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Invited Review

Anisakis – A food-borne parasite that triggers allergic host defences

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ABSTRACT

Anisakis is a parasitic nematode which infects fish and marine invertebrates, including crustaceans and molluscs. Ingestion of contaminated seafood can cause acute gastrointestinal diseases. Infection can be accompanied by severe allergic reactions such as urticaria, angioedema and anaphylaxis. Diagnosis of allergy due to *Anisakis* currently relies on the detection of serum IgE antibodies to allergenic proteins and a history of reactions upon exposure to fish. *Anisakis* proteins demonstrate considerable immunological cross-reactivity to proteins of related nematodes and other invertebrates such as crustaceans and house dust-mites. In contrast, very limited molecular associations with other parasite groups are observed, including trematodes and cestodes. This review outlines current knowledge on *Anisakis* as a food-borne parasite, with special focus on the underlying immunological mechanisms resulting in allergic host defence responses.

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1. Introduction

Anisakis species are marine roundworms (nematodes) which use marine mammals such as dolphins and whales as primary hosts. The L3 of *Anisakis* infects fish and other seafood such as squid, and consequently humans may become accidental hosts for *Anisakis* if they consume raw or undercooked fish (Sakanari and McKerrow, 1989). Infection is known as anisakiasis (or anisakidosis) and is often associated with acute gastrointestinal symptoms such as abdominal pain, diarrhoea, nausea and vomiting. However patients range from being asymptomatic to requiring emergency room care. In addition, IgE mediated allergic reactions to a range of allergenic proteins are often reported. Some of these allergens, tropomyosin and paramyosin, demonstrate strong molecular and immunological cross-reactivity to other invertebrates, including crustaceans and mites, but are only distantly related to trematodes and cestodes. Since 1960 when anisakiasis was first described, thousands of cases have been reported from Japan and hundreds from Europe as well as from other parts of the world (Audicana et al., 2002).

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2. Biology and life cycle

Anisakis spp. belong to the subfamily Anisakinae, family Anisakidae, superfamily Ascaridoidea, suborder Ascaridina, order Ascarida (Smith and Wootton, 1978). Phylogenetic studies indicate that the human parasite to which *Anisakis* is most closely related is *Ascaris* (Blaxter et al., 1998; Nielsen, 1998). As a genus, *Anisakis* is found world-wide, but *Anisakis* spp. are differentially distributed geographically and utilise different host species (Mattiucci et al., 1997; Paggi et al., 2001; Mattiucci and Nascetti, 2006; Jabbar et al., 2013). Species recognised include the three sibling species of the *Anisakis simplex* complex (*A. simplex sensu stricto*, *A. simplex C* and *Anisakis pegreffii*), as well as the morphologically different *Anisakis typica*, *Anisakis ziphidarum*, *Anisakis schupakovi*, *Anisakis physeteris* and *Anisakis brevispiculata* (Paggi et al., 1985, 2001; Nascetti et al., 1986; Mattiucci et al., 1997, 2001; D'Amelio et al., 2000). With the advent of molecular approaches, it is now possible to identify anisakid nematodes to the species level and to reveal cryptic species (Mattiucci and Nascetti, 2006, 2008). A number of studies have demonstrated that the first and second internal transcribed spacers (ITS-1 and ITS-2, respectively) of nuclear ribosomal DNA (rDNA) provide suitable genetic markers for the identification of anisakid species, irrespective of their developmental stage (Zhu et al., 1998, 2007; Zhang et al., 2007; Shamsi et al., 2011a,b; Jabbar et al., 2012, 2013) and PCR-coupled mutation scanning of the ITS-1 and/or ITS-2, combined with targeted sequencing (Gasser et al., 2006) and phylogenetic analysis (Jabbar et al., 2012, 2013).

provides a powerful approach for exploring the genetic composition of anisakid populations and for investigating their biology. The other genera in the subfamily Anisakinae, collectively known as the anisakids, are *Pseudoterranova*, *Contracaecum* and *Hysterothylacium*.

All of these nematodes appear to have similar life-cycles, although their host species vary (Ishikura et al., 1993; Mattiucci et al., 1997; Audicana et al., 2002). The primary/definitive hosts of anisakids are marine mammals such as whales, dolphins, seals and sea lions, as well as aquatic birds and turtles (Fig. 1). *Anisakis* spp. utilise cetaceans such as dolphins and whales as primary hosts. Eggs are passed into the sea via the faeces. The first moult (L1 to L2 takes place inside the egg, releasing free-swimming L2s that are ingested by tiny crustaceans such as krill (e.g. *Euphausia*, *Tysanoessa*), which are the first intermediate hosts (Smith and Wootton, 1978; Audicana et al., 2002). The crustaceans in turn are eaten by second intermediate hosts, which are fish, larger crustaceans or cephalopods. Inside these hosts, the larvae moult into L3s and become encapsulated on the surfaces of organs or muscles. Larger fish may become infected by eating smaller fish, leading to

an accumulation of larvae with the age of the fish. All L3-infected seafood including fish, crustaceans and molluscs, can cause anisakiasis when ingested by humans. Humans are an “accidental host” in which the larvae cannot complete their life-cycle; other accidental hosts include bears, otters and cats (Davey, 1971; Sohn and Chai, 2005; Torres et al., 2004). In the natural cycle, the L3s in fish are ingested by cetaceans and moult into L4s and then adults. They cluster inside the stomachs of the cetaceans, where the female adult worms are fertilised and lay eggs, completing the cycle. Occasionally, anisakids moult into L4s in humans, but do not progress into adults. *Pseudoterranova* spp. are more likely to moult into L4s than *Anisakis* spp. In a rare case, an adult male worm of *Pseudoterranova* was found in a patient and is regarded as an exception (Kliks, 1983; Ishikura, 1989).

