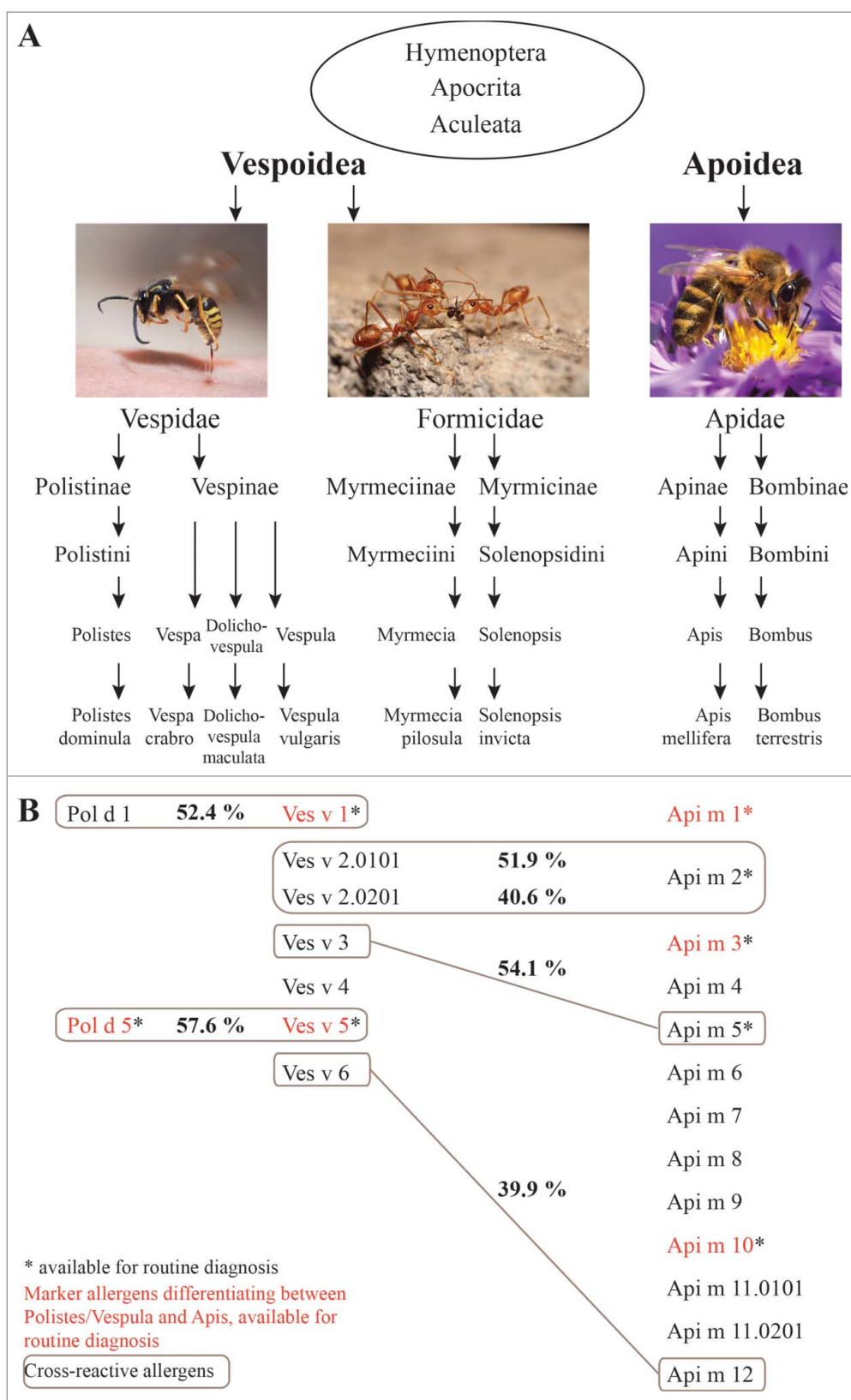


Figure 1. Relevant species and allergens in hymenoptera venom allergy. A, Taxonomy of hymenoptera,^{154,155} with examples of prominent species, which are relevant elicitors of venom allergy. B, Identified allergens of the allergy-relevant hymenoptera species *Polistes dominula*, *Vespula vulgaris* and *Apis mellifera*. Allergens which are marked with an asterisk are available for routine diagnosis. Indicated in red are commercially available marker allergens, used to discriminate between allergies against *Polistes/Vespula* and honeybee venom allergy. Cross-reactive allergens and their sequence identity (in percent) are shown in gray boxes.

IgE-dependent¹¹ or cell-mediated,¹⁰ although previous work described them to be independent of detectable IgE (with the detection limit at that time).¹² It is believed that only

very few patients that suffer from LLRs develop more severe reactions when they are re-stung by the same insect, hence, they are not a predictor of the severity of

allergy.¹²⁻¹⁷ Due to the low risk of systemic reactions, immunotherapy is not recommended for patients experiencing only LLRs.^{18,19}

Systemic or generalized reactions (SR) or anaphylaxis include cutaneous urticaria, angioedema, pruritus, flush, unusual nephropathy, central and peripheral neurologic syndromes, idiopathic thrombocytopenic purpura, rhabdomyolysis, vascular or respiratory symptoms, bradycardia, arrhythmia, angina, myocardial infarction, abdominal cramps, gastrointestinal tract and/or uterine smooth muscle contraction.¹ SRs usually begin 10 to 30 minutes after the sting, but can also arise faster (i.e. in patients with mast cell disorders) or slower (1–4 h) although being less life threatening in the latter case.¹ It is assumed, that 0.4–0.8% of children and 3% of adults show potentially life threatening systemic reactions after an insect sting.^{20,21} Anaphylactic reactions due to stings of hymenoptera can cause a rapid death, since cardiorespiratory arrests can be observed in a median time of 15 min after the sting, a fact that leaves people at risk of severe allergic reaction in great anxiety.²² The only therapeutic options for venom allergy are the prescription of emergency medication (adrenaline/epinephrine auto-injector, anti-histamines, corticosteroids) or, as the only curative treatment, venom-specific immunotherapy (VIT, also named allergen-specific immunotherapy).

