



Global View on Ant Venom Allergy: from Allergenic Components to Clinical Management

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Abstract

Hymenoptera venom allergy is characterised by systemic anaphylactic reactions that occur in response to stings from members of the Hymenoptera order. Stinging by social Hymenoptera such as ants, honeybees, and vespids is one of the 3 major causes of anaphylaxis; along with food and drug exposure, it accounts for up to 43% of anaphylaxis cases and 20% of anaphylaxis-related fatalities. Despite their recognition as being of considerable public health significance, stinging ant venoms are relatively unexplored in comparison to other animal venoms and may be overlooked as a cause of venom allergy. Indeed, the venoms of stinging ants may be the most common cause of anaphylaxis in ant endemic areas. A better understanding of the natural history of venom allergy caused by stinging ants, their venom components, and the management of ant venom allergy is therefore required. This article provides a global view on allergic reactions to the venoms of stinging ants and the contemporary approach to diagnose and manage ant venom allergy.

Keywords Allergy · Anaphylaxis · Stinging ant · Venom

Introduction

Ants (order Hymenoptera, family Formicidae) are very bio-diverse organisms with 21 recognised subfamilies and more than 13,000 described species [1, 2]. Sixteen subfamilies of ants and 1900 living species have a stinging apparatus and use injected venom for defence against predators, prey capture, and social communication [3, 4]. However, most stinging ants are not aggressive and rarely sting large organisms. There are approximately nineteen genera of ants that

have been associated with venom allergy in humans, but only members of four genera (Fig. 1), namely *Solenopsis* spp., *Myrmecia* spp., *Pachycondyla* spp., and *Pogonomyrmex* spp., have caused anaphylactic reactions and fatalities frequently enough to be considered important medical problems. The risk of envenomation and anaphylactic reaction by a particular species of stinging ant depends on complex interactions between the likelihood of human contact, species aggressiveness, efficiency of the venom delivery apparatus, and venom allergenicity [5, 6]. Individuals with venom allergy will have IgE specific for the venom allergens (sIgE) and if exposed to venom (usually via a sting) can develop symptoms of anaphylaxis that range from urticaria through to respiratory and cardiovascular collapse and death [7].

Causative Species

Solenopsis spp

The genus *Solenopsis* comprises small to medium sized ants of 2 to 6 mm in length. They are commonly referred to as fire ants due to the fierce burning pain inflicted by their sting [8]. There are over 60 known species of fire ants within this genus [9], but only five have been associated

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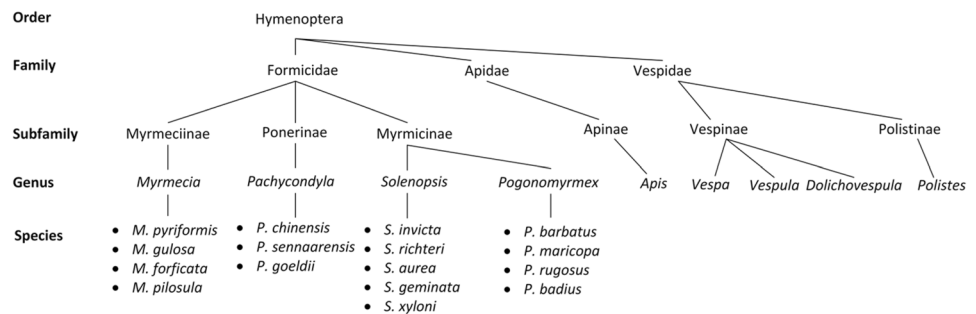
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Fig. 1 Schematic diagram depicting phylogenetic relationships of social Hymenoptera, with an emphasis on stinging ants frequently linked to anaphylactic reactions and death in human



with sting allergy. Fire ants are usually found in large colonies of 250,000 ants or more, and up to 600 colonies per acre may be found in heavily infested areas [8]. Fire ants use pheromones to recruit large numbers of worker ants to sting intruders *en masse* [10], thus achieving a significant antigenic load despite the small amount of venom delivered from an individual sting. The medically important species of fire ants are *S. richteri*, *S. invicta*, *S. aurea*, *S. geminata*, and *S. xyloni*.

S. richteri, the black fire ant, was introduced into the United States of America (USA) from Uruguay or Argentina in 1918 by cargo vessels through the port of Mobile, Alabama [11], while *S. invicta*, the red fire ant, was introduced to the USA from Brazil around the time of World War II [12]. They are often referred to as Imported Fire Ant (IFA) to distinguish them from the native North American fire ant species, *S. aurea* (desert fire ant), *S. geminata* (tropical fire ant), and *S. xyloni* (southern fire ant). The red IFA, *S. invicta*, has also been established in Asia and Australia, while the black IFA, *S. richteri* has been carried as far as Saudi Arabia [10, 13–17]. The tropical fire ant, *S. geminata*, commonly found in Mexico, Central America, and the Caribbean islands [18], has become a serious pest and is the cause of ant sting allergy in Mexico, Southeast Asia, the United Arab Emirates, and on some Pacific islands including Okinawa and Guam [19–22].

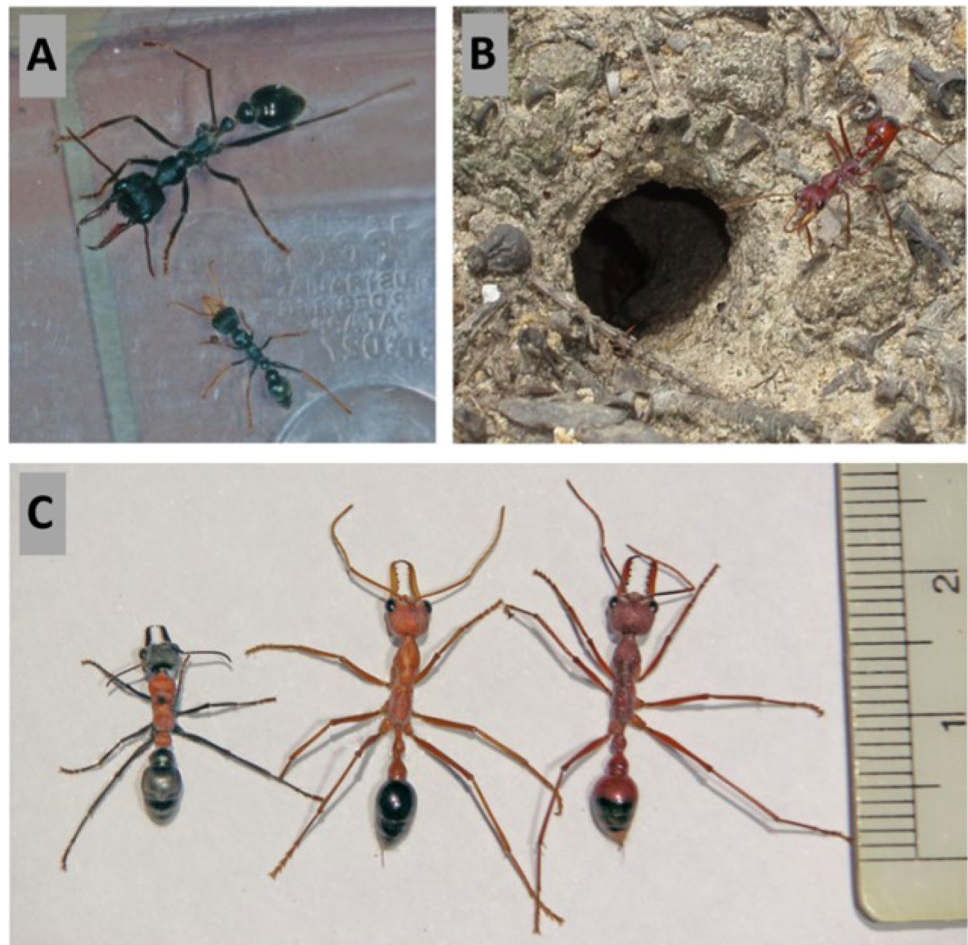
Myrmecia spp

The genus *Myrmecia* is one of the most primitive subfamilies of ants and is widely distributed in and virtually endemic to Australia, with only one species found in New Caledonia [23, 24]. A species of *Myrmecia* ant, *M. gulosa*, was among the earliest Australian insects to be discovered by Joseph Banks and Daniel Solander at Botany Bay in 1770 when they were on shore from Captain Cook's "Endeavour" [6]. *Myrmecia* spp. are highly aggressive ants [25, 26], characterised by a prominent pair of powerful mandibles and large eyes that enable them to use visual cues to navigate [27]. There are 89 recognised species within this genus, which can be broadly divided into two groups based on their morphological characteristics [23, 28]. The first group comprises ants

commonly known as "bulldog ants", "bull ants", "bulljoe ants", "giant bull ants", or "inchman ants" (Fig. 2). Species in this group all share a large body length between 20 to 30 mm [28]. Three of the common types of "bulldog ant" are *M. pyriformis*, which is brown in colour and is common in open sclerophyll woodland in South-Eastern Australia [29], *M. gulosa*, a slender ant with long legs and a variety of colours [28, 30], and the "inchman ant", *M. forficata*, with a purple-brown body and black abdomen that predominates in Tasmania [28].