3. Infection and treatment

Live *Anisakis* L3s are capable of infecting humans to cause a disease known as anisakiasis. This disease normally presents as

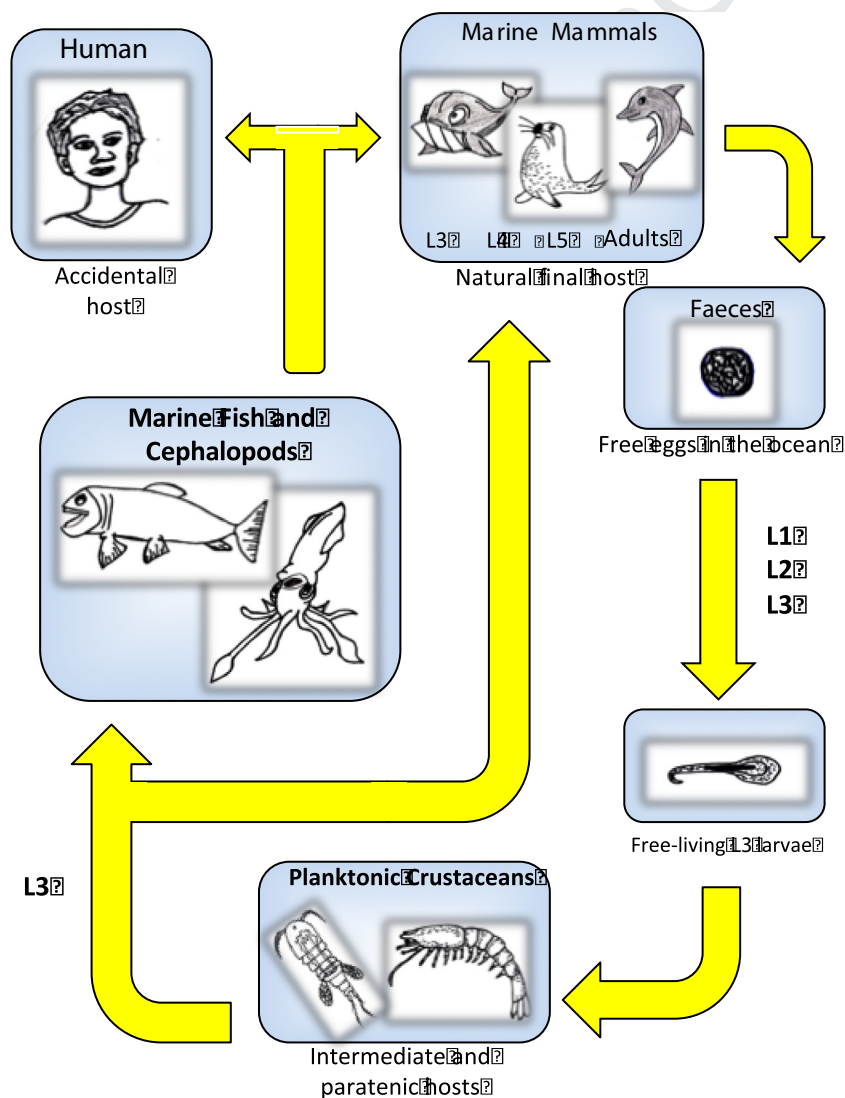


Fig. 1. Life cycle of *Anisakis simplex* including accidental human hosts. Adult parasites live in the stomach of marine mammals and unembryonated eggs are expelled with the faeces. These eggs develop and hatch, releasing free-living *A. simplex* L3s. These L3s are ingested by krill (euphausiid) and copepods, which form the intermediate hosts. Marine fish and cephalopods, which are paratenic hosts, contribute to the dissemination of this parasite by ingesting crustaceans, fish and cephalopods infected with L3s. The infective L3s are mostly embedded in the viscera and muscle and transferred to the final hosts (marine mammals) by ingestion of infected fish, cephalopods or krill. The L3 develops to the adult in the final host, closing the life cycle of this parasite by producing and releasing eggs. Ingestion of raw fish or cephalopods infected with L3s by humans, who are accidental hosts since the larvae do not develop further, can generate adverse reactions through activation of various host-defence responses.

mild to severe abdominal pain, nausea, vomiting and/or diarrhoea within 48 h, and is in some cases accompanied by allergic reactions such as urticaria (hives), angioedema, bronchospasm and even severe anaphylaxis (Audicana et al., 1995; Del Pozo et al., 1997; Daschner et al., 2005). The disease therefore demonstrates an intriguing interaction between parasitic infection and allergic response. Many facets of the immune response to helminths and allergens are similar, but the two diseases do not usually coalesce and the underlying immunological mechanisms are detailed below.

Apart from allergic symptoms occurring during acute infection with *Anisakis*, there are also several case reports of allergy to *Anisakis* proteins occurring in occupational or domestic settings, with symptoms such as asthma, rhinitis, conjunctivitis and dermatitis (Carretero Anibarro et al., 1997; Anibarro and Seoane, 1998; Armentia et al., 1998; Pulido-Marrero et al., 2000; Scala et al., 2001; Nieuwenhuizen et al., 2006; Lopata and Jeebhay, 2013). There are also some cases where consumption of cooked or canned fish appears to have led to *Anisakis*-specific allergic reactions (Del Pozo et al., 1996). Skin prick testing (SPT) with deep-frozen and heat-treated or boiled extracts has demonstrated the resistance of *Anisakis* allergens to cooking, and many *Anisakis* allergens are also resistant to degradation by the digestive enzyme pepsin (Audicana et al., 1995; Caballero and Moneo, 2004). This suggests that immunological reactions might occur after exposure to *Anisakis* antigens alone (versus live larvae) (Audicana et al., 2002).

The management of anisakiasis involves physically removing the larvae, if possible, or treating the patient with antihelminthics, anti-inflammatories and analgesics (Moore et al., 2002; Pacios et al., 2005). *Anisakis* larvae cannot survive or reproduce in humans, but if the larvae are not removed, the disease can become chronic as inflammatory cells surround the larval remains and lead to symptoms which can mimic dyspepsia, Crohn's Syndrome, appendicitis, Irritable Bowel Syndrome, diverticulitis, non-specific eosinophilic enteritis, or even gastric cancer (Audicana et al., 2002). As many cases of anisakiasis have occurred after consumption of freshly caught fish that appeared well-cooked but was not sufficiently heated throughout to kill larvae, ingestion of raw seafood should not be the only factor meriting further investigation.

4. Diagnosis and allergenic proteins

Currently, the diagnosis of *Anisakis* allergy relies on a clear history of potential exposure to *Anisakis* and symptoms of gastroallergic anisakiasis together with *Anisakis*-specific IgE and a positive *Anisakis* SPT (Daschner et al., 2000; Baeza et al., 2001). However, because many allergens of *Anisakis* are heat stable, exposure to *Anisakis* proteins in fish on an ongoing basis can also cause symptoms such as chronic urticaria, contact dermatitis, asthma and rhinoconjunctivitis (Kasuya et al., 1990; Carretero Anibarro et al., 1997; Montoro et al., 1997; Anibarro and Seoane, 1998; Armentia et al., 1998; Scala et al., 2001; Daschner et al., 2005). In this case, the clinical history may be less clear since patients may be exposed to many agents in their environment at the same time. The use of specific IgE alone to diagnose *Anisakis* allergy is confounded by the fact that even asymptomatic individuals can have *Anisakis*-specific IgE due to immunologic cross-reactivity with other helminths (e.g. *Ascaris*, hookworm) or invertebrates such as dust mites, cockroaches and shrimp (Pascual et al., 1997; Kennedy et al., 1988; Asturias et al., 2000). Studies in Spain have found that a large number of asymptomatic individuals have *Anisakis*-specific IgE, some related to subclinical sensitisation and others due to false positive results as a result of cross-reactivity (Moneo et al., 1997, 2000; Pascual et al., 1997).