Prevalence of venom allergy

Epidemiologic studies by Biló et al.²³ showed that most of the venom-allergic patients suffer from LLRs (ranging from 2.4 to 26.4% in the general population) and that this number can be as high as 38% in beekeepers. Between 0.3 and 7.5% of the population studied, have experienced systemic anaphylaxis (self-reports), whereas the number of severe systemic reactions is as high as 14–43% among beekeepers. The prevalence of venom-allergic reactions in children is only 0.15–0.3%. The estimated number of annual mortalities ranges from 0.03 to 0.45 fatalities per one million inhabitants. But this number could be underestimated as many deaths due to anaphylactic reactions to insect stings probably remain undetected.²³ A survey of the European network of severe allergic reactions (NORA) found that 48.2% (> 18 years) and 20.2% (in children) of severe anaphylactic reactions occur due to insect stings.⁹

Risk factors for severe allergic reactions

Main risk factors for severe allergic reactions to insect venoms seem to be an elevated serum tryptase concentration²⁴ (independent of mast cell disorders) and mastocytosis.²⁵ Nevertheless, it could be shown that if tryptase concentration is above a certain threshold, the risk of severe insect venom-allergic reactions declines.²⁵ The prevalence of severe allergic reactions was found to be 50% in patients with a tryptase level of 20.4 to 29.9 $\mu\text{g/mL}$ and this prevalence is lower than 10% in patients with a tryptase level below 6.1 $\mu\text{g/L}$ and above 191 $\mu\text{g/L}$. However, this prevalence is still higher than in the general population.²⁵ Further risk factors include older age, male sex,

medication of hypertension (ACE, β blockers), diagnosed vespid allergy and preceding stings with systemic reactions.²⁶ Of note, there is no significant difference in the frequency of hymenoptera venom allergy in the non-atopic and atopic population.^{27,28}

Diagnosis and selection of patients for venom-specific immunotherapy

The correct identification of the allergy-relevant insect is of major importance for accurate therapy of venom-allergic patients, as *de novo* sensitizations to the wrong therapeutic extract are possible.²⁹ Besides the thorough and important clinical history, several additional tools exist to analyze sensitization and/or allergy against insect venoms *in vivo* and *in vitro*. According to the guidelines of the allergy academies of Europe and the USA, there is a unique diagnostic algorithm for each patient which has to be gone through in different ways, depending on the diagnostic results.¹⁹ During diagnosis and the decision process for venom-specific immunotherapy, clinicians are advised to discuss the risks and benefits of therapy for each individual case.¹⁹

History

Standard diagnosis begins with the detailed survey of the medical history of the patient, including previous sting reactions (time course and severity of the reaction, number of stings, all associated symptoms and treatments), the assessment of potential risk factors (such as medication, cardiovascular risks and other diseases) and the identification of the insect causing the allergic reaction.¹ Unfortunately, most of the patients have difficulties to correctly identify the insect that has stung and caused the allergic reaction.^{30,31} Additionally, the severity of stings is often under- or overestimated because of fear, panic, exercise, heat, alcohol or underlying cardiorespiratory disease. Furthermore, if the sting reaction was experienced already a long time ago, sometimes it is only poorly remembered.¹ Therefore, in most of the cases further diagnostic tests are needed to correctly identify the allergy-relevant insect.

Skin testing

If a patient has had previous systemic reactions or severe dermal reactions (systemic cutaneous or LLRs) after insect stings, further diagnostic tests are recommended. Additionally, under special circumstances such as frequent exposure or a certain lifestyle, further diagnosis and therapy is considerable.^{19,32} Despite improvements in molecular *in vitro* diagnosis, skin testing is the gold standard to diagnose insect venom allergy. There are 2 options for skin testing, skin prick testing and intradermal testing, whereas the intradermal testing is the more sensitive procedure. Skin testing is safe³³ and should be performed at least 2 weeks after the sting reaction to avoid possible false-negative results during the refractory period, in which sIgE can be exhausted. Ideally, skin tests should be repeated one to 2 months later.³⁴

Baseline serum tryptase

It is recommended to determine the basal serum tryptase concentration in all patients with a history of a severe reaction after a hymenoptera sting. Adult patients with mastocytosis and/or elevated baseline serum tryptase are at risk to experience more severe reactions following stings, as well as for severe side effects during venom immunotherapy.^{25,26} In addition, proper diagnosis of venom allergy in mastocytosis patients can be affected since results of sIgE-testing might be more often negative compared with venom allergic patients without mastocytosis.³⁵

Specific IgE

Candidates for VIT can be considered for complementary *in vitro* testing.¹⁹ This can supplement the information of the clinical history and skin testing, even though testing for specific IgE (sIgE) to the hymenoptera venoms in serum is a little lower than (intradermal) skin testing.³⁴ Before the late 1970s testing for sIgE was done with whole insect body extracts,³⁶ but sensitivity could be increased by using purified venoms. The sensitivity could even be increased further by the use of natural or recombinant single allergens.³⁷ Limitations of *in vitro* testing are demonstrated by the fact, that 15% of a population studied by Mosbech et al.³⁸ showed sensitization to wasp and/or bee venom, but only 31% of those had reactions to stings. In a smaller study group of sensitized patients (n = 94), which were additionally diagnosed by sting challenge, only 5.3% showed systemic reactions.³⁹ Nevertheless, the increasing knowledge of single allergen components in the venom of hymenoptera is able to increase the diagnostic sensitivity and specificity in many (unclear) cases by component-resolved diagnosis (see below).^{40,41}

Allergens of hymenoptera venoms

The increasing knowledge of the exact composition of hymenoptera venoms created added value for accurate diagnosis of venom allergy. Moreover, it turns out that the understanding of all allergen components, not only in the venom, but also in therapeutic extracts used for specific immunotherapy, might influence the outcome of the therapy.^{42,43}