The second group includes smaller-sized ants, typically 10 to 12 mm in length, which are commonly known as jumper ants (Fig. 2). The most notable species and an archetype for this group is *M. pilosula*, an exceptionally aggressive stinging ant with highly developed vision that is attracted to movement [5, 27]. *M. pilosula* has a jet black body and yellow or orange mandibles and leg tips. These ants move in short jerks and jumps, leading to the colloquial names "jumper ant", "hopper ant", "jumping jack", "black jumper", "jackie jumper", and "jack jumper" [31, 32]. *M. pilosula* is a "species complex" which is comprised of several closely related sibling species with subtle morphological differences [33–35]. Components of the species complex have been recently reviewed, illustrated, and keyed. The six known members of the complex are *M. banksi* sp. nov., *M. croslandi* Taylor 1991, *M. haskinsorum* sp. nov., *M. imaii* sp. nov., *M. imparternata* sp. nov., and *M. pilosula* Fr. Smith 1858 [32]. *M. pilosula* form small colonies of 100 to 1000 ants and their nests range from a single hole to mounds up to a meter in diameter with multiple entrances (Fig. 2), typically surrounded by a scattering of fine gravel [5, 31, 36]. *M. pilosula* is found in sandy soiled areas starting from the north of Brisbane through to the higher altitude and rural areas of South-Eastern Australia and west to the vicinity of Denmark in Western Australia [29, 30, 37]. Several ant species from this genus are an important cause of anaphylactic sting reactions in Australia [6, 28]. Allergy is common, particularly to the venom of *M. pilosula* in Tasmania, regional Victoria, the Adelaide Hills and southern New South Wales [6, 31, 36, 38]. Unlike the massive ant attacks typical of IFA, single or few stings from *M. pilosula* are sufficient to cause anaphylactic reactions in humans [39].

Fig. 2 Pictures of some *Myrmecia* ant species. **a** Large bulldog ant, *Myrmecia pyriformis*, and a smaller jack jumper ant, *Myrmecia pilosula*. **b** A *Myrmecia* ants' nest showing a single entrance. **c** A jumper ant, *Myrmecia nigrocincta*, and two bulldog ants of the *Myrmecia gulosa* species group



***Pachycondyla* spp**

Ants of the genus *Pachycondyla* originate from the neotropics, but have been spread to numerous locations by commerce, including to South-Eastern USA [40–42]. The genus comprises approximately 200 species, most of which appear to be predators who subdue their prey with their venom [43]. Two species, *P. chinensis* and *P. senaarensis*, have been recurrently reported to cause anaphylactic sting reactions in humans [44–49], while another species, *P. goeldii*, has been associated with ant sting anaphylaxis in a single case report [50].

P. chinensis (Chinese or Asian needle ant), originally referred to as *Brachyponera chinensis* until the early 1990s [51, 52], originate from Far East Asia, but are now widely distributed throughout Asian-Pacific countries [41, 53]. *P. chinensis* are 4 to 6 mm in length, with a shiny brownish-black body, orange-brown legs and a well-defined stinger [40]. Similar to *M. pilosula*, *P. chinensis* is also a species complex [51]. Colonies are composed of a few hundred workers and nests tend to be found in damp areas near mature forest, within rotten wood, or in old wooden houses [48, 54]. Although not an aggressive species, *P. chinensis* is

known to have a sting that results in sharp, low-pain intensity that persists for 30 min or more [9, 54–56].

P. senaarensis (samsam or sword ant) has a black and relatively slender 4 to 6 mm long body and is widely distributed throughout the African tropics, Arabian Peninsula, and Southern Iran [42, 55, 57]. Colonies of *P. senaarensis* typically contain several thousand workers in underground nests that are approximately one meter deep [43, 58]. *P. senaarensis* requires an environment with high humidity, so they are commonly found around human settlements with a strong presence in gardens and buildings. *P. senaarensis* is an “opportunistic” ant; in its natural habitat, it has a mainly granivorous diet during the dry season and feeds on animal prey during the rainy season, but in urban areas, the ants scavenge on human food refuse [42, 55, 59]. *P. senaarensis* has a reputation for its aggressiveness and extremely painful sting, with pain that can persist for up to 4 h [20, 43, 57, 60].

***Pogonomyrmex* spp**

Certain members of the genus *Pogonomyrmex* have been reported to cause ant sting anaphylaxis in the USA [53, 61, 62]. Commonly referred to as the harvester ant, they

have large mandibles to collect and feed on seeds from their native habitat [61, 63]. They are 5 to 9 mm in length and reddish to blackish in colour [64]. Harvester ant colonies typically contain more than 20,000 ants, and their nests are usually constructed in sand or soil and may be more than 6 m deep [65]. The genus comprises over seventy species, and they are typically found in arid grasslands and deserts of North and South America [66]. Three species, *P. barbatus* (red harvester ant), *P. maricopa* (Maricopa harvester ant), and *P. rugosus* (rough harvester ant), have been reported to cause anaphylactic sting reactions in humans, particularly around the South-Western North American desert [61, 63, 67]. Although less aggressive than members of the genus *Solenopsis*, harvester ants also sting *en masse* and the sting has long been recognised for its extreme painfulness that may last for up to 24 h [9, 64, 66, 68]. The pain has been described as “ripping muscles or tendons” and like “turning a screw in the flesh” [3]. The venom causes localised sweating and piloerection at the sting site and is accompanied by pain and tenderness in nearby lymph nodes [69]. It is these

properties that have earned them the reputation of being dangerous adversaries to humans [70].

Other Ant Species Associated with Allergic Reactions

In addition to the four genera of stinging ants described above, at least 16 other species have also been associated with allergic reactions in humans (Table 1). One of these, *Rhytidoponera metallica*, is a frequent cause of ant sting anaphylaxis in Queensland [28, 71–73], and some cases have also been reported in country New South Wales [28, 74]. *R. metallica* is a native Australian ant species of the subfamily Ectatomminae [53], which is widely distributed throughout Australia [29]. It is an omnivorous diurnal predator that inhabits open and moderately wooded areas, including metropolitan parks and gardens [75], feeds on insects and invertebrates [76], and forages on seeds and honeydew [75]. The worker ant is 5 to 7 mm long with variation in a metallic colour of green, purple, red, and violet [75].

Table 1 Other ant species reported to cause allergic reactions in humans

Species	Common name	Geographic location of incident	Number of incident(s)	Remarks	References
<i>Dinoponera gigantea</i>	False tocantera ant	Bonito town, Brazil	1	Patient recovered	[77]
<i>Hypoponera punctatissima</i>		Storrington, USA	1	Patient recovered	[53, 78]
		Roosevelt Island, USA	1	Patient recovered	[53]
<i>Odontoponera denticulata</i>		Thailand	Not specified		[79]
<i>Odontomachus bauri</i>	Trap-jaw ant	Caracas, Venezuela	1	Patient recovered	[80]
<i>Odontomachus simililimus</i>	Dalakadiya	Kurunegala, Sri Lanka	1	Patient died	[81]
<i>Camponotus compressus</i>	Common Indian black ant; Kulpe	Karnataka, India	1	Patient recovered	[82]
<i>Cataglyphis bicolor</i>	Sahara desert ant	Benha District, Egypt	Not specified		[83]
<i>Cerapachys spp.</i>	Cannibal ant	Australia	Not specified		[10]
<i>Crematogaster matsumurai vagala</i>		Iksan, Korea	1	Patient recovered	[84]
<i>Formica rufa</i>	Wood ant	Central Europe	1	Patient recovered	[53, 85]
<i>Oecophylla smaragdina</i>	Weaver ant; green tree ant	Northern Australia	Not specified		[86]
<i>Pseudomyrmex ejectus</i>	Twig ant; oak ant	Georgia, USA	1	Patient recovered	[53, 78]
	Twig ant; oak ant	Florida, USA	1	Patient recovered	[53]
<i>Rhytidoponera metallica</i>	Greenhead ant; pony ant	Queensland, Australia	47	Cases spanning 17-year period	[28, 53, 71, 72, 78, 87]
<i>Tetraponera rufonigra</i>	Bi-coloured arboreal ant	Bangkok, Thailand	1	Patient recovered	[79, 88]
	Hathpolaya	Kurunegala, Sri Lanka	1	Patient recovered	[81]
<i>Tetramorium caespitum</i>	Pavement ant	South Carolina, USA	1	Patient recovered	[53, 78, 89]
<i>Heteroponera carinifrons</i>		Chile	Not specified		[90]