Since immunological cross-reactivity between related proteins can cause false positives in SPT and specific IgE tests, some authors have used IgE immunoblotting to differentiate anisakiasis/*Anisakis* allergy from asymptomatic *Anisakis* sensitisation (Del Pozo et al., 1997; Garcia et al., 1997; Moneo et al., 1997). One study found that patients with confirmed *Anisakis* allergy had IgE directed at several proteins of medium molecular weight as well as low molecular weight proteins, while patients with no allergy or doubtful symptoms were more likely to recognise either a single medium molecular weight protein of approximately 40 kDa (possibly *Anisakis* tropomyosin) or a few medium molecular weight proteins (Garcia et al., 1997). Another study also found that asymptomatic blood donors with specific IgE to *Anisakis* frequently detected a single protein of 42 kDa whereas truly sensitised patients recognised multiple allergens in the crude extract (Moneo et al., 1997).

The ideal diagnostic test for *Anisakis* allergy should include all clinically relevant *Anisakis* allergens. Currently, ImmunoCAP-radioimmunoassay (CAP-RAST) and SPTs use whole *Anisakis* extracts, while the latest allergen microarrays (ImmunoCAP™-ISAC) encompass *A. simplex* antigen 3 (Ani s 3) (tropomyosin). The determination of specific IgE against allergenic components is central in the recent application of allergen microarray technology. Currently 103 allergenic molecules are fixed on a solid support, allowing the accurate quantification of IgE antibody using only 30 µl of serum. This technology allows a more precise diagnosis of molecular sensitisation and reactivity to specific allergens (Sanz et al., 2011). Once a patient has confirmed with *Anisakis* allergy, after excluding fish allergy and taking into consideration cross-reactivity to other helminths (e.g. *Ascaris*, hookworm) or invertebrates such as dust mites, cockroaches and shrimp, identifying which allergens are recognised by the patient will assist in making dietary recommendations (Moneo et al., 2005). Many patients with *Anisakis* allergy are able to tolerate a diet of frozen or well-cooked fish (Garcia et al., 2001), but a small percentage of patients are particularly sensitised to heat-stable allergens and can react badly to cooked or canned fish (Audicana et al., 2002; Baeza et al., 2004; Moneo et al., 2005). The different allergenic proteins are discussed in detail below.

Currently 12 allergens have been identified in *A. simplex* (Table 1). Patients may be exposed primarily to somatic antigens from dead larvae in food, excretory–secretory (ES) antigens when there is expulsion or surgical removal of the intact larvae, or both, in cases where the larva penetrates the tissue, is killed by the host, and subsequently degenerates inside the host (Audicana and Kennedy, 2008). Many allergens of *Anisakis* are heat- and/or pepsin-resistant (Caballero and Moneo, 2004; Moneo et al., 2005; Caballero et al., 2008) and most of them are present in ES products.

Identified and biochemically characterised allergens are named using the systematic nomenclature of the Allergen Nomenclature Sub-Committee of the World Health Organization (WHO) and International Union of Immunological Societies (IUIS) (Chapman et al., 2007). The system uses abbreviated genus and species names and an Arabic number to indicate the chronology of allergen purification. In the case of *A. simplex*, the very first allergen purified and characterised is named Ani s 1. The major allergens of *Anisakis* (recognised by more than 50% of patients analysed) are considered to be Ani s 1 and Ani s 7 (Anadon et al., 2009), although in one study Ani s 5 was recognised by 49% of patients (41/84). The 24 kDa Ani s 1 is recognised by 67–87% of patients with gastroallergic anisakiasis and is not detected by asymptomatic individuals (Moneo et al., 2000; Shimakura et al., 2004). This allergen is secreted by the worm and shows homology to serine protease inhibitors. A 21 kDa isoform of Ani s 1 also exists (Shimakura et al., 2004). Ani s 1 is heat stable and can act as a food allergen, causing reactions after ingestion of cooked fish. The other major allergen, Ani s 7, is also an ES product of 139 kDa and is a novel glycoprotein

Table 1
Characterised allergens of *Anisakis simplex* (Ani s).

Allergen	MW (kDa)	Compartment	Function	IgE reactivity (%)	Major allergen	Panallergen
Ani s 1	24	ESP	Kunitz-type trypsin inhibitor	85	Yes	
Ani s 2	97	Somatic	Paramyosin	88	Yes	Yes
Ani s 3	41	Somatic	Tropomyosin	4		Yes
Ani s 4	9	ESP	Cystatin	27		
Ani s 5	15	ESP	SXP/RAL protein	25–49		
Ani s 6	7	ESP	Serpin	18		
Ani s 7	139	ESP	Glycoprotein	83–100	Yes	
Ani s 8	15	ESP	SXP/RAL protein	25		
Ani s 9	14	ESP	SXP/RAL protein	13		
Ani s 10	22	Somatic?	?	39		
Ani s 11	55	Somatic?	?	47		
Ani s 11-li	?	Somatic?	?	?		
Ani s 12	?	?	?	57	Yes	

ESP, excretory–secretory products; ?, denotes unknown function, source or molecular weight; bold panallergens are further analysed in Figs. 2 and 3.

(Rodríguez et al., 2008). It was recognised by 100% of patients with *Anisakis* allergy (Rodríguez et al., 2008). However, Ani s 7 has cross-reactive O-glycans and is better for diagnostic tests when deglycosylated (Iglesias et al., 1997).