According to the WHO/IUIS Allergen Nomenclature Subcommittee,⁴⁴ protein components of one allergen source (in this case venom) are considered as allergen if specific IgE binding from at least 2 out of 10 subjects with allergy to the source and no IgE binding from subjects without allergy to the source can be shown. This IgE binding should preferably be demonstrated to the purified (natural or recombinant) protein as well as to the extract of the source. In general, hymenoptera venoms are composed of low molecular weight substances like biogenic amines, basic peptides and proteins of higher molecular weight, of which most have an enzymatic activity. One of the best characterized venoms is that of the honeybee *Apis mellifera*, for which detailed analyses of venom proteins are available.⁴⁵ More recently 113 proteins and peptides were identified in honeybee venom.⁴⁶ These detailed analyses were also able to reveal seasonal changes of venom composition.⁴⁷ Together with the proteomic data, the unraveling of the genomic information of the honeybee⁴⁸ simplified the production of single recombinant allergens, advancing the field of component-resolved diagnosis. Recently, also the genome of the invasive species *Polistes dominula* was sequenced,⁴⁹ a fact that will probably simplify the discovery of novel allergens of *Polistes* species in the future. Unfortunately, detailed proteomic venom analyses comparable to those for the honeybee are still missing for *Vespula* species. An overview of identified honeybee, yellow jacket and *Polistes* allergens is given in Table 1.

Table 1. Identified venom allergens from honeybee (*Apis mellifera*), yellow jacket (*Vespula vulgaris*) and paper wasp (*Polistes dominula*). MW, molecular weight; CRP, carbohydrate-rich protein; DPP IV, Dipeptidyl peptidase IV; DW, dry weight; MRJP, major royal jelly protein.

Allergen	Name/Function	MW (kDa)	% of DW	Potential N-glycosylation
Honeybee (<i>Apis mellifera</i>)				
Api m 1	Phospholipase A2	17	12	1
Api m 2	Hyaluronidase	45	2	3
Api m 3	Acid phosphatase	49	1–2	2
Api m 4	Melittin	3	50	0
Api m 5	Allergen C/DPP IV	100	<1	6
Api m 6	Protease inhibitor	8	1–2	0
Api m 7	Protease	39	?	3
Api m 8	Caarboxylesterase	70	?	4
Api m 9	Carboxypeptidase	60	?	4
Api m 10	CRP/icarapin	55	<1	2
Api m 11.0101	MRJP 8	65	?	6
Api m 11.0201	MRJP 9	60	?	3
Api m 12	Vitellogenin	200	?	1
Vespula (<i>Vespula vulgaris</i>)				
Ves v 1	Phospholipase A1	35	6–14	0
Ves v 2.0101	Hyaluronidase	45	1–3	4
Ves v 2.0201	Hyaluronidase (inactive)	45	?	2
Ves v 3	DPP IV	100	?	6
Ves v 5	Antigen 5	25	5–10	0
Ves v 6	Vitellogenin	200	?	4
Polistes (<i>Polistes dominula</i>)				
Pol d 1	Phospholipase A1	34		1
Pol d 4	Protease	33		6
Pol d 5	Antigen 5	23		0

The best characterized allergen of honeybee venom is the phospholipase A2 or Api m 1.^{50,44} In contrast, the venoms of yellow jackets contain phospholipase A1 (Ves v 1, Pol d 1), which differs in sequence and substrate specificity.⁵⁰ Honeybee and yellow jacket venoms contain hyaluronidases (Api m 2 and Ves v 2).⁵⁰ Recently, for the yellow jacket it was shown, that the hyaluronidase exists in 2 isoforms, Ves v 2.0101 and Ves v 2.0201, whereby the latter is an inactive, but surprisingly the predominant isoform.⁵¹ Not described in other species so far, are the acid phosphatase (Api m 3)^{52,53} and the small peptide melittin (Api m 4) of honeybee venom. However, melittin is an allergen of minor importance.⁵⁴ In contrast, Api m 3 was recently identified as a major allergen.⁴¹

Shared between almost all *Vespoidea* venoms (except *Myrmecia*), is an allergen named “antigen 5” (i.e., Ves v 5, Pol d 5). Moreover, it has been shown, that there exists an antigen 5-like protein also in honeybees, which is only expressed in the winter and probably shows no IgE reactivity with allergic-patients’ sera due to missing sensitizing stings in winter.⁴⁷ The antigen 5 protein is of high abundance in wasp venoms, however, its function is still unknown (antigen 5 of *Vespa mandarinia* was shown to be neurotoxic at the neuro-muscular junctions of lobster legs).⁵⁵ Since antigens 5 share a high degree of sequence similarity, and thus of protein epitopes, they are highly cross-reactive and recognized by many *Vespoidea*-allergic patients, independent of the sensitizing species.⁵⁶

Other major allergens of honeybee and yellow jacket venom are the dipeptidyl peptidases IV (DPP IV, Api m 5 and Ves v 3), which also exhibit cross-reactivity due to sequence identity.⁵⁷ Additional minor allergens include the putative protease inhibitor Api m 6,^{58,59} the protease Api m 7,⁶⁰ the esterase Api m 8, the peptidase Api m 9, the major royal jelly proteins (MRJP) 8 and 9 (Api m 11.0101, Api m 11.0201)⁶¹ and the cross-reactive vitellogenins Api m 12 and Ves v 6.⁶²

Another clinically relevant protein of honeybee venom is Api m 10 (named icarapin).⁶³ This carbohydrate-rich and unstable allergen of unknown function with at least 9 known transcript isoforms⁶⁴ was recently identified as major allergen with important implications for diagnostics and therapy.^{41,43,65} Api m 10 is not only a marker allergen for honeybee venom allergy but also underrepresented in some therapeutic extracts that are commonly used for honeybee venom immunotherapy.^{43,65}

Component-resolved diagnosis (CRD)

In the last years it has become more and more evident that testing for specific IgE reactivity against several single allergens (molecular allergology⁶⁶ or component-resolved diagnosis, CRD) is evolving as a superior tool to support classical allergy testing. For a deep review of developments in the field of CRD in insect venom allergy we refer the reader to a recent review by Ollert and Blank.⁴²