Incidence and Prevalence of Ant Sting Allergy

Solenopsis spp

Stings by the IFA are a major cause of systemic allergic reactions in South and Central America as well as in South-Eastern USA [19, 91], and occasional cases have also been reported in Australia, Canada, Saudi Arabia, Spain, and Switzerland [10, 17, 92–94]. In heavily infested areas in Southern USA, sting attack rates range from 13 to 58% of the population per year with the highest attack rates occurring between April and September [8, 12, 95]. In these endemic areas, the incidence of systemic anaphylaxis due to IFA stings exceeds that of other species of Hymenoptera [70, 96]. Rhoades et al. estimated nearly four per 100,000 population develop new systemic allergic reactions to IFA stings per year [97]. In one survey of 29,205 physicians, it was reported that 20,755 patients per year were treated for IFA sting reactions, of whom 413 (2%) had life-threatening anaphylaxis [98]. Other surveys reported that between 0.6 and 16% of those stung by IFA developed anaphylaxis [99]. Over 80 fatal anaphylactic reactions caused by IFA stings have been reported [99–101]. While the overwhelming majority of allergic sting reactions to fire ants are caused by IFA, cases of systemic allergic reactions caused by the less common species of fire ants, *S. aurea*, *S. geminate*, and *S. xyloni* have also been described in India, Indonesia, Thailand, and USA [18, 21, 53, 67, 102–105].

Myrmecia spp

Myrmecia spp. is the predominant cause of ant sting allergy in Australia, being implicated in 89% of anaphylaxis cases due to ant stings [28]. The prevalence of systemic allergic reaction to the venom of *Myrmecia* ants is 0.8% in Queensland, 2.4% in regional Victoria, and 2.7% in Tasmania [38, 72, 106]. Nationally, 60.8% of the 347 hospitalised cases involving ant sting allergy between 2002 and 2005 were attributed to *Myrmecia* spp. [107]. The jumper ant *M. pilosula* is the principal cause, accounting for 57% of all ant sting anaphylaxis incidents in one large nationwide study [28]. In Western Australia the bulldog ants *M. gratioiosa* and *M. nigriscapa*, and a jumper ant, *M. ludlowi*, have been documented as being responsible for 10 cases of ant sting anaphylaxis [73]. In Tasmania, where 12% of the population is exposed to *M. pilosula* stings every year [106], it accounts for 15.4% of the total allergic reactions and cases of anaphylaxis, and between 21 and 25% of anaphylaxis cases treated with epinephrine (adrenaline) at the Emergency Department

of the Royal Hobart Hospital, the only public teaching hospital in Southern Tasmania [31, 108]. Around 1% of the population in Tasmania is at risk of severe and potentially life-threatening anaphylactic reactions to *M. pilosula* stings [106].

Fatal anaphylaxis from *Myrmecia* spp. stings has also been documented. Three ant-sting related fatalities were recorded in New South Wales in 1931 and one fatality was recorded from Tasmania in 1963 [109, 110]. Additionally, six documented cases of ant sting-related fatalities were attributed to *M. pilosula*, *M. pyriformis*, or *M. forficata* in Australian Coronial records between 1980 and 1999; all the deceased were living in either New South Wales or Southern Tasmania [31, 39]. Examination of the National Mortality Database maintained by the Australian Institute of Health and Welfare between January 1997 and December 2005 showed 6 fatalities attributed to *Myrmecia* spp. and arthropods other than honeybees and vespids [111]. In another study using data extracted from the Australian National Coronial Information System database, 2 deaths were recorded between January 2000 to December 2013 that were caused by ant-sting related anaphylaxis [112, 113]. Taken together, the incidence of fatalities due to ant sting anaphylaxis in Australia occurred at a rate of approximately 1 case per 5 years.

Pacycondyla spp

Numerous incidences of allergic sting reactions to the venom of *P. chinensis* have been reported, mostly from South Korea, but cases from Japan and South-Eastern USA have also been reported [41, 44, 45, 48, 114–120]. There have been multiple reports of allergic sting reactions caused by *P. sennaarensis* in Saudi Arabia, South-Eastern Iran and the United Arab Emirates (UAE), including five fatal reactions in the UAE [20, 46, 47, 49, 57, 121–126]. A single case of ant sting anaphylaxis caused by *P. goeldii* was recently reported in Brazil [50]. The proportion of the population that experienced systemic allergic and life-threatening anaphylactic reactions to *P. chinensis* stings in an ant-endemic area of South Korea have been reported to be 2.1% and 1.2%, respectively [115], which is comparable to those seen in the endemic areas for IFA and *Myrmecia* spp. in USA and Australia, respectively.

Pogonomyrmex spp

Stings from ants of the genus *Pogonomyrmex* occasionally result in allergic reactions. At least two deaths and a single anaphylactic reaction were attributed to stings of the red harvester ant, *P. barbatus*, between 1950 and 1974 [67, 127, 128]. During this period, harvester ant stings were also implicated in severe allergic reactions in at least 27 people in

Texas [129]. Further cases of anaphylactic reactions in four patients and large local reactions in another four patients caused by either *P. maricopa* or *P. rugosus* were reported in Arizona in 1977 [61]. More recently, three cases of anaphylactic reactions by *P. barbatus*, *P. maricopa*, and *P. rugosus* were reported from Arizona and Texas in the early 2000s [53, 105, 130]. The Florida harvester ant, *P. badius*, has a venom that appears capable of causing allergic reactions (see following section), but anaphylactic reactions to this species have not been reported [131].

Ant Venom Components, Venom Allergens, and their Immunological Cross-Reactivity Potential

Knowledge of the composition of ant venoms, particularly venom allergens and their cross-reactivity potential, is a prerequisite for the accurate diagnosis and treatment of ant venom allergy. However, despite the rapid progress in the fields of genomics, transcriptomics, and proteomics over the last decade, there has been seldom comprehensive analysis of venom allergens of stinging ants. The limited amount of venom present in the venom sac limits its analysis and consequently limits the available data on ant venom chemistry and composition [132, 133]. The currently available information shows that ant venoms include a complex mixture of chemicals including proteins, peptides, biogenic amines, hydrocarbons and other organic molecules [3, 70, 132–134]. However, only those venoms that contain proteins and large peptides have been associated with allergic reactions [135].

Compared to other Hymenoptera venom allergens, only a limited number of stinging ant venom allergens have been characterised and named according to the International Union of Immunological Societies (IUIS) criteria for allergen nomenclature [136]. Under this system, allergens are designated according to the accepted Linnéan taxonomic name of their source as follows: the first three or sometimes 4 letters of the genus, space, the first one or two letters of the species, space, followed by an Arabic numeral, which is assigned to individual allergens in the order of their identification [137]. Similar allergen molecules that share common biochemical properties, such as amino acid sequence identity, molecular size and biologic function, are designated as isoallergens or variants [136]. Where isoallergens and variants of an allergen group have been identified, they are designated by suffixes of a period followed by four Arabic numerals. The first two numerals (01 to 99) refer to a particular isoallergen, and the two subsequent numerals (01 to 99) refer to a particular variant of a particular isoallergen [136]. Additionally, allergens may also be referred to as major and minor depending on whether allergen specific

IgE can be detected in more or less than 50% of allergic patients, respectively [138].

Solenopsis spp

The venom of IFA is composed of 2 phases, like that also seen in *S. xyloni* [139], which includes a non-aqueous phase that comprises 90–95% of the total volume, which contains non-allergenic piperidine alkaloids named Solenopsins, primarily of the trans stereoisomer of 2,6-dialkylpiperidines [140–144], and an aqueous phase that contains 10–100 ng of protein [145]. The Solenopsin alkaloids are responsible for local sting reactions and the development of a characteristic sterile pustule formation at the sting site [146]. Forty-six proteins with molecular weights of < 10 to 44 kDa have been identified in the venom of *S. invicta*, 21 of which are toxins. These proteins include various neurotoxins, myotoxin 2-like proteins, and poneracin-like peptides [147]. Immunoblot studies identified five IgE-binding bands in *S. invicta* venom, two of which are major allergens [148]. Four distinct allergenic proteins (Sol i 1–4) have been isolated, sequenced and characterised [145, 149].