Another important allergen is Ani s 4, a heat stable nematode cystatin that is recognised by only 27–30% of patients but appears to be particularly important in eliciting anaphylaxis (Moneo et al., 2005). Heat stable allergens such as Ani s 4 are important even if they are classified as minor allergens due to their frequency of recognition, because these allergens are associated with allergic reactions to cooked or canned fish (Rodríguez-Pérez et al., 2008). Therefore, frequency of recognition is not always equal to clinical relevance. Other minor allergens include Ani s 5 (15 kDa), Ani s 8 (15 kDa) and Ani s 9 (14 kDa), which share homology and are all members of the SPX/RAL-2 family, which is specific to nematodes (Kobayashi et al., 2007). They are all heat-stable ES products, although Ani s 9 is reportedly more abundant in crude extracts, and their biological function is unknown (Caballero et al., 2008; Kobayashi et al., 2007; Rodríguez-Pérez et al., 2008). Another minor allergen, Ani s 6 (7 kDa), is homologous with serine protease inhibitors, including the honeybee allergen Api m 6 (Kobayashi et al., 2007). Four additional allergens have been identified, Ani s 10–12 and a 21 kDa protein with homology to nematode troponin that has been identified as an allergen but has never been named (Arrieta et al., 2000). However very little is known about their function or origin. In addition a novel haemoglobin from *A. pegreffii* was recently characterised, which demonstrated strong immuno reactivity and phylogenetic similarity to other invertebrates (Nieuwenhuizen et al., 2013).

The remaining two allergens, Ani s 2 (100 kDa) and Ani s 3 (42 kDa) are the muscle proteins paramyosin and tropomyosin, respectively, and are thought to be primarily responsible for cross-reactivity between *Anisakis* and other invertebrates (Asturias et al., 2000; Pérez-Pérez et al., 2000; Guarneri et al., 2007). The muscle protein tropomyosin is an important source of cross-reactivity with other invertebrates. The ability of tropomyosin to withstand heat treatment and most known forms of food processing techniques can be attributed to its exceptionally stable alpha helical coiled-coil secondary structure (Lopata et al., 2010). A phylogenetic comparison of tropomyosin amino acid sequences, among and between different invertebrate and vertebrate species, demonstrates that tropomyosins of crustaceans, insects and mites are closely related (Fig. 2). Within the nematodes, tropomyosin from *Anisakis* and *Ascaris* are closely related as expected. In contrast, tropomyosins from cestodes and trematodes seem to be more closely related to those of molluscs than those of nematodes. The close molecular relationship of nematode tropomyosin with insect and mite tropomyosins indicates possible immunological

cross-reactivity. It has recently been suggested that nematodes have a very close phylogenetic relationship to crustaceans and mites, which form the group of pan-crustacea. The group Ecdysozoa is a group of protostome animals (Telford et al., 2008) including arthropod (insects, crustaceans), nematode and other smaller phyla, defined by phylogenetic trees using 18S rRNA genes (Aguiñaldo et al., 1997). These data were subsequently supported by Dunn et al. (2008), suggesting the Ecdysozoa as a clade with a common ancestor. Indeed, we recently demonstrated by allergen microarray analysis that all patients with specific IgE antibodies to *Anisakis* tropomyosin (Ani s 3) also recognised tropomyosin of shrimp, dust-mite, cockroach and snail (Lopata, unpublished data). Whether *Anisakis* tropomyosin is a clinically relevant allergen is however controversial. Asturias et al. (2000) suggested that tropomyosin is not an important allergen as asymptomatic patients were sensitised to it whereas symptomatic patients were not (Asturias et al., 2000). Other researchers suggest that *Anisakis* tropomyosin could play a role in eliciting food allergy after ingestion of cooked seafood, because it closely resembles the heat-stable shrimp tropomyosin, an important allergen in seafood allergy (Guarneri et al., 2007). The other pan-allergen, paramyosin, demonstrates a very similar molecular phylogenetic relationship (Fig. 3), with nematode paramyosins being more closely related to paramyosins of mites and insects, whereas trematode and cestode paramyosins are more closely related to paramyosins of other invertebrates such as molluscs.

Purified *Anisakis* allergens have proven useful in diagnosis, especially in combination. In one study, 95% of 64 *Anisakis*-allergic patients tested positive for Ani s 1 and/or Ani s 4 by immunoblotting (Moneo et al., 2007) and in a follow-up study, only 12% of patients (10/84) did not recognise one or both of these allergens (Caballero et al., 2008). Including Ani s 5 in the panel of specific allergens tested raised the sensitivity to 94%, with 79/84 patients recognising one or more of the three *Anisakis* allergens.

5. Prevention of anisakiasis

The best means of avoiding infection with *Anisakis* or related nematodes is to ensure that all fish to be consumed raw or partially cooked is deep-frozen (–20 °C) for at least 24 h (García et al., 2001), or that cooked fish reaches a temperature of at least 60 °C throughout for 10 min or longer (Audicana et al., 2002). The number of cases of anisakiasis in the Netherlands has dropped to almost zero since these measures were included in legislation (Feldmeier et al., 1993). Other countries have taken similar measures. Current European Community regulations require visual examination of fish with removal of heavily parasitised specimens from the market, and extraction of visible larvae in less heavily