Natural venom extracts used for diagnosis can lead to “false positive” results due to cross-reactivity of IgE directed against antigenic carbohydrate determinants (cross-reactive carbohydrate determinants, CCDs). Up to 75% of double-positive *in vitro* test results with honeybee and yellow jacket venom are caused by IgE to CCDs⁶⁷ and only a minor portion by true

allergy to both venoms. IgE antibodies directed against glycostructures of insect and plant proteins were shown to be of high affinity⁶⁸ but their clinical relevance seems to be low, meaning that to an unknown reason they are causing no clinical symptoms.⁶⁹ Measuring sIgE to CCD markers (MUXF3, horseradish peroxidase, bromelain, ascorbate oxidase) is able to confirm the presence of CCD-specific IgE antibodies as reason of multiple positive test results. However, since specific IgE directed against both (CCD and protein epitopes might be present), the detection of CCD-specific IgE alone does not allow the exclusion of sensitization to protein epitopes of multiple venoms.^{66,67}

To circumvent the problem of CCDs, allergens that cannot be recombinantly produced in *E.coli* (without glycosylation) due to complex 3-dimensional folding, can be expressed in Sf9 (*Spodoptera frugiperda*) insect cells without the naturally occurring insect glycosylation (CCDs).⁷⁰ In contrast to other hymenoptera venoms, venom allergens of *Polistes* species show no immunologically detectable CCD-reactivity.⁷¹

Currently, commercially available hymenoptera allergens for component-resolved allergy testing include rApi m 1, rApi m 2, rApi m 3, rApi m 5 and rApi m 10 for honeybee venom as well as rPol d 5, rVes v 1 and rVes v 5 for yellow jacket venom for the ImmunoCAP platform (Phadia/Thermo Fisher Scientific) and rApi m 1, rApi m 2 and rVes v 5 for the Immulite platform (Siemens Healthcare Diagnostics). EUROIMMUN Medizinische Labordiagnostika AG offers a different test system for rVes v 1, rVes v 5, rApi m 1, rApi m 2, rApi m 10 and in the near future rPol d 1 and rPol d 4. All allergens available for routine diagnosis are produced in a CCD-free form.

By using all of the commercially available allergens for the diagnosis of honeybee venom allergy, it is possible to detect sIgE reactivity in 94.4% of the patients.⁴¹ Köhler et al⁴¹ additionally calculated the contribution of sIgE to single honeybee venom allergens to sIgE reactivity to whole honeybee venom. It was shown, that there is high contribution of Api m 1 (19.6%) and Api m 10 (14.4%), medium contribution of Api m 2 (7.6%), Api m 3 (7.2%) and Api m 5 (8.9%) and low contribution of Api m 4 (2%) and CCDs (2.5%). However, until now there is no clinically meaningful interpretation of such data. The diagnostic sensitivity for yellow jacket (*Vespula vulgaris*) venom allergy was increased to a sensitivity of almost 100% by the use of the 2 recombinant marker allergens Ves v 1 and Ves v 5.^{35,72} Therefore, in most cases where the venom extract-based diagnostics do not allow the differentiation between honeybee and yellow jacket venom allergy due to clinically irrelevant cross-reactivity, the newly available component-resolved diagnostics, using CCD-free allergens, enable the detailed characterization of sensitization profiles and the identification of the venom causing clinical symptoms. Moreover, for patients who are difficult to diagnose due to very low levels of sIgE, component-resolved sIgE testing shows a higher sensitivity compared with venom extract-based testing.⁷²⁻⁷⁶

The situation for the diagnostic differentiation between allergies to the different wasp species is more difficult, as cross-reactivity, independent from CCD-reactivity,^{35,71} is frequently observed between *Vespula* and *Polistes* species, when sIgE to venom extracts⁷⁷ or single allergens⁷⁸ is assessed. This cross-

reactivity is not limited to *Polistes* and *Vesputula* species and shows the need for novel marker allergens.⁵⁶

In the last years component-resolved approaches were also used to analyze products used for venom immunotherapy. These analyses revealed that particular allergens are underrepresented in some therapeutic extracts⁶⁵ and this might be a reason for therapeutic failures.⁴³ An overview of clinically relevant allergens and allergens which are relevant for molecular diagnostics is given in Fig. 1B.

Basophil activation test (BAT)

In line with component-resolved diagnostics is the testing for the activation of patient-derived basophils by allergens (basophil activation test, BAT). In this test, whole blood is stimulated with venoms (or single allergens) and the subsequent activation of basophils is measured by the detection of CD63 upregulation (as a consequence of cell degranulation) on the surface of basophils by flow cytometry.

Diagnosis with the BAT is only recommended if all other diagnostic methods fail to detect an allergy against venom, despite a convincing history of an allergic reaction.^{19,23} A study could demonstrate that 81% out of 21 patients without detectable sIgE could be diagnosed using BAT.⁷⁹ This method can even be useful to gain more detailed information about the insect, the patient is allergic to.⁸⁰ Unfortunately, detailed clinical information whether if BAT can predict the severity of a reaction to an insect sting is missing due to ethical limitations on diagnostic sting challenges.⁸¹ Moreover, there is some evidence, that BAT can support the diagnosis of venom allergy in mastocytosis patients with negative sIgE and low total IgE,⁸² whereas other studies are not able to show clear results for the diagnosis of this patient group using BAT.^{83,84} Nevertheless, it was demonstrated that BAT can be useful for monitoring the effectiveness of VIT.¹⁹

Allergen-specific immunotherapy and monitoring of venom hypersensitivity

History of VIT

The first described case of specific immunotherapy of venom allergy was published by Braun in 1925, reporting the desensitization of one patient sensitive to bee stings.⁸⁵ This therapeutic approach was based on the administration of a body extract of the abdomen. Whole insect body extract-based therapy was continued for many years, until a randomized trial proved its ineffectiveness. In 1978, the first randomized controlled study using whole venom, extracted from venom sacks, was published and demonstrated high effectiveness.⁸⁶

Treatment protocols for VIT

Modern therapeutic products for specific immunotherapy of venom allergy are venom extracts which are purified by a standardized procedure. The production process of these products is regulated by country-specific guidelines. Moreover, due to confidential manufacturer-specific processing, the venom extracts produced might differ in terms of composition and

allergen activity. Products for VIT are available as aqueous (or lyophilized) extracts or as depot preparations adsorbed to aluminum hydroxide.