Sol i 1 is a major allergen in IFA venom, but comprises only 2 to 4% of the total venom protein [7, 145]. It is a 37 kDa phospholipase A₁ protein, with 35% sequence homology to those found in vespid venom [4, 150, 151]. It contains N-linked carbohydrates, but a study using recombinant carbohydrate-free Sol i 1 indicated that IgE reactivity is directed against the protein determinants [152]. Some honeybee and vespid venom-sensitised individuals produce IgE antibodies that cross-react with Sol i 1 [153], and as such, these individuals may experience allergic reactions to their first IFA sting [135].

Sol i 3 is the second major allergen in IFA venom and makes up about 15 to 25% of the venom protein [19]. It has a molecular weight of 24 kDa and is a member of the antigen 5 family of vespid venom [7, 13]. Sol i 3 does not contain N-linked carbohydrates and displays 47% homology to antigen 5 from vespid venom (Ves v 5) [149], but the three-dimensional structure of Sol i 3 shows very little surface conservation relative to antigen 5 [13, 154], which is consistent with the lack of immunological cross-reactivity [135].

Sol i 2 is a disulfide-linked homodimeric protein of 28 kDa that represents 67% of the total protein content of IFA venom [8, 63, 155]. The three-dimensional structure of Sol i 2 suggests that it is related to pheromone-binding proteins [156]. Sol i 4 is a monomeric protein of 13.3 kDa [91]. It represents about 9% of the IFA venom protein and is the most basic protein component in the venom, with an isoelectric point (*pI*) of 10.08 [99]. It has approximately 35% sequence homology and one less cysteine residue than Sol i 2 monomers, which prevents dimerisation [63, 135, 145,

[155]. Sol i 2 and Sol i 4 are minor allergens and neither contain N-linked carbohydrates [7, 13].

The Sol 2 antigen group shows a lower degree of sequence conservation among different fire ant species, while the Sol 1 and Sol 3 antigen groups appear to be more conserved [13, 150, 157]. However, studies on sera from patients allergic to the native fire ant species *S. aurea* and *S. xyloni* show some evidence of immunological cross-reactivity with the Sol i 2 and Sol r 2, suggesting that similar IgE-binding epitope(s) is/are present in the venom of these species [18, 158]. *S. richteri* venom does not contain a protein analogous to Sol i 4, but a homologous allergen Sol g 4 is present in the venom of *S. geminata* [13].

***Myrmecia* spp**

Studies on the venom components of *Myrmecia* spp. have been performed on *M. pilosula*, *M. gulosa*, and *M. pyriformis*. The venoms of *M. pilosula* and *M. pyriformis* contain phospholipase A₂, phospholipase B, hyaluronidase, acid and alkaline phosphatases, histamine-releasing activities, and a heat-sensitive haemolytic factor [159–163]. Analysis of *M. gulosa* venom shows the presence of 13 unique peptides named MIITX-Mg and six proteins, including a venom antigen 5, a venom dipeptidyl peptidase IV, a phosphatase, an esterase, and a hyaluronidase [164–166]. Histamine has been found in all three *Myrmecia* venoms, with *M. pyriformis* venom containing a significantly higher proportion than honeybee venom [161, 162, 165], and *M. forficata* venom is also exceedingly rich in histamine [167]. Pharmacological studies on *M. pilosula* and *M. pyriformis* venoms showed that they are able to promote the synthesis of arachidonic acid metabolites [162], which is most likely related to the presence of phospholipase A₂ [168].

Three peptides in *M. pilosula* venom have been recognised as allergens by the International Union of Immunology Societies (Myr p 1, Myr p 2 and Myr p 3 [169]), and another histamine-releasing peptide named Pilosulin 5 [170]. Myr p 1 is a monomer peptide of 6067 Da (also called Pilosulin 1 and M-myrmeciotoxin-Mp1a); Myr p 2 is a disulfide linked heterodimer of 5608 Da (also called Pilosulin 3 and delta-Myrtoxin-Mp1a); Myr p 3 is a disulfide linked homodimer of 8198 Da (also called Pilosulin 4.1); and Pilosulin 5 is a disulfide linked homodimer of 8546 Da [170–174]. All four peptides are highly basic and multiple isoforms of these peptides have been identified by genetic analysis or mass spectrometry [175]. Myr p 2 is the only major allergen in *M. pilosula* venom and is identified by 80% of the population with *M. pilosula* venom allergy [169, 176]. The three dimensional structures of Myr p 1 and Myr p 2 have been determined by NMR spectroscopy [133, 173]. In addition to the Myr p peptides, a number of components of > 15 kDa with IgE-binding capacity have also been identified (Table 2) [176].

Table 2 Components with IgE-binding capacity in *M. pilosula* venom

Component	Estimated molecular weight (kDa)
Acidic phospholipase A2 isoform X2	22–23
Phosphoglycerate mutase 1	26
Venom allergen 3-like	28
Hyaluronidase-like	33
Arginine kinase isoform X2	40
Esterase FE4-like	62
Venom dipeptidyl peptidase 4	90

The cross-reactivity potential of *Myrmecia* ant venoms has been studied. Using 32 sera from subjects allergic to the stings of *Myrmecia* ants and venom extracts from six *Myrmecia* ants species (*M. pilosula*, *M. pyriformis*, *M. gulosa*, *M. nigrocincta*, *M. tarsata*, and *M. similima*), it was shown that some degree of cross-reactivity exists between the different *Myrmecia* ant venoms analysed [177]. Another study using sera from patients with IFA venom and *Myrmecia* venom allergies demonstrated a low degree of cross-reactivity between IFA and *Myrmecia* venoms [157]. Except for *M. pilosula* venom, IgE-binding components in other *Myrmecia* ant venoms have not been characterised.

***Pachycondyla* spp**

Preliminary evidence indicated that the venom of *P. chinensis* likely contains hyaluronidase, phospholipase A₂, histamine, formic acid, and terpenes [114]. One-dimensional polyacrylamide gel electrophoresis (PAGE) analysis of *P. chinensis* venom extract showed the presence of at least 10 proteins with molecular mass ranging from < 14 to 90 kDa, with the most intense band at approximately 29 kDa [45, 178]. Immunoblot analysis showed the presence of two IgE-binding bands with an apparent molecular weight of 27 and 29 kDa [45]. The presence of these IgE-binding bands was confirmed in a separate study, which also identified six additional IgE-binding bands with apparent molecular weights of 12, 22, 25, 31, 46, and 58 kDa [44]. Results from ELISA inhibition studies failed to detect cross-reactivity between *P. chinensis* and IFA venom, but demonstrated some cross-reactivity with yellow jacket venom and a very high degree of cross-reactivity with *P. solitaria* venom [44, 45, 115].

Analysis of *P. sennaarensis* venom showed the occurrence of a volatile compound phenol-2,4-bis(1,1 dimethylethyl), a few terpenes including an alarm pheromone trimethyl pyrazine, and a small number of unidentified peptides that were in low abundance [43, 58, 179]. An immunoblot study using *P. sennaarensis* venom identified five IgE-binding proteins, two of which had an apparent molecular

weight of 16 and 24 kDa [57, 121]. A cross-reactivity study using immunoblot and sera from patients sensitised to *P. sennaarensis* venom showed some cross-reactivity with IFA venom. All sera tested bound to the 24 kDa allergen Sol i 3 and some of the sera also bound the 13 kDa allergen Sol i 4 [121, 135]. Characterisation of a 23 kDa IgE-binding band in *P. chinensis* venom resulted in the identification of Pac c 3 [178]. Pac c 3 is a member of the antigen 5 family of proteins and has 54% homology to Sol i 3. Pac c 3 was added to the official list of Hymenoptera venom allergens in 2017 [180].

There have been 15 novel peptides with antibacterial, insecticidal, and haemolytic properties identified in the venom of the arboreal ant *P. goeldii*, and these have been collectively named Ponericins [181]. When subjected to two-dimensional PAGE analysis, approximately 45 protein spots were observed—these proteins had an apparent molecular weight between 10 and 66 kDa and *pI* between 4 and 10 [50]. Immunoblot of the resolved proteins using a serum sample from an allergic patient revealed four IgE-binding protein spots with apparent molecular weights between 30 and 45 kDa [50].