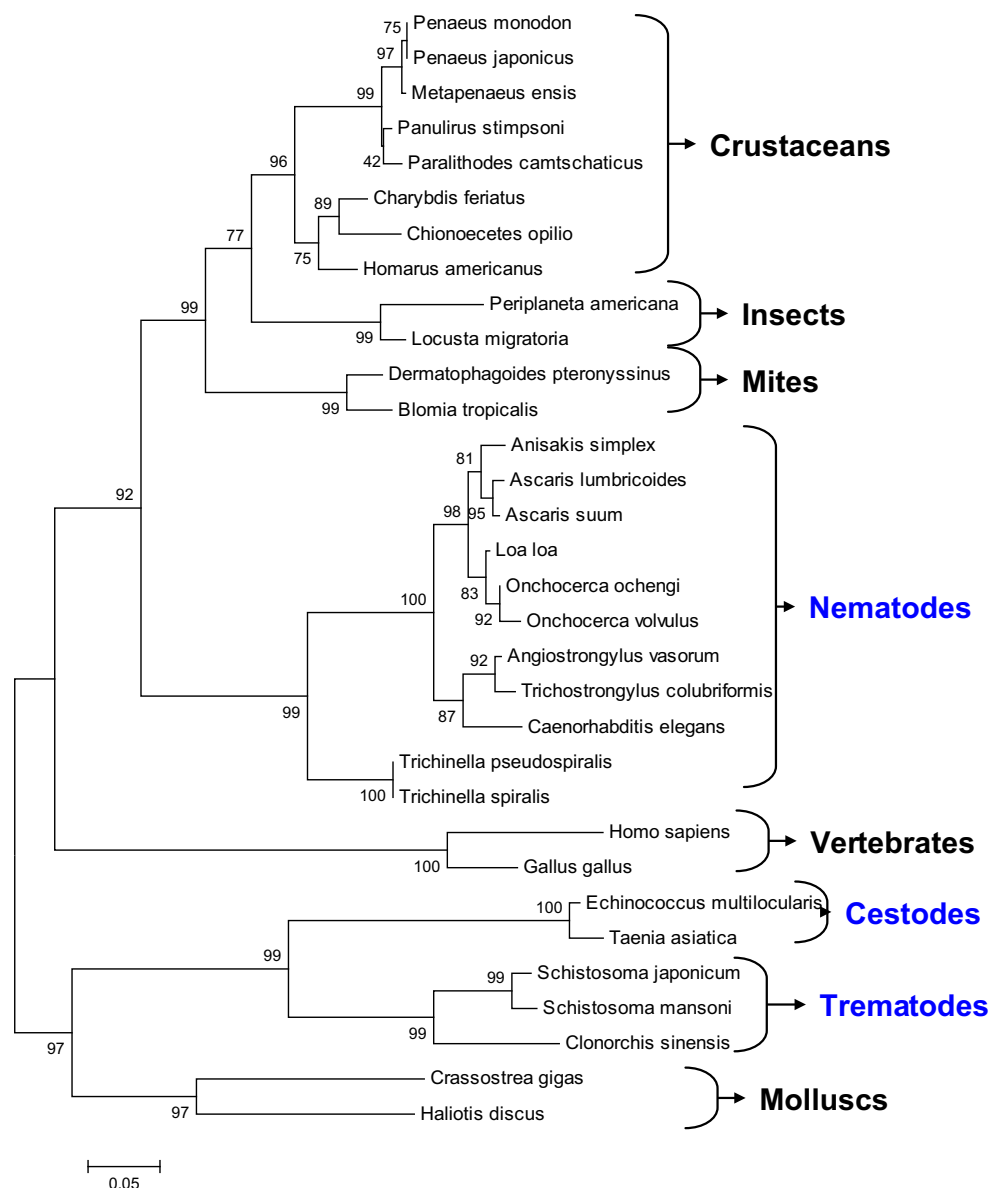


Fig. 2. The evolutionary history of a variety of tropomyosin proteins was inferred using the Neighbour-Joining method. The bootstrap consensus tree inferred from 10,000 replicates is taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 32 tropomyosin amino acid sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5.

parasitised specimens, as well as freezing of fish (Audicana et al., 2002). Food and Drugs Administration (FDA; <http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/Seafood/ucm091704.htm>) regulations in the United States require that all fish and shellfish that will not be cooked or processed at temperatures above 60 °C are blast-frozen to –35 °C or below for 15 h or frozen at –23 °C or below for 7 days. While such measures may make commercially available frozen fish safe, fish can be bought fresh or caught by consumers, and such stringent procedures may not be followed in the domestic environment. Fresh fish that was presumed to be well-cooked is often the source of infection in reported cases (Audicana et al., 2002). Furthermore, freezing of fish alters the flavour of sushi and other fish delicacies, so the practice may be intentionally avoided (Ishikura, 1989). This means that *Anisakis* infections are likely to continue to occur in the future despite food safety legislation.

6. Immunology of helminth infections and anisakiasis

Immune responses to pathogens are characterised by innate immune responses, which are the first line of defence and are rapidly activated, and adaptive immune responses, which develop more slowly and provide a tailored response directed against specific antigens (Langrish et al., 2004). Human epidemiological studies and experimental mouse models both support the idea that repeated infection by helminths can lead to acquired protective immunity in the host (Finkelman et al., 1997, 2004; Allen and Maizels, 2011). CD4⁺ T helper (Th) cells play an important role in mediating the adaptive immune response by the secretion of a wide variety of cytokines, and are known to be essential for resistance to nematodes (Else and Finkelman, 1998). While immune defences against viruses, bacteria, protozoan parasites and fungi rely principally on T helper (Th)1 and Th17 mediated effector responses that

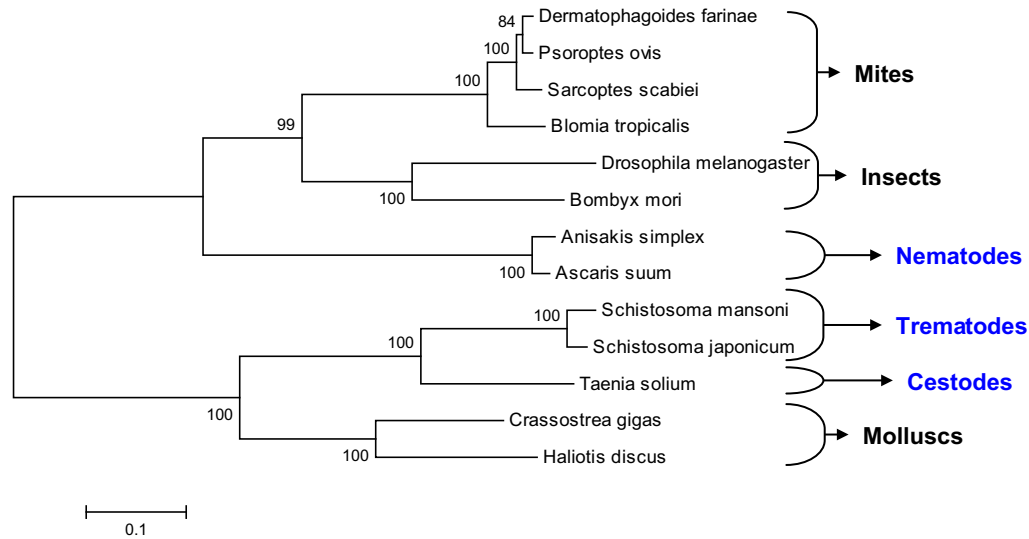


Fig. 3. The evolutionary history of a variety of paramyosin proteins was inferred using the Neighbour-Joining method. The bootstrap consensus tree inferred from 10,000 replicates is taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 13 paramyosin amino acid sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5.