Current guidelines for VIT of the American Academy of Allergy and Clinical Immunology (AAAAI) recommend the treatment of patients who had a history of a systemic sting reaction.¹⁹ Guidelines of the European Academy of Allergy and Clinical Immunology (EAACI) further specify the indication for VIT and include children and adults who had systemic reactions including respiratory and cardiovascular symptoms upon stings of hymenoptera.²³ Additionally, both guidelines demand the definite diagnosis either by skin tests and/or specific IgE tests. Patients only suffering from LLRs should not be treated.

The injection for specific immunotherapy represents a medical task and should thus be performed by the physician and done with a 1 ml syringe with fine graduation down to 0.01 ml with an injection needle (size 14–18, short bevel, sufficient length). The injection is made strictly subcutaneously into a lifted skin fold a hand's width above the olecranon on the extensor side of the upper arms.⁸⁷ The guidelines for VIT^{19,23} recommend a starting dose of 1 μ g which is contradictory to package inserts of therapy products (starting dose of 0.1 μ g), as it was shown, that this dose is well tolerated.⁸⁸ The starting dose is then gradually increased up to a maintenance dose of 100 μ g, chosen out of historical reasons, as it was assumed that this is the equivalent venom amount of 2 honeybee stings. To reach the suggested maintenance dose, several protocols exist. The conventional protocol gradually increases the amount of injected venom during 8 to 16 weeks treatment, by injecting increasing volumes and/or concentrations of therapy product every week. The detailed protocol can be found on package inserts or in the guideline for venom immunotherapy.¹⁹ Faster protocols reach the maintenance dose within one week, 2 to 3 d or in up to 4 to 8 hours in rush or ultrarush treatment protocols, respectively.⁸⁹ In clustered or modified rush up-dosing protocols, 2 or 3 injections are given in 30 minutes intervals every 3 to 7 d.³⁴ All up-dosing protocols are safe in most of the patients, with the only exception of ultrarush protocols, where the probability of systemic reactions is somewhat increased.⁹⁰ Fast up-dosing protocols are used in cases, when patients do not have access to specialists for treatment (maintenance treatment can be given by general practitioners) or when there is need for fast protection.^{19,23}

Several studies could show by sting challenge tests, that clinical protection is reached as soon as the maintenance dose is reached^{91,92} and that the treatment interval can be gradually increased from monthly injections to a treatment course of every 6 to 8 weeks without loss of clinical protection.⁹³ In some cases it was shown, that the interval can even be increased to treatment every 3 months.^{19,23} After its introduction, VIT was thought to be a lifelong treatment or a treatment that should be performed until serum IgE and skin tests turn negative. As the majority of patients will stay positive in diagnostic screenings, studies could show that 80–90% of patients are protected from having SR to sting challenge tests or field stings when VIT is discontinued after 3–5 y.^{19,23} Of note, stopping VIT after 3 y might only be feasible for patients with good prognosis (patients with mild reactions and a reduction of sensitivity to venom in response to VIT, either measured by skin tests or

sIgE serum tests) and should not be performed when sting challenge during therapy cannot be performed.⁹⁴ Extending the treatment to 5 y protects the majority of unselected patients.⁹⁵ Several specific risk factors such as older age, very severe reactions to previous stings or during VIT (injection or sting), treatment of less than 5 years, elevated basal serum tryptase and/or mastocytosis, honeybee venom allergy, cardiovascular disease, concomitant treatment with angiotensin-converting enzyme inhibitors or β -blockers, exposure to repeated stings and high skin sensitivity at the time point of stopping VIT are associated with the loss of clinical protection after discontinuation of VIT.⁹⁶ However, all patients continue to have a 10% chance of having a reaction to a future sting, even if venom skin tests become negative.⁹⁷ Hence, it should be kept in mind that that longer treatment periods are associated with a lower risk of relapse⁹⁸ and that the only way to keep the risk down to 2% is to remain on maintenance immunotherapy.⁹⁴ Therefore, to keep the risk of relapse after stopping VIT as low as possible, thought must be given to prolonging treatment or even maintaining it lifelong, especially for high-risk patients. Considering life-long treatment regimens it surely would be worth to investigate if treatment intervals can be extended to longer than 3 month.^{19,23}

Immunological mechanisms of VIT

Venom-specific immunotherapy aims to induce a shift from pro-allergic and pro-inflammatory Th2 conditions, present in an allergic individual, toward a tolerating state of the immune system (Fig. 2). The induction of this tolerogenic reaction to venom allergens during VIT is characterized by several changes within cellular and humoral parameters.

Whereas in an allergic individual upon a sting, venom-specific Th2 cells are thought to induce and initiate the allergic reaction, venom-specific regulatory T cells (Treg) are thought to be induced during VIT.⁹⁹ These Tregs are able to suppress the pro-allergic Th2 cells. Further, the Th2 suppression leads to the reduction of the secretion of particular cytokines such as IL-3, IL-4, IL-5, IL-9 and IL-13, reduces the activation and degranulation of mast cells, eosinophils and basophils and, therefore, dampens the inflammation.⁹⁹

During the last years, the beneficial role of changes within the B cell compartment during VIT, became more and more obvious.¹⁰⁰ A switch from IgE-producing pro-allergic B cells to IgG4-producing B cells with a regulatory phenotype (secretion of IL-10 and TGF- β) was shown to be important, as blocking IgG4 antibodies are supposed to have a protective anti-inflammatory role.¹⁰¹ These regulatory B cells are also able to suppress venom-specific T cell proliferation¹⁰² and to induce additional Tregs,^{103,104} and thus amplify the shift toward a tolerogenic phenotype of the immune system (Fig. 2). During the course of immunotherapy, specific IgE increases at the beginning and gradually decreases starting some month after the initiation of immunotherapy. Nevertheless, this time course of sIgE does not correlate with the clinical improvements of allergy.¹⁰⁵