***Pogonomyrmex* spp**

The venom of the harvester ant *P. badius* is primarily an aqueous solution with proteins constituting 73% of dry venom weight [13, 63]. Separation of the venom using one-dimensional PAGE revealed the presence of at least nine protein bands [182]. In one comparative study, the venoms of *P. maricopa* and *P. rugosus* were found to contain primarily basic proteins and a pair of highly expressed glycoproteins with a molecular weight of approximately 40 kDa [183]. A biochemical study on *P. badius* venom showed that it contained an exceptionally high concentration/amount of phospholipase A₂, hyaluronidase, acid phosphatase, and lipase, and also contained phospholipase B, alkaline phosphatases and several esterases [3, 69, 184, 185]. Histamine and free amino acids were also found, while kinin-like activity and

kinin agonists were detected in the venom of *P. maricopa* [70, 184, 185]. A study on *P. barbatus* venom described a 34 amino acid polypeptide (molecular weight 3.5 kDa) with a *pI* of 10.0, which was named Barbatolysin [186]. Another polypeptide with a *pI* of 8.0 and a molecular weight of about 8 kDa was also reported in this study [186].

Overall, *P. maricopa* and *P. rugosus* venoms exhibit similar immuno-profiles [183], which is consistent with immunological studies of patients sensitised to *P. maricopa* and *P. rugosus* that demonstrate antigenic cross-reactivity to the venom of seven other harvester ant species [61, 187]. Thus, an individual sensitised to one species of *Pogonomyrmex* is likely to react to a sting from another species. Cross-reactivity studies of *Pogonomyrmex* whole-body extract (WBE) by immunodiffusion with commercial WBE of IFA, *Camponotus* spp. (carpenter ant), *Formica* spp. (wood ant), and a stinging insect mixture of honeybee and vespids did not identify any cross-reactivity potential [61]. The venom allergens in *Pogonomyrmex* spp have not been characterised thus far [66, 135, 150]. The current known allergens in stinging ant venoms are summarised in Table 3.

Diagnosis of Ant Venom Allergy

The symptoms of anaphylaxis following an ant sting are comparable to anaphylaxis caused by other stinging Hymenoptera species. The interval between an ant sting and symptoms is typically less than half an hour [91]. Symptoms of acute allergic reactions can manifest in the skin as itching, flushing, urticaria, or angioedema; gastrointestinal symptoms include abdominal cramps, nausea, vomiting, and diarrhoea; respiratory symptoms include dyspnoea, hoarseness, asthma, or pulmonary oedema; and cardiovascular symptoms include dizziness, hypotension, arrhythmia, collapse, and loss of consciousness [7]. These acute symptoms usually disappear within several hours, but delayed or biphasic anaphylactic reactions to insect stings have also been reported

Table 3 Allergen components of stinging ant venoms

Species	Allergen name (IUIS nomenclature)	Molecular weight (kDa)	Glycosylated	Comments	References
<i>Solenopsis invicta</i>	Sol i 1	37	+	Member of phospholipase A1 protein	[151, 152]
	Sol i 2	28	-	Member of pheromone-binding protein family	[155, 156]
	Sol i 3	24	-	Member of antigen 5 protein family	[7, 13]
	Sol i 4	13.3	-	Member of Sol i 2 protein family	[91, 155]
<i>Myrmecia pilosula</i>	Myr p 1	6.1	-	Monomer peptide	[169, 171]
	Myr p 2	5.6	-	Heterodimer peptide	[169, 171]
	Myr p 3	8.2	-	Homodimer peptide	[169, 172]
<i>Pachycondyla chinensis</i>	Pac c 3	23	-	Member of antigen 5 protein family	[178, 180]

IUIS International Union of Immunological Sciences

[188, 189]. Lasting morbidity such as myocardial or cerebrovascular infarction following severe reactions as well as fatalities may occasionally occur [190–192].

Patients with anaphylaxis to the venom of stinging ants should be referred to an allergist. Evaluation of patients presenting with a potential ant sting allergy must include a detailed clinical history [4, 193]. Effort should be made to identify the offending species, but this may present considerable challenges [194, 195]. Other useful clinical clues to the diagnosis of ant sting allergy may include the description of a typical nest in the vicinity of the sting incident and the development of typical symptoms, e.g. sterile pustules with IFA sting, presence of jumping ants in sandy soiled area with *M. pilosula* sting, piloerection and sweating around the sting site with *Pogonomyrmex* spp. sting, etc. As sensitisation to an allergen does not indicate an allergy to it, such tests should only be performed if there is a definitive clinical history of systemic allergic reaction following a sting [196]. The diagnosis of ant allergy is determined by correlation of the clinical manifestation of sting reactions with sIgE as determined by skin and/or serological testing [197].

Diagnostic Tools

Skin Testing

Skin testing is one of the 2 primary confirmatory tests for the presence of sIgE antibodies that are commonly used for the diagnosis of allergic disease [198] and is the gold standard for the diagnosis of ant venom allergy [199]. A skin prick or intradermal injection can be used to apply venom extract to the skin. However, skin prick testing with Hymenoptera venom is much less sensitive than intradermal injection and is often omitted in favour of the intradermal test [193, 200, 201]. The test concentrations that provide optimal sensitivity and specificity with intradermal testing typically range between 0.001 and 1 µg/mL [200]. Venom concentrations higher than 1 µg/mL may give improved sensitivity but can also cause false-positive results because of local irritative effects [202]. However, if higher concentrations of venom extract (e.g. 10 µg/mL) are required to achieve a greater diagnostic accuracy, dialyzed venom extract, which has naturally occurring histamine removed, can be used to reduce these irritative effects [198, 203]. Skin testing should be performed at least 6 weeks after the sting reaction to avoid possible false-negative results during the refractory period, in which allergen-specific IgE antibody can be exhausted [199].

Skin testing for venom allergy to IFA uses whole body extract (WBE), which is prepared by homogenising whole insect bodies [204, 205]; although venom extract obtained from homogenates of dissected venom apparatuses might be

superior [8, 206], it is not commercially available. Commercial preparations of *S. invicta* and *S. richteri* WBE for skin testing are available from Stallergenes-Greer (Lenoir, NC, USA) and Hollister-Stier Laboratories (Spokane, WA, USA) [10, 57]. Purified extract of *M. pilosula* venom, prepared by the Tasmanian Jack Jumper Allergy Program at the Royal Hobart Hospital is available [207, 208], and has been used in intradermal and in vitro testing for the diagnosis of *M. pilosula* venom allergy [209–211]. WBE of *P. sennaarensis* for skin testing, prepared by Allerbio Laboratory (Varenes en Argonne, France), has been developed for the diagnosis of allergy to *P. sennaarensis* venom [57]. Both WBE formulated in glycerine and venom extracts of *P. chinensis* have been used in skin testing and in *P. chinensis* venom allergy research [44, 45, 115, 178]. Commercial preparations of *P. rugosus* WBE were available from Greer Laboratories, Hollister-Stier Laboratories and Meridian Biomedical Inc. until late 1970s [61, 62].

Serological Testing

Serological (in vitro) based tests are an alternative diagnostic method commonly employed by physicians when skin testing is not possible, e.g. in patients with extensive skin disease, when patients are unable to discontinue medications with anti-histaminergic activity, or due to lack of available reagents or trained staff, or when the skin test is not safe to conduct, e.g. during pregnancy [212]. Currently available in vitro test methods for the diagnosis of ant venom allergy are primarily based on allergosorbent assays for the detection of allergen-specific IgE antibodies. The accuracy of these assays may be affected not only by the activity of the many allergens in each venom but also by their binding characteristics for the solid phase used in the assay [198, 213].

The radioallergosorbent test (Phadebas RAST, Phadia, Uppsala, Sweden) was the first commercial in vitro assay developed for the detection of allergen-specific IgE antibodies [214]. Its original method used allergen coupled to a cyanogen bromide-activated paper disc (allergosorbent) to bind allergen-specific antibodies of all isotypes from serum. After a buffer wash to remove unbound serum proteins, bound allergen-specific IgE antibodies were detected with a radiolabelled anti-human IgE [215]. With technological advancements, the RAST assay has evolved into autoanalyzer-based assays, such as the ImmunoCAP system by Phadia, etc., that mimics the RAST's solid phase chemistry [198]. However, unlike RAST these assays do not use radiolabelled anti-human IgE and are calibrated by means of interpolation of response data from a heterologous serum IgE calibration curve that has been referenced to the World Health Organization IgE serum standard 75/502 [198, 216]. However, the most significant advancement of these next-generation assays was the development of

an encapsulated cellulose-based carrier polymer to which the allergen of interest was covalently coupled [216, 217]. This polymer was three-dimensionally configured into the shape of a small cup and called a CAP. Its use in the Phadia ImmunoCAP system improved the allergosorbent's overall IgE-binding capacity, which led to more rapid assay kinetics and enhanced assay sensitivity [215, 218–220]. The Phadia ImmunoCAP system is commercially available for detecting allergen-specific IgE to *S. invicta* WBE [221, 222], and to *M. pilosula* venom [223, 224]. In addition to the ImmunoCAP system, an enzyme-linked immunosorbent assay (ELISA) or comparable in vitro assay for measurement of allergen-specific IgE has been developed for *S. invicta* [225], *M. pilosula* [223], and *P. chinensis* venom [45]. No in vitro-based assay is currently available commercially for the diagnosis of *Pogonomyrmex* spp. venom allergy.