induce direct killing of these pathogens or the cells they infect, defences against large multicellular organisms such as parasitic nematodes require Th2 mediated effector responses which promote removal or expulsion through physical means (Allen and Maizels, 2011; Palm and Medzhitov, 2013). Interestingly, this type of immune defence also appears to be involved in protection against ectoparasites such as ticks (Wada et al., 2010) and it was recently suggested that the so-called type 2 immune responses evolved not only as a response to helminths but also to venoms, fluids from haematophagous insect vectors, and environmental irritants and toxins (Diaz, 2007; Palm and Medzhitov, 2013). Th2 driven responses include the production of IgE and IgG1 (mouse) or IgG4 (human) antibodies, the recruitment of eosinophils, mast cells and basophils, smooth muscle cell constriction, goblet cell hyperplasia, epithelial sloughing and vascular fluid release that aids in expulsion of parasites, as well as the promotion of alternative activation of macrophages, which may be important in tissue repair post infection (Horsnell and Brombacher, 2010; Allen and Maizels, 2011; Palm and Medzhitov, 2013). In mouse models, resistance to helminths is associated with the activation of Th2 cells and the production of the Th2 type cytokines IL-4, IL-5, IL-9, IL-10 and IL-13, while the activation of Th1 cells and the presence of IFN- γ are associated with prolonged or chronic infection (Urban et al., 1992; Else et al., 1994; Grecis, 1996; Finkelman et al., 1997; Bancroft and Grecis, 1998; Else and Finkelman, 1998; Hayes et al., 2004). Similarly, in humans helminths induce Th2 type responses, which are associated with reduced worm burdens (Cooper, 2000; Turner et al., 2003). In addition, helminth infections are often associated with Treg cells, regulatory B cells and the production of IL-10 (Nagler-Anderson, 2006). The associated immunosuppression may protect parasites against inflammatory host defences and benefit humans to an extent by limiting harmful inflammation (Yazdanbakhsh et al., 2001, 2002; Schopf et al., 2002; Turner et al., 2003; Cooper, 2004; Maizels et al., 2004; Maizels, 2005; Araujo and de Carvalho, 2006; Carvalho et al., 2006; Nagler-Anderson, 2006). While IL-10 is often associated with Th2 responses in helminth infections (Schopf et al., 2002; Nagler-Anderson, 2006), there is some evidence that IL-10-associated Th2 responses are modified, with production of IgG rather than IgE, compromised

specific T cell proliferation, decreased levels of IL-5 and eosinophils, and inhibition of mast cell degranulation and cytokine production (Yazdanbakhsh et al., 2001; Maizels, 2005; Araujo and de Carvalho, 2006).

6.1. Immunology of anisakiasis

A spectrum of responses can be seen after infection with *Anisakis* larvae. While allergic symptoms are not frequent in helminth infections, they can occur in zoonotic infections such as anisakiasis (Daschner and Cuellar, 2010). Sometimes gastrointestinal symptoms occur without any allergic symptoms, whereas in other cases there are acute allergic symptoms (ranging from urticaria or angioedema to anaphylaxis) with no or minor gastrointestinal symptoms (Daschner et al., 2000; Gonzalez-Munoz et al., 2010). In the latter cases, termed “gastroallergic anisakiasis” (Daschner et al., 2000) the larvae are normally expelled by the violent allergic reactions and gastroscopic removal is not necessary, whereas in the purely gastrointestinal cases it is often necessary to remove the larvae (Daschner and Cuellar, 2010). The immunological response against *Anisakis* larvae resembles responses to other helminths, however expression of allergic symptoms is more frequent. This could also be due to the relatively low numbers (1–2 larvae) to which most patients are exposed (Daschner et al., 2000). It is postulated that low but continuous exposure to allergenic proteins is more likely to result in the generation of specific IgE antibodies whereas high exposure may instead trigger IgG production.

When histological samples from patients with anisakiasis are examined, the extent of tissue destruction and inflammation resulting from infection with *Anisakis* is out of proportion to the small size of the parasite (1–3 cm long), indicating that host-parasite interactions are responsible for the pathology of anisakiasis (Sakanari and McKerrow, 1989). Infection is primarily associated with intense eosinophilic infiltrates around the larvae, with some patients additionally showing leukocytosis with increased neutrophils and others demonstrating peripheral blood eosinophilia. Studies in guinea pigs and rabbits have demonstrated that *Anisakis* extract is strongly chemotactic for eosinophils but not neutrophils (Tanaka and Torisu, 1978; Iwasaki and Torisu, 1982). It is