These protective shifts in the T and B cell compartment together with the resulting changes in cytokine profiles and antibody classes are useful characteristics which should be

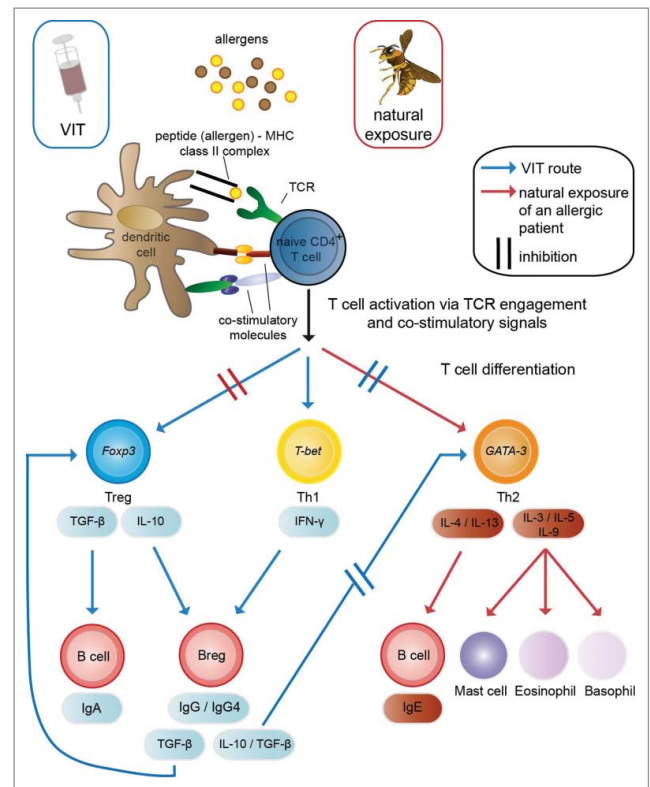


Figure 2. Mechanisms of venom-specific immunotherapy (VIT) compared with the allergic immune response. Venom allergens are injected into the skin, either by the allergy-causing insect (natural exposure) or by subcutaneous injection during VIT. Skin-resident dendritic cells take up the allergens, process them and present derived peptides in a complex with MHC class II molecules to allergen-specific naive CD4⁺ T cells. In a venom-allergic individual this leads to their differentiation into Th2 cells (key transcription factor: GATA-3), which secrete the cytokines IL-3, IL-5 and IL-9, contributing to the activation and degranulation of mast cells, eosinophils and basophils, as well as IL-4 and IL-13 which induce the production of IgE by B cells. These inflammatory processes elicit the allergic reaction and suppress a tolerogenic phenotype of the immune response, which can be observed in individuals not allergic to insect venom. A shift of this Th2-directed reaction toward a tolerogenic reaction is observed during VIT, characterized by the differentiation of allergen-specific naive T cells to Tregs (key transcription factor: Foxp3) and Th1 cells (key transcription factor: T-bet). The effector cytokines of Tregs and Th1 cells then lead to the suppression of Th2 cells and their inflammation-promoting functions, therefore causing desensitization of mast cells and basophils, as well as the induction of IgA-producing B cells and Bregs. These Bregs then produce protective blocking IgG4 antibodies, further enhance the differentiation of Tregs via TGF- β and can inhibit the differentiation of Th2 cells via IL-10 and TGF- β .^{99,100,102-104} Breg: regulatory B cell; Foxp3: Forkhead Box P3; GATA-3: GATA binding protein 3; Ig: Immunoglobulin; IL: interleukin; MHC: major histocompatibility complex; T-bet: T-Box 21; TCR: T cell receptor; TGF- β : Transforming growth factor β ; Th: T helper cell; Treg: regulatory T cell; VIT: venom-specific immunotherapy.

monitored during VIT studies, to be able to define predictive markers for clinical outcome of the therapy in the future.

Putative options for therapy monitoring

Until now there is no laboratory test available to predict the safe end point of VIT. In most cases, this is examined by sting challenge tests in a clinic situation.¹⁰⁶ This procedure can be quite stressful for patients because of the risk to develop severe systemic reactions. Hence, there is ongoing research effort to find predictive biomarkers to monitor VIT to decide if the therapy has been successful. One big drawback of most of the studies is the lack of sting challenges during or after VIT, to be able to correlate any of the biomarkers to the success of therapy.

Starting in 1983, studies of IgG (and IgE) antibody concentrations in serum against venoms were conducted, but they could not correlate the amount of IgG to the protection against allergy after stings.^{107,108} Nevertheless, monitoring the thresholds of skin reactivity, total serum IgE, specific IgE, specific IgG and specific IgG4 in patients that completed VIT showed that no severe reactions upon field stings were observed when VIT induced changes in at least 3 of these parameters.¹⁰⁹

Studying restimulated PBMCs of patients before, during and after VIT showed a shift from Th2 to Th1 cells¹¹⁰ or a reduction of ICOS upregulation¹¹¹ and an increase in IL-10 producing T-cells.¹¹² None of the mentioned studies correlated these findings to the clinical outcome of VIT. In line with these results, an induction of regulatory T cells during VIT could be observed. This finding also correlated with a shift of venom-specific IgG4 and IgE, but no correlation to the clinical outcome was made.¹¹³

B cell responses of patients on VIT showed clear similarity to non-allergic beekeepers. This includes the expansion of IL-10 producing BR1 cells, plasmablasts and as Api m 1-specific class-switched memory B cells within the IgG4 producing cells.¹¹⁴ However, there are no conclusive data sets that show whether these parameters could be useful markers of clinical successful VIT.