Sting Challenge Test

A deliberate sting challenge using live insects may be considered as a reference method to confirm ant venom allergy [226, 227]. In this method, the offending insect species is allowed to intentionally inject its venom into the patient's skin under medical supervision [196, 228]. However, the sting challenge test is generally not recommended for routine use in the diagnostic workup of a suspected ant venom allergy, due to the risk that deliberate stings could boost the already diminished sensitisation, cause severe systemic/life-threatening allergic reactions, sensitise or re-sensitise the patient to the venom allergens [229]. Instead, the sting challenge test is frequently employed in research environments where informed consent, placement of intravenous access pre-challenge and appropriate medical personnel with experience in treating anaphylactic reactions are available [196]. In this setting, the sting challenge test is typically used for assessing the effectiveness of venom immunotherapy (see following section) and to identify those who are not yet desensitised by the treatment under study. The sting challenge test with live ants has been used to confirm *P. chinensis* venom as the cause of anaphylaxis [117, 230], and to assess the protection offered by venom immunotherapy against *M. pilosula* venom allergy [210, 211].

Management of Anaphylactic Reactions to Ant Venoms

The treatment of ant venom anaphylaxis is similar to the treatment of Hymenoptera venom anaphylaxis or other causes of anaphylaxis and depends on the manifestations of the reaction. However, the rapid onset of venom-induced anaphylactic reactions requires an aggressive treatment that must be started early. The acute management of anaphylaxis

comprises the prompt use of epinephrine (adrenaline) injection, and potentially, intravenous fluids and/or supplemental oxygen in the presence of hypotension and respiratory symptoms, respectively [231]. Cold compresses, antihistamines, and analgesics may be used to treat swelling, pruritus, and local pain as required [231]. In patients with a high risk of severe anaphylactic reactions from future ant stings, long-term management using a disease-modifying approach called venom immunotherapy (VIT) is indicated [189, 227, 232]. VIT involves subcutaneous administration of increasing amounts of ant venom extract (usually beginning with a dose of 0.001 to 1 µg and gradually increasing during initiation phase to a maintenance dose of 100 µg) for a period of 3 to 5 years to induce and maintain long-term immunological tolerance to the offending venom allergens [227, 233].

Efficacy and Adverse Events Associated with Stinging Ant Venom Immunotherapy

The high efficacy of VIT to prevent future sting anaphylaxis caused by social Hymenoptera was shown in multiple systematic reviews and meta-analyses [234–237]. However, to date there is only one randomised double-blinded, placebo-controlled trial of VIT against stinging ant allergy, whereby efficacy was confirmed with a deliberate sting challenge test [7, 209, 211, 238]. This trial showed that treatment with *M. pilosula* VIT is highly effective at preventing life-threatening anaphylaxis secondary to a deliberate sting with live ants. An objective systemic sting reaction rate of less than 5% was observed in patients in the VIT arm compared to 72% in the placebo arm ($p < 0.0001$) [209].

As VIT involves injecting patients with a venom extract to which they have sIgE-mediated allergy, the most common side effects of VIT are systemic allergic reactions, which more often occur during the up-dosing phase of treatment [234, 239]. Unlike reactions to wild stings, reactions to venom extract given during VIT are usually mild in nature (e.g. itching, swelling and erythema at injection site, urticaria) due to the gradual up-dosing/slow initiation phase. However, in some instances, reactions to VIT can be severe (e.g. hypotensive reactions, anaphylaxis) that they require treatment with epinephrine and/or intravenous fluids [239, 240]. Recurrent systemic reactions can prolong the time required to achieve maintenance dose. Systemic reactions can also be uncomfortable and/or distressing to patients, and when combined with the need for regular visits for treatment continuation, this can reduce patient compliance and premature discontinuation of treatment. For *M. pilosula* VIT, the risk of adverse reactions is influenced by the rate of initiation and target maintenance dose [210, 238]. Higher rates of systemic reaction occur with fast initiation “ultra-rush” compared to slower initiation “semi-rush” with lower maintenance doses.

While there are only WBE extracts available for IFA immunotherapy and no randomised controlled trials, studies investigating IFA WBE immunotherapy have shown an efficacy of greater than 95%, when using a dose of 0.5 mL of 1:10 – 1:100 weight/volume dilution [97, 241–246]. Updated ‘Stinging Insect Hypersensitivity Practice Parameters’ state that *S. invicta* alone or a mix of *S. invicta* and *S. richteri* can be used [204]. Rush IFA WBE immunotherapy has also been performed with a high efficacy rate of > 98% and low systemic reaction rate (5.2%) [243].

Mechanisms of Stinging Ant Venom Allergy and Venom Immunotherapy

The allergic response to Hymenoptera venom, including stinging ants, is initiated by an exposure of antigen presenting cells (APCs) in the skin (including Langerhans cells in the epidermis and dendritic cells (DCs) in the dermis) to venom allergens after sting(s). Under the influence of the cytokine interleukin (IL)-4, APCs present venom allergens to polarise naïve CD4 + T helper (Th) cells to differentiate to the pro-allergic CD4 + phenotype, T helper type 2 (Th2) cells [247]. Interleukin 4 and IL-13 cytokines produced by the activated Th2 cells cause B cells to class switch antibody production to generate sIgE. This sIgE binds to high-affinity FcεR1 IgE receptors on the surface of inflammatory effector cells (mast cells and basophils) and DCs, as well as to low-affinity FcεR2 IgE receptors on the surface of B cells, thus “priming” these cells [248, 249]. Upon subsequent sting exposure(s), venom allergen(s) cross-link receptor-bound sIgE on mast cells and basophils, leading to cell degranulation and the release of inflammatory allergy mediators (including histamine, leukotrienes and prostaglandins) and inflammatory cell enzymes (e.g. mast cell tryptase). Additionally, sIgE forms allergen-IgE complexes that more effectively bind to the IgE receptors on the surfaces of DCs and B cells. This further increases the processing and presentation of venom allergen to naïve Th cells, resulting in enhanced Th2 cell activation and secretion of Th2 cytokines. Eosinophilic activation, maturation, survival and infiltration into tissues occurs in the presence of Th2 cell derived IL-4, IL-5 and IL-13 [247, 248]. This allergic response by ant venom has recently been demonstrated in vivo in venom-sensitised mice using IFA venom proteins devoid of the Solenopsin alkaloids [250].

VIT works through complex immunological mechanisms, which are not completely understood, but appear to affect the fine balance between allergen-specific regulatory T (Treg) cells and Th2 cells, Th1 cells, or both. VIT is thought to induce immunological tolerance by the generation of allergen-specific Treg cells, characterised by production of IL-10 and/or transforming growth factor (TGF)-β.

These cytokines modify the response of Th2 cells to directly or indirectly influence effector cells of allergic inflammation (mast cells, basophils and eosinophils) and B cells, suppress sIgE production and induce the production of ‘blocking’ IgG4 and IgA antibodies against venom allergens [247, 251, 252]. These immunological changes are summarised in Fig. 3. The modulation of IL-4, sIgE and sIgG4 production during VIT was shown in the clinical trials of patients with *M. pilosula* venom allergy [210, 211].

Venom Collection and Purification

Current diagnostic tests for stinging ant venom allergy and the preparations used in VIT are based on extracts of the allergen source materials. Consequently, a fundamental prerequisite is the development of appropriate methods to collect venom extracts. Stinging ants possess a highly variable quantity of dried venom per individual, with the largest ants (e.g. *Myrmecia forficata*) yielding up to 300 µg and the smallest (e.g. IFA) as low as 10 ng of venom protein [145, 182]. In comparison, individual spiders, scorpions, and snakes typically yield between 1 and 100 mg of venom [182]. As stinging ants must typically be collected from their nests in the field, obtaining ant venom in reasonable quantities and purities is associated with practical difficulties [3, 253]. The two main methods of collecting venoms from stinging ants are by manual dissection of their venom sac and by electrostimulation [254].