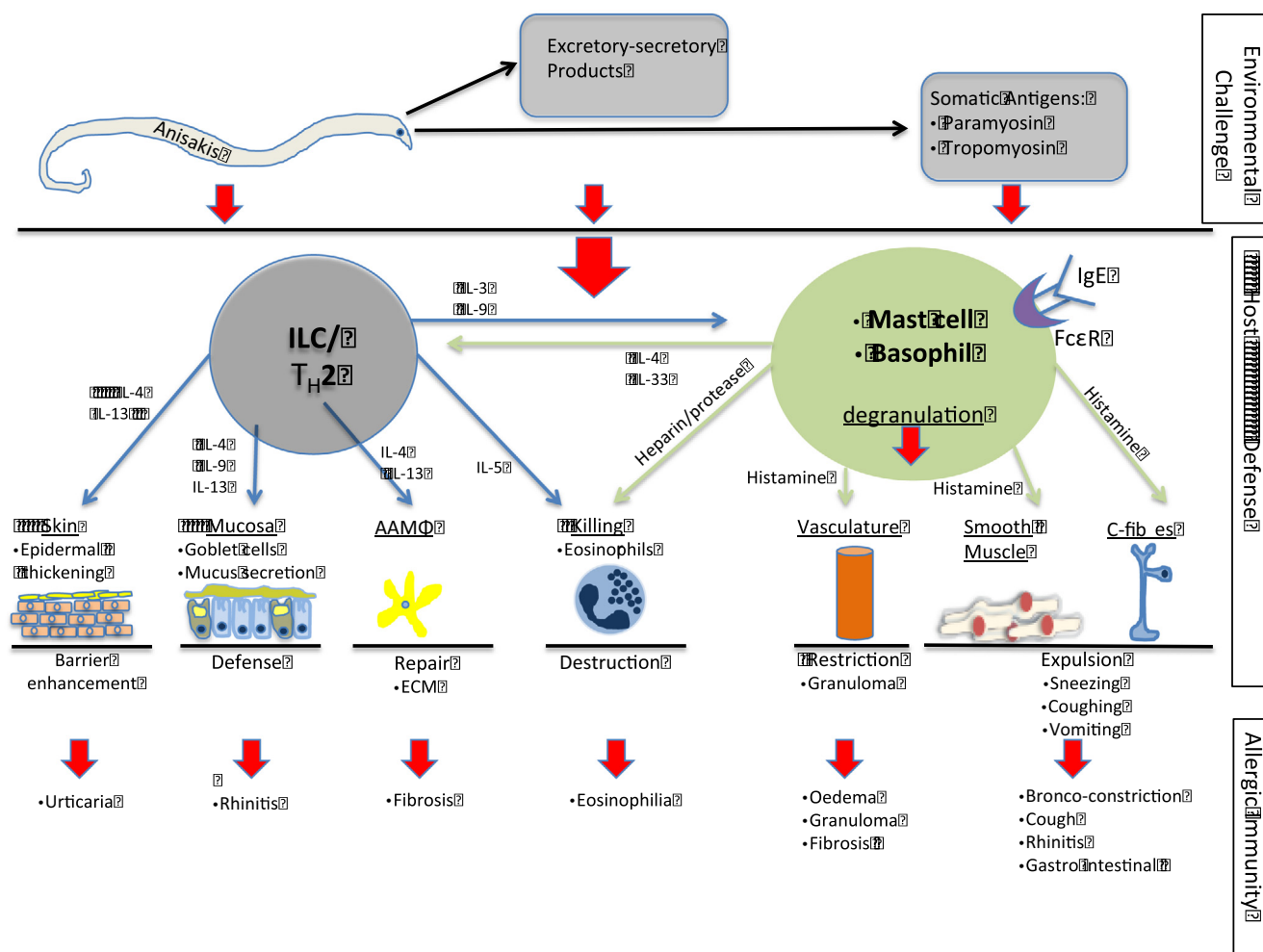


Fig. 4. Functional module of allergic host defence response. Various stimuli activate the allergic host defence response to *Anisakis*: the live helminth itself, a range of excretory–secretory products and somatic antigens released during killing. A variety of effector modules constitute the allergic host defence response. Keratinocyte and goblet cell hyperplasia (mucus production) enhance the barrier functions to restrict helminth entry, feeding and growth. Alternative activated macrophages can restrict helminth spread and tissue repair through extracellular matrix deposition and barrier restoration. Eosinophils can mediate direct helminth killing, while heparin and proteases from activated mast cells and basophil deactivate various proteins. Activation of mast cells and basophils induces vascular restriction to reduce spread of the parasite, while sneezing, coughing, vomiting and diarrhoea are direct means of expelling the parasite. Central to the activation of these defence mechanisms to *Anisakis* and its different antigens is the activation of innate lymphoid cells and Th2, leading to the secretion of specific cytokines, including IL-4, IL-5, IL-9 and IL-13. These cytokines promote cell hyperplasia and mucus secretion, while IL-5 induces eosinophil recruitment to kill *Anisakis*. IgE antibodies to specific *Anisakis* allergens bind to receptors (FcεR) on the cell surface of mast cells and basophils, and upon interaction with allergens trigger degranulation and release of various mediators including proteases and histamine. Interesting crosstalk between these two modules, the innate lymphoid cells/Th2 and mast cells/basophils, is generated via specific cytokines. Th2 cells produce IL-3 which stimulates mast cell/basophil production, while IL-9 recruits mast cells. In turn mast cells produce innate lymphoid cells-activating IL-33, and basophils IL-4, which induce Th2 cells.

thought that tissue damage and not the worm itself evokes the neutrophil response (Feldmeier et al., 1993). *Anisakis* extract injected into guinea pigs causes a large accumulation of eosinophils within 1 h and reached a peak 8 h after injection. In patients, serum levels of eosinophil cationic protein (ECP) are raised in the first 72 h after gastrointestinal infection (Dominguez-Ortega et al., 2003). Eosinophil major basic protein (MBP), known to damage parasites, has been detected in eosinophils of the inflammatory infiltrate of biopsies from patients with anisakiasis (Del Pozo et al., 1999). Inducible nitric oxide synthase (iNOS), another molecule that aids in parasite killing, is also expressed in the tissue and is thought to be produced by the eosinophils. In vitro, *Anisakis* extract down-regulates NO production by macrophages (Cuellar et al., 1998).

The release of ECP, MBP and NO, as well other products such as peroxidases and eosinophil-derived neurotoxin, are probably responsible for worm death but also cause local tissue damage (Feldmeier et al., 1993). The larvae usually die within a few days

(Sakanari and McKerrow, 1989) and degenerate in approximately 8 weeks (Hsiu et al., 1986), during which time they are surrounded by necrosis, oedema and massive eosinophilia mixed with neutrophils, macrophages and lymphocytes (Ishikura, 1989; Bouree et al., 1995). A granuloma forms around the larval debris, with deposition of fibrotic tissue and formation of foreign body giant cell lymphocytes. In some patients this gradually disappears (leading to the term “vanishing tumours” (Ishikura, 1989; Fujisawa et al., 2001), while in others clearance can take longer and the continuing inflammation results in symptoms of chronic anisakiasis. An examination by reverse-transcriptase PCR of intestinal resections from anisakiasis patients containing infiltrates of eosinophils and lymphocytes demonstrated expression of Th2 cytokines (IL-4, IL-5) and T cell receptor (TCR) in 100% of the tissues, but no IFN-γ or IL-2, indicating a Th2 type profile and suggesting that Th2 cells mediate the immunological effector mechanisms in anisakiasis (Del Pozo et al., 1999). IL-4 stimulates IgE production, while IL-5 is responsible for inducing eosinophil proliferation, differentiation

and activation (Takatsu, 1998). These data therefore fit with the features of the disease.

Several authors have used animal models of anisakiasis in an attempt to further elucidate the immunological mechanisms of disease (Jones et al., 1990; Iglesias et al., 1993; Perteguer and Cuellar, 1998; Perteguer et al., 2001; Kim et al., 2005; Cho and Lee, 2006; Cho et al., 2006). Jones et al. (1990) surgically implanted *Anisakis* larvae into the abdominal cavity of CBA/J mice and looked at pathological changes at one, two and 3 weeks p.i. (Jones et al., 1990). After 1 week, neutrophils had accumulated around the larvae. By 2 weeks, most larvae were still viable and were surrounded by granulocytes, occasional multinucleate giant cells, and mature granulomata consisting of eosinophils, fibroblasts and collagen. At 3 weeks p.i. the larvae had been invaded by inflammatory cells and were dead. Surrounding granulomata consisted primarily of connective tissue with scattered eosinophils, with multinucleate giant cells adjacent to the larvae and eosinophils adjacent to larval debris. Systemically, mice had varying degrees of neutrophilia, which began to return to normal values by week 3. Peripheral blood eosinophils were slightly decreased at weeks 1 and 2, but also returned to normal by week 3, suggesting homing of the eosinophils to the site of the larvae during acute infection. In a similar model using intraperitoneal injections with *Anisakis* larvae, Iglesias et al. (1993) measured antibody production during the first 8 weeks p.i. in BALB/c × CBA/J mice (Iglesias et al., 1993). The majority of injected larvae were shown to remain viable up to 2 weeks, producing ES products that stimulated an immune response. The larvae later died and were broken up, releasing internal components that induced a secondary response, with IgG1 the predominant IgG isotype produced (Cuellar et al., 1998). Increased IL-4 was detected between days 6 and 12 and again at 3 weeks post oral infection, indicating the likely cause of the skewing towards type 2 antibody production (Perteguer et al., 2001). Studies in rats showed production of specific IgE that reached higher levels after a second infection, with a peak at 3 weeks after re-infection, indicating that an allergic state could be caused by multiple infections with *Anisakis* larvae (Cho et al., 2006).