Studies of basophil activation before, during and after VIT show that basophil activation sensitivity at low allergen concentrations decreases during VIT.^{115,116} Moreover, recent studies demonstrate that there is an increase of basophil sensitivity during therapy, but this increase declines again after 18 months of therapy.¹¹⁷ The lower sensitivity of basophil activation correlates with the increase of serum specific IgG4.¹¹⁸ Correlations to the clinical outcome of therapy were not made in studies with basophil activation. Nevertheless, it could be shown that a higher sensitivity before VIT was associated with more severe side effects during the up-dosing phase of VIT. There is evidence, that no changes in basophil sensitivity can be correlated with a positive sting challenge after therapy.¹¹⁶

Other changes during VIT include the increased expression of osteopontin on mRNA¹¹⁹ and protein level in serum,¹²⁰ changes of miRNA expression (lower expression of miRNAs involved in allergic inflammation and higher expression of those involved in tolerance induction) during VIT up-dosing¹²¹ and the decreased spontaneous release of prostaglandin E2 and an increase of lipoxin A4 at the beginning and a decrease after 6 months of therapy.¹²² None of the studied biomarkers were linked to clinical outcomes. These studies are needed, to find predictive markers, so the use of potentially dangerous sting challenge tests are not needed anymore.

Studies of effectiveness and safety

Side effects

Comparable to allergen-specific immunotherapy for other allergens, there is a significant risk of systemic reactions (14.2% honeybee VIT and 2.8% wasp VIT).¹²³ Nevertheless, a study by Mosbech et al.¹²⁴ showed that most of these systemic reactions do not need medical treatment, proving that the side effects are not severe. The development of LLRs at the site of injection is

experienced by 12.7%, 15.2% and 11.5% of patients undergoing bee, wasp and bee and wasp VIT, respectively.¹²³ Such reactions can easily be managed by premedication with anti-histamines or glucocorticoids.¹²⁵⁻¹²⁷ There is even evidence, that premedication with anti-histamines can improve the efficacy of VIT.¹²⁸ If systemic reactions occur repeatedly, especially before reaching the maintenance dose, the change from standard treatment protocols to rush up-dosing protocols, optionally together with premedication, can be very helpful.¹²⁹

Premedication may include the use of Omalizumab, a recombinant humanized monoclonal anti-IgE antibody approved as add-on therapy for severe allergic asthma and chronic spontaneous urticaria. Omalizumab binds free IgE and, hence, prevents IgE binding to FcεRI on mast cells, basophils and eosinophils, consequently leading to its downregulations.¹³⁰ In patients with repeated systemic reactions to VIT it is difficult to reach the maintenance dose. In several published case reports Omalizumab has been successfully used for the pre-treatment of patients who experienced systemic reactions to VIT, including patients with indolent systemic mastocytosis.¹³¹⁻¹³⁵ Most of these patients were able to tolerate VIT after Omalizumab pre-treatment. However, the duration of therapy and optimal dosing schedule in this clinical setting is not clearly established and a wide variety of different approaches were used. The dosing schedule in most cases was obtained from the approved table for asthma therapy, which is based on body weight and total IgE. While in some cases a single injection before initiation of VIT was used¹³¹ others used 3 to 5 injections.^{132,136} In other cases Omalizumab therapy and VIT were combined for several months^{132,133,135} or even an unlimited pre-treatment before every maintenance dose was administered.¹³⁴ This suggests, that the optimal treatment schedule with Omalizumab depends on the individual response to VIT administration.

Risk factors for severe side effects during VIT include honeybee venom allergy and elevated basal serum tryptase or mastocytosis.¹³⁷ The use of antihypertensive medication is controversially discussed as a risk factor, as it is a risk factor for anaphylaxis to stings.^{19,26} Nevertheless, a prospective study of patients taking antihypertensive medication during VIT could show that there is no adverse effect on safety and efficacy.¹³⁸ The current guidelines recommend, that the start of VIT as well as the up-dosing phase should be avoided during pregnancy, however, maintenance dose treatments should and can be continued.¹⁹

Efficacy

The first controlled trial on the efficacy of venom-specific immunotherapy in 1978⁸⁶ was already able to show, that the risk of subsequent systemic reactions caused by re-stings can be reduced to less than 5%. The same study observed that sting reactions that happened during VIT are usually milder than those before treatment. In general, venom immunotherapy is 75–98% effective in preventing sting anaphylaxis,¹³⁹ whereby efficacy of yellow jacket venom-specific immunotherapy is higher compared with honeybee venom-specific immunotherapy.¹⁴⁰ To date it is not known why VIT is not successful in a minor population of venom-allergic patients. So far, the only

identified risk factor for treatment failure in honeybee venom allergy is a predominant sensitization (> 50% of sIgE to HBV) to the major allergen Api m 10,⁴³ which is present in the venom as well as in therapeutic extracts only in minimal amounts.^{63,65} If patients still react to the venom of hymenoptera stings in a sting challenge setting, increasing the dose used for therapy can protect these patients from further reactions.¹⁴¹ The analysis of 7 controlled clinical trials of venom immunotherapy could not show a statistically significant reduction of fatalities with or without treatment due to their rarity, but a significant reduced risk of systemic reactions to a future sting after VIT could be demonstrated.¹²³ Nevertheless, the analysis of the studies mentioned before, suggested that venom immunotherapy is efficient concerning the prevention of allergic reactions of different severities. Furthermore, the authors analyzed 2 non-blinded studies in this context. Here, they could show an significant improvement of quality of life.¹²³ Even after short treatment times of one year, patients had a positive view on VIT, reduced anxiety and less limitations to activities that cause fear of insects.¹²³

Novel approaches to improve immunotherapy

Allergen-specific immunotherapy in general

Allergen-specific immunotherapy is the only curative treatment of allergies of all kinds and despite all efforts to improve patient's quality of life, treatment protocols have not changed in the last years. The patient is treated with increasing dosages of allergen up to a certain maintenance concentration and the treatment continued for several years. The treatment is supposed to re-educate the immune system toward a tolerogenic response to the allergen source. Despite of studies showing significant reduction of symptom scores after allergen-specific immunotherapy,¹⁴² this therapy is still facing challenges. The probability of side effects is high and the long duration of therapy (3–5 years) leads to high costs and low patient adherence. Hence, there is a need for novel adjuvant systems to improve the efficacy of therapy and further attempts to increase the safety of allergen-specific immunotherapy.