The venom sac dissection (VSD) technique was first introduced in 1964 and has been used for a variety of different arthropods [255], including stinging ants [28, 29, 45, 50, 68, 177, 186, 209]. This method of venom collection begins with the collection of live insects from their nests, before the insects are snap-frozen at −20 °C or lower. The frozen insects are then thawed for species identification and quality controlled for parasites, dirt and other contaminants, before the venom sac is removed. The collected venom sacs are lightly crushed, homogenised and filtered to purify the venom [256–258]. This basic VSD technique was developed further for commercial purposes by scientists at Vespa Laboratories together with scientists at the Hollister-Stier Laboratories [259]. The VSD procedure requires good hand–eye coordination and patience as the venom sacs are extremely fragile and any rupture during dissection will lead to venom loss [260]. The obvious disadvantage of VSD is that the ants must be sacrificed. This method has been employed to extract venoms from *Myrmecia* [28, 29, 163, 177, 209], *Pachycondyla* [43, 45, 50], and *Pogonomyrmex* ants [68, 69, 184, 186], and is the current method used to obtain venom from *M. pilosula* (the only commercially available standardised ant venom extract) [208].

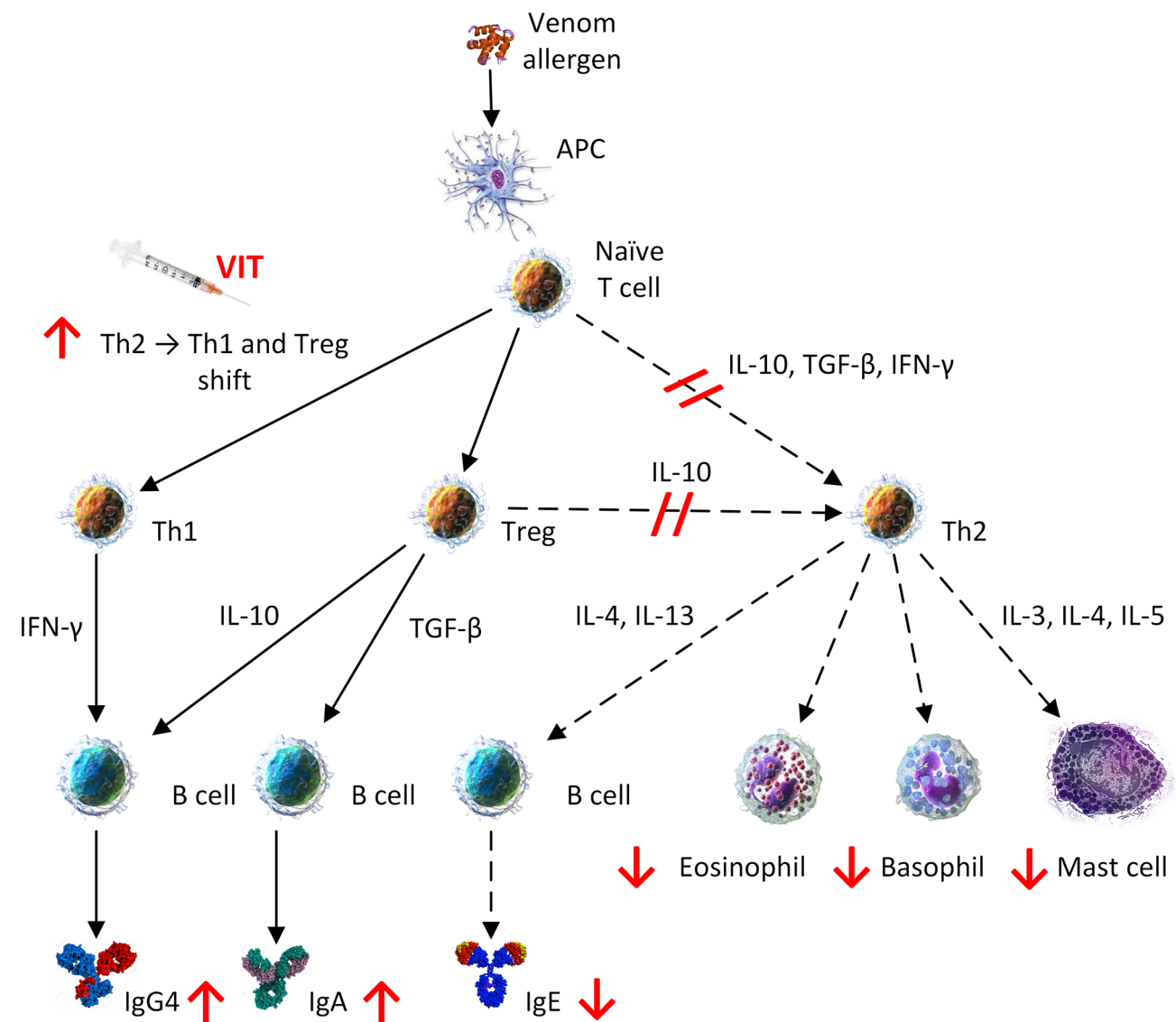


Fig. 3 Proposed immunological mechanisms involved in venom immunotherapy. Symbols in red indicate immunological changes and CD4+ shift which occur as a result of venom immunotherapy. APC antigen-presenting cell, IFN- γ interferon gamma, Ig immunoglobulin, IL interleukin, TGF- β transforming growth factor beta, Th

CD4+ T-helper cell, Treg regulatory T cell, VIT venom immunotherapy. Illustrations remain copyright of: www.ebi.ac.uk (allergen); www.123rf.com (antibodies); www.kisspng.com (APC); www.blausen.com (leukocytes)

The electrostimulation method was first described by Markovic and Molnar in 1954 for obtaining venom from honeybees (*Apis mellifera*) [200] and was refined by Benton et al. in 1963 [261]. The procedure is relatively simple and has been adapted to collect venom from stinging ants [61, 254, 262]. The potential advantages of electrostimulation are that it may give a higher yield of venom if performed *en masse*, is less time consuming and keeps the insect alive for subsequent venom extraction [261]. However, in practice, electrostimulation is not particularly effective for collecting venom from ants, because unlike honeybees, they do not

readily release their venom when stimulated [3]. Regardless, it has been used to collect venoms from IFA [148], *M. pilosula* [171, 208], *M. gulosa* [166], and *Pogonomyrmex* ants [3].

An alternative method of venom collection is to “milk” the venom from individual live ants into glass rods, capillary tubes, or through membranes [150, 257]. The venom is then collected by washing it with an appropriate buffer [260]. Small to moderate amounts of relatively pure venom can be collected, but the method is tedious and inefficient for commercial use [256]. For safety, it is recommended

that ants be chilled and pinned, and handled with the pins while the ants are being “milked” [150].

After extraction from the source material, the resulting venom extract is clarified to separate solid contaminants from aqueous extract. Typically, a series of graded filters is used for clarification, but other techniques such as dialysis and centrifugation may also be used. Venom extract is then filtered through a 0.2 µm filter for sterilisation and is usually freeze-dried and stored frozen (to maximise venom integrity and stability) [256].

Problems do exist in the methods of venom collection and venoms may contain small amounts of non-venom proteins [263]. The main contaminants of venom obtained using electrostimulation are stinger lancets, mouth secretions, faecal material and body hairs [3, 150]. Venom extracts obtained via VSD contain most of the proteins that are in electrically stimulated venom, but also other proteins that are usually contaminants from the venom sac, including structural and cellular proteins [254, 264]. While venom produced by electrical stimulation may give a more genuine representation of the venom components, the method may not be suitable for collecting venom from aggressive ants such as *Myrmecia* [253, 265]. Venom collection by this method can be dangerous as the electrically stimulated species will release alarm pheromones and volatile sting substances, causing relevant species in the area to react simultaneously, resulting in uninvolved persons being attacked and stung [150, 265]. Although both the electrostimulation and VSD methods have been used in the past to obtain venom extract from *M. pilosula* [171, 208], venom from this highly aggressive ant species is best collected using the VSD method for the reasons described above.

The venom sac of each IFA contains about 40 nanolitres of venom solution [142]. This can be laboriously collected by either having live ants directly stinging into capillary tubes or by hand-milking venom from dissected venom sacs into micro-capillary pipettes under a dissecting microscope [8, 148]. Both methods are extremely laborious and the amount of venom obtained is small. More recently, an alternative method for the extraction of IFA venom has been described, which involves soaking large quantities of fire ants in a dual-phase mixture of non-polar organic solvent and water [266]. However, solvent extractions are bound to select for classes of compounds based on their relative solubility and may result in venom losses during the evaporation step [267]. A proprietary modified electrical stimulation technique was developed by Vespa Laboratories for the collection of a prototype commercial-grade IFA venom [148, 150]. However, the currently available commercial products are still prepared from whole body extracts [200].