Different strains of mice have different responses to *Anisakis*, demonstrating the importance of the genetic background of the host in the immune response to *Anisakis* (Perteguer and Cuellar, 1998). Human patients show a wide range of disease caused by *Anisakis*, ranging from asymptomatic to inflammatory to allergic. After infection, IL-4Rα^{-/-} mice, which mounted a Th1 response, had increased levels of infiltrating immune cells in the intraperitoneal cavity compared with wildtype BALB/c controls, which mounted a Th2 response (Nieuwenhuizen et al., 2006). Splenocyte numbers were similarly increased in IL-4Rα^{-/-} mice compared with wildtype controls and splenomegaly was observed in this strain. However when mice were re-infected after resolution of the primary infection, specific IgG and IgE levels rose dramatically in wildtype BALB/c mice but not in IL-4Rα^{-/-} mice. After oral challenge with *Anisakis* crude extract, wildtype mice exhibited allergic signs such as itching, diarrhoea and increased mucus and cell infiltration in the lungs while IL-4Rα^{-/-} mice did not. This seems to reflect the findings of a human study in which patients with predominantly gastrointestinal symptoms and weak or no allergic symptoms showed raised IFN-γ and lower IgE while patients who presented primarily with urticarial-angioedema and/or anaphylaxis but mild or absent gastrointestinal symptoms had higher total and specific IgE levels and higher production of the Th2 cytokines IL-4 and IL-5 (Gonzalez-Munoz et al., 2010).

In addition we demonstrated that exposure *Anisakis* allergens is associated with the development of protein contact dermatitis. In particular, IL-13 seems to play a central role in protein contact dermatitis associated with repeated epicutaneous exposure to

Anisakis extract, whereas IL-4 drives systemic sensitisation and resultant anaphylactic shock (Nieuwenhuizen et al., 2009).

Intranasal sensitisation induced airway hyperresponsiveness (AHR) in wild-type mice only, showing that AHR was IL-4/IL-13 dependent. Unexpectedly, infection with *Anisakis* larvae induced AHR in both wild-type and IL-4Rα-deficient mice. IL-4Rα-independent AHR was mediated by IFN-γ, as evidenced by the fact that in vivo neutralisation of IFN-γ abrogated AHR. Subsequent studies on the intranasal sensitisation with *Anisakis* extract and the induction of AHR demonstrated that both infection with larvae and inhalational exposure to *Anisakis* proteins are potent routes of allergic sensitisation to *Anisakis*, explaining food- and work-related allergies in humans (Kirstein et al., 2010). Moreover, depending on the route of sensitisation, AHR can be induced either by IL-4/IL-13 or by IFN-γ.

This suggests that the different manifestations of anisakiasis may be largely dependent on the host immune responses, with strong Th2 responders able to effectively expel the larvae through allergic reactions while individuals prone to Th1 responses develop a more inflammatory response associated with gastrointestinal pain. Patients with allergic reactions typically do not recall a previous episode of infection, but must have had one in order to produce IgE antibodies, indicating that the primary infection was asymptomatic (Daschner et al., 2000). Patients with gastroallergic anisakiasis typically expel the parasite quickly through their violent allergic reaction (vomiting, diarrhoea) and do not need gastroscopic removal of the parasites, suggesting that the allergic response functions as an immune defence mechanism (Daschner and Cuellar, 2010).

Interestingly, a patient found to be parasitised with an exceptional number of worms (over 200) had relatively low IgE levels (compared with patients with gastroallergic anisakiasis) but increased neutrophils at the time of admission (Jurado-Palomo et al., 2010). In another case a woman parasitised with 56 larval nematodes also presented with epigastric pain and nausea (Kagei and Isogaki, 1992). However, in an experimental infection model, rats infected with a high number of *Anisakis* larvae developed weaker IgE responses than those infected with low numbers of larvae (Amano et al., 1995). It was suggested that this could reflect the ability of high burden worm infections to stimulate a suppressive regulatory response. This is interesting as it suggests that it could be the level of tissue damage due to the infecting helminth (which would often correlate to the parasite burden) that determines whether regulatory responses are induced. An interesting study (Romero et al., 2013) recently demonstrated that *A. pegreffii*, the species predominantly causing infections in Europe (D'Amelio et al., 1999; Umehara et al., 2007; Mattiucci and Nascetti, 2008; Fumarola et al., 2009), has less penetrative power in vivo than *A. simplex*, the principal agent of anisakiasis in Japan (Arizono et al., 2012). Based on recent studies (Suzuki et al., 2010; Quiazon et al., 2011; Arizono et al., 2012) demonstrating that *A. pegreffii* was less able to penetrate fish muscle and solid agar and to survive artificial gastric juices than *A. simplex*, the authors measured the pathogenic potential of the different larval species in vivo, which they defined as its ability to cause lesions, attach itself to the gastric or intestinal wall or penetrate through the walls into the abdominal cavity. The results indicated that *A. pegreffii* was significantly less pathogenic. More cases of gastroallergic anisakiasis are reported in Europe, where *A. pegreffii* infection is most common, whereas gastrointestinal symptoms are more commonly reported from Japan, where *A. simplex* is the dominant species found (Romero et al., 2013), suggesting the possibility that there is a correlation between the amount of tissue damage caused by a helminth and its ability to induce regulatory immune responses that suppress allergy. Helminths can cause extensive tissue damage while migrating through the host, which may explain in evolution-

ary terms why wound-healing pathways are strongly connected to anti-helminth responses (Allen and Maizels, 2011; Allen and Wynn, 2011).

7. Summary

Helminth infections typically induce both Th2 effector responses that aid in their expulsion, and regulatory responses, which down-regulate immune pathology. Allergic reactions are often considered to be misdirected Th2 responses to environmental antigens that cause immune pathology. Zoonotic infection with the marine nematode *Anisakis* gives rise to a relatively unique situation in which some patients demonstrate severe allergic reactions that appear to aid in expelling the parasites, perhaps due to a lack of helminth induced regulatory responses (Fig. 4).

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