The most common adjuvant for allergen-specific immunotherapy is aluminum hydroxide (75%) which is in use since 80 y for this therapy.^{143,144} In products for VIT an excess of aluminum hydroxide is mixed with the venom extract and the mixture injected subcutaneously. Unlike for prophylactic vaccines, the manufacturers of products for allergen-specific immunotherapy are not required to specify the amount of aluminum in their summary of product characteristics or package leaflets.¹⁴⁴ In Europe, 1.25 mg aluminum per injection is considered as the maximum value permitted.¹⁴⁵ The efficacy of aluminum-based adjuvants is not subject to discussion, but it was shown that they are able to stimulate the immune system into the unwanted Th2 direction and to induce the production of IgE.^{146,147} In allergen-specific immunotherapy the cumulative aluminum dose can be more than 50 times higher, compared with the vaccination against hepatitis B.¹⁴⁴

Alternative adjuvants, which are approved for immunotherapy, include L-tyrosine (depot effect) and the Toll-like receptor agonist monophosphoryl lipid A (MPL). Further adjuvants like

LPS, CpG-oligodeoxynucleotides and imiquimod/resiquimod (R837, R848) are currently investigated. Novel delivery systems are liposomes, virus-like particles or biodegradable polymeric carriers.¹⁴⁸

For the reduction of side effects through IgE-mediated adverse reactions to injected allergens and to increase the safety of immunotherapy, allergens can be chemically modified. This includes the use of recombinant hypoallergenic molecules, dimers, trimers, fusion proteins or peptides. For an overview of novel approaches in the field of allergen-specific immunotherapy the reader is referred to a recent review.¹⁴⁸

Venom-specific immunotherapy

Even though novel treatment approaches were tested already in 1987¹⁴⁹ (passive and active immunization to overcome severe side effects), other new methods to improve venom immunotherapy are scarcely investigated and were only tested in animal models. This includes treatment with T-cell epitopes, already published in 1998,¹⁵⁰ and the mucosal pretreatment with a single yellow jacket allergen, a procedure that reduced sensitization of mice to yellow jacket venom.¹⁵¹ Moreover, it was shown that novel delivery systems such as PLGA microspheres and microbubbles could be efficiently loaded with venom or venom allergens.^{152,153} The microbubbles were even used to prevent sensitization in a honeybee venom allergy model in mice. Issues of study design, efficacy and safety for allergen products being developed for specific immunotherapy of allergic diseases are addressed by guidelines of national and international medical agencies.^{87,145}

Conclusion

Venom-specific immunotherapy is proven to be highly effective in improving the patient's quality of life and to significantly reduce the risk for systemic sting reactions during and after therapy. The increasing knowledge about the molecular composition of hymenoptera venoms has created added clinical value over the last decade. Component-resolved diagnostics using recombinant CCD-free allergens enables the differentiation between cross-reactivity and true allergy, and thus in many patients improves the selection of the appropriate immunotherapeutic intervention. Nevertheless, there is still a lack of information about the relevant allergens present in the venoms of different hymenoptera species. This knowledge would help to further increase the sensitivity and specificity of diagnostics and to improve therapeutic extracts used in the clinics. The aim of current developments is to increase efficacy of therapy, and therefore to reduce treatment times and to increase patient compliance. In line, there is a need to identify biomarkers that can efficiently predict the end point of therapy.

As a consequence of the development of component-resolved diagnostic approaches, enabling the identification of various different sensitization profiles to particular venom allergens, a patient-tailored recombinant immunotherapy would be highly desirable. Such a therapy would allow treating the patient only with adequate amounts of the allergens he is sensitized to. Considering the complex sensitization profiles of venom allergic-patients and the unequal distribution of

relevant allergens in therapeutic extracts, such a therapy might have a high potential for superior efficacy and, moreover, would avoid *de novo* sensitizations to additional allergens. However, due to current regulatory requirements for the approval of novel products for allergen-specific immunotherapy and associated costs, such developments might be in the distant future.

Abbreviations

AAAAI	American Academy of Allergy, Asthma and Immunology
ACE	angiotensin-converting enzyme
Api m	<i>Apis mellifera</i>
BAT	basophil activation test
Breg	regulatory B cell
CCD	cross-reactive carbohydrate determinant
CD	cluster of differentiation
CRD	component-resolved diagnosis
DPP	dipeptidyl peptidase
EAACI	European Academy of Allergy and Clinical Immunology
<i>E. coli</i>	<i>Escherichia coli</i>
Foxp3	Forkhead Box P3
GATA-3	GATA binding protein 3
ICOS	inducible T cell costimulator
Ig	Immunoglobulin
IL	interleukin
LLR	large local reaction
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MPL	monophosphoryl lipid A
MRJP	major royal jelly protein
PLGA	poly lactic-co-glycolic acid
Pol d	<i>Polistes dominula</i>
r	recombinant
Sf9	<i>Spodoptera frugiperda</i>
sIgE	specific Immunoglobulin E
SR	systemic (generalized) reaction
T-bet	T-Box 21
TGF- β	transforming growth factor β
Th	T helper
Treg	regulatory T cell
Ves v	<i>Vespula vulgaris</i>
VIT	venom-specific immunotherapy

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MS has received travel support from ALK-Abelló and Bencard. MO has received consultancy fees from Siemens Healthcare, Hitachi Chemical Diagnostics and Bencard; has received lecture fees from Thermo Fisher Scientific, Bencard and Siemens Healthcare; is co-founder of PLS-Design GmbH. CBS-W has received grants from Allergopharma, Leti, PLS-Design and Regeneron; is member of the scientific advisory board of Leti and Bencard; has received consultancy fees from Leti, GLG Consultancy, Allergopharma and Bencard. SB has received speaker's honorarium and/or travel support from ALK-Abelló, Bencard and Thermo Fisher Scientific; has received consultancy fees as an advisory board member and research support from Bencard. AG declares to have no conflict of interest.

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