Standardisation and Stability of Ant Venom Extracts for Human Use

Comparable to other venomous animals, the synthesis and chemical composition of venoms from various stinging ants vary over time and are influenced by endogenous and exogenous factors [268–273]. Likewise, seasonal variation in allergenic composition and activity has also been shown for venom extracts of the IFA, *S. invicta* [274]. Accordingly, allergenic composition in different batches of venom extracts is expected to vary and the use of non-standardised venom extracts in the diagnosis of ant venom allergy and/or VIT is likely to lead to variations in treatment outcomes. To minimise this potential variability, venom extracts must be prepared and standardised according to regulatory requirements for the manufacture of allergen products [275–278]. Although the standardisation of allergen products varies for each allergen extract, the process generally consists of the following steps: (i) characterising prospective reference allergen extract preparations, (ii) identifying the major and minor allergenic components in the allergen extract, (iii) measuring the biological potency of the reference allergen extract in human subjects, (iv) developing and validating in vitro assays to predict biological potency using the reference allergen extract as the standard, (v) obtaining manufacturing licenses for commercial distribution, and (vi) establishing a quality system to monitor the regulatory compliance for each manufactured allergen product [213].

The concept of allergen extract standardisation, which is one of the cornerstones of allergen immunotherapy, was first introduced by Noon in the early 1900s [279]. Allergen extracts are highly heterogeneous mixtures that contain multiple allergenic components as active ingredients in addition to several non-allergenic components [280]. Thus, owing to their complex nature, the standardisation of allergen extracts is challenging. The modern concept of allergen extract standardisation became a reality in the 1970s when the US Food and Drugs Administration (FDA) released the criteria of allergenic potency and allergen composition [281]. Central to allergen extract standardisation is the use of reference standards [282]. Manufacturers of “standardised” allergen products for commercial distribution in the USA must use US reference standards and serum pools specified by the FDA Centre for Biologics Evaluation and Research (CBER) [275, 278]. Many allergenic products such as aeroallergens and animal dander are released and labelled in common bioequivalent allergy units (BAUs) [281], but Hymenoptera, including stinging ant, venom products have units of micrograms of venom protein [157]. In the absence of a US reference standard, the product is exempted by regulation from

potency and stability testing, and is labelled as a “non-standardised” allergen product [275]. On the contrary, those manufacturers of allergen products that follow the quality requirements specified in the European Pharmacopoeia monograph on Allergen Products must prepare their own in-house reference (IHR) standard and create their own allergen extract units accordingly [283–285].

Allergen extract batches manufactured for commercial use must be compared with the reference standard using validated qualitative and quantitative in vitro assays before batch release [256]. Established in vitro assay methods for allergen standardisation must be able to measure allergenic potency and activity, ensure purity and consistency in allergen composition, and demonstrate the stability of relevant allergens in the extracts [277, 286]. Storage conditions, particularly temperature and allergen extract dilution, can affect product quality and potency [287–290]. Endogenous proteases, frequently present in allergen extracts, can also lead to the degradation of some allergens during storage [291–293]. Thus, stability studies must be performed to enable proper expiration dating of allergen products [200, 294].

Initial estimations of allergenic extract potency are made by measuring total protein content in the extract using the FDA-approved Lowry [200], or Bradford, ninhydrin, or bicinchoninic acid methods [258, 280]. During the commercial development of Hymenoptera venom extracts in the late 1970s, the enzymes phospholipase and hyaluronidase were discovered to be major allergenic proteins in Hymenoptera venoms [200]. Functional assays for enzymatic activity confirmed the presence of these enzymes in Hymenoptera venoms and these assays became part of the Hymenoptera venom extract potency testing [200, 278, 295, 296].

The discovery of IgE antibodies by Ishizaka et al. in 1966 [297], and the development of immunoassays that measure allergen-specific IgE, led to the use of RAST inhibition and later, ELISA inhibition assay, in potency testing of allergen extracts [278, 298]. These assays rely on high-affinity binding interactions between allergens and IgE antibodies derived from human allergic sera [280]. However, the immunoassay approach is highly dependent on the ability of sIgE to bind the allergens, and variability of the specificity and concentration of sIgE in serum from the selected patients [213, 299]. The identification, purification, and characterisation of relevant allergenic components from various allergen sources have allowed the direct quantification of specific allergen content in allergen extracts [213]. Assay methods that are based on monoclonal and polyclonal antibodies that recognise specific allergens, such as the sandwich ELISA assay, have been employed for the standardisation of allergen extracts [280, 300]. Unlike the RAST and ELISA inhibition

assays, this approach is not dependent upon variation in the specificity of patients' allergen-specific IgE antibodies [213].

When specific allergen content cannot be quantified, qualitative assay methods can be employed to monitor the identity, consistency and purity of allergen extracts [213]. Additionally, qualitative assays can also serve to complement quantitative analyses [280]. In these methods, banding patterns and allergen profiles of allergen extract components, usually produced after electrophoretic separations in porous gels are compared to reference standards to establish the identity and protein composition in the allergen extract batches. Electrophoresis techniques such as isoelectric focusing (IEF), polyacrylamide gel electrophoresis (PAGE), and crossed immunoelectrophoresis (CIE) have been employed to confirm the identity, consistency and purity of allergen extracts [280, 301]. Allergen profiles in extracts can be assessed using techniques such as PAGE coupled with immunoblotting, crossed radio-immunoelectrophoresis, or mass spectrometry [299, 302].

Presently, there are two stinging ant-derived products available to diagnose and treat ant venom allergy. The IFA WBE are “non-standardised” commercial products available in the USA, which are neither standardised for potency nor for the presence of the Sol i allergens [157]. The concentration of these products is labelled as 1:10 weight/volume of whole-body extracts [256], but an evaluation of three IFA WBE commercial products by crossed immunoelectrophoresis and RAST inhibition assays showed major differences in their antigen contents [303]. Furthermore, batch-to-batch variability and stability of these products are not known [200]. Purified *M. pilosula* venom extract is available in Australia [238]. This product, in contrast, is standardised and labelled with the concentration of venom protein in mg/mL [208, 304]. The standardisation procedure, consisting of five complementary assays (Table 4), satisfies the requirements described in the European Pharmacopoeia monograph for Allergen Products [284]. Information on batch-to-batch variability of this product is available [305], and the stability of *M. pilosula* venom extract and diluted preparations containing as low as 0.1 µg/mL of venom protein has been studied [304, 306].

While there are no other commercially available ant venom extracts, there was one case of successful *P. sen-naarensis* desensitisation using locally made WBE in the Middle East [126], but due to lack of access to this extract no further people have currently commenced desensitisation. There have also been cases of locally made *Pachycondyla* (*P. sen-naarensis*, *P. chinensis*) and *Pogonomyrmex* (*P. rugosus*, *P. maricopa*) WBE but these have only been used for allergy diagnosis, not VIT [44, 57, 61].

Table 4 Analytical methods employed in the standardisation of *M. pilosula* venom extracts intended for venom allergy diagnosis and treatment

Quality requirements	Method	Acceptance criteria	References
Quantitative assays			
Protein content	Bicinchoninic acid	80–120% of IHR	[208]
Allergenic potency	ELISA inhibition	50–150% of IHR	[208, 284]
Relevant allergen content	HPLC–UV	50–200% of IHR	[171, 208]
Qualitative assays			
Protein profile	SDS–PAGE	Comparable protein banding patterns to IHR	[172, 208]
Allergen profile	SDS–PAGE immunoblot	Comparable IgE-binding patterns to IHR	[169, 208]

ELISA enzyme-linked immunosorbent assay, *HPLC–UV* high-performance liquid chromatography with ultraviolet detection, *IHR* in-house reference, *SDS–PAGE* sodium dodecyl sulphate–polyacrylamide gel electrophoresis

Conclusion

Stinging ant venom allergy is a significant public health concern in parts of the world where the ants are endemic. Although current data on the molecular components involved in ant-venom allergy are limited, the venoms are increasingly studied. Diagnosis and clinical management of ant-venom allergy is similar as for other Hymenoptera venom allergies, and as such, if available, VIT should be offered to patients with a high risk of severe anaphylactic reactions from stinging ant venom.

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Declarations

Ethical Approval This paper does not contain any studies with human participants or animals performed by any of the authors.

Conflict of Interest Troy Wanandy, Emily Mulcahy, Wun Yee Lau and Simon Brown are employees or consultants to the Tasmanian Health Service, the organisation owning the intellectual property over *Myrmecia pilosula* Venom Immunotherapy. All other authors declare no conflict of interest.

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