Immunity of the lugworm *Arenicola marina*: cells and molecules

MN Berlov\textsuperscript{1,2}, AL Maltseva\textsuperscript{3}

\textsuperscript{1}\textit{Department of General Pathology and Pathophysiology, Institute of Experimental Medicine, St Petersburg, Russia}
\textsuperscript{2}\textit{Department of Biochemistry, St Petersburg State University, St Petersburg, Russia}
\textsuperscript{3}\textit{Department of Invertebrate Zoology, St Petersburg State University, St Petersburg, Russia}

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Abstract

Immune responses of invertebrate animals are mediated through innate mechanisms, among which phagocytosis, encapsulation, production of ROS and antimicrobial peptides. Although polychetes represent an evolutionary interesting group closely related to presumable common ancestor of other celomates, their immune mechanisms still remain scarcely investigated. Here we discuss immune responses of the polychete *Arenicola marina*, the lugworm. Besides an overview of diversity of celomocytes and cellular responses, we present the synopsis on antimicrobial peptides arenicins: their structure, function and therapeutic potential.

Key Words: immunity; celomocytes; phagocytosis; antimicrobial peptides; arenicins; polychetes; lugworm; *Arenicola marina*

Introduction

Polychetes are segmentary celomate invertebrate animals inhabiting marine or estuarine habitats being either benthic or pelagial. Polychaeta is an ancestral group within the phylum Annelida (Struck \textit{et al.}, 2011). The molecular data unambiguously place it within Lophotrochozoa animals (together with Lophophorates and Molluscs (Halanych \textit{et al.}, 1995). The long held morphological view closely relates Annelids with Arthropods within Articulata as both being segmented (Dohle and Scholtz, 1995). In accordance to any of these views polychetes are considered among taxa closest to a putative common ancestor of other protostome bilaterians (Balavoine and Adoutte, 2003), and so, are of particular evolutionary importance.

Immunity is among crucial evolutionary factors for speciation, surviving or extinction (Loker, 2012). Identification of the immunological features typical for the high-rank taxa (e.g., phylum or class) is an essential problem for comparative immunology. This can help in the understanding of the immune system history in macroevolution. Elucidation of immune mechanisms in a particular species is also very important - it allows to estimate diversity of immune patterns within a phylum. This also may clarify some aspects of the species history. Several comprehensive reviews of immunity of Annelids are available (Stein and Cooper, 1983; Salzet \textit{et al.}, 2006; Tasiemski, 2008; Vetvicka and Sima, 2009), but there are still few immunological syntheses, done on particular polychete species. Here we discuss the available data concerning aspects of immune responses of polychete *Arenicola marina*.

*A. marina* is a common inhabitant of sand flats in Northern Europe. It lives within the burrow, in permanent contact with surrounding sediment. *A. marina* is sensitive to quality of sediment in term of biochemical properties and toxicity (Morales-Caselles \textit{et al.}, 2008, 2009) and has been suggested as a test-species for ecotoxicological studies (Ramos-Gómez \textit{et al.}, 2011). Living in habitats rich with microbes and spending their lives engulfing and disgorging the sediments, these animals obviously need an efficient immunity. Like other invertebrates it relies on innate immune system. Traditionally immunity is considered as a concord of cellular and humoral branches, so we will discuss known facts about cells and molecules involved in the lugworm’s defensive reactions.

Receptors

Several classes of pathogen recognizing receptors (PRR) were identified in annelids: TLR, NLR, and lectins (Ozeki \textit{et al.}, 1997; Hirabayashi \textit{et al.}, 1998; Molchanova \textit{et al.}, 2007; Davidson \textit{et al.}, 2008; Cuvillier-Hot \textit{et al.}, 2011; Škanta \textit{et al.}, 2013).
Among them, only one lectin was recognized in A. marina as a putative PRR - AML-1 (Vitashenkov et al., 2012). AML-1 showed no homology with any known lectin or other protein families, and is a representative of a new family of lectins with specificity directed towards N-acetylated carbohydrates (e.g., chitin). It was able to agglutinate different mammalian erythrocytes in vitro with clear preference to rabbit cells. This data are in line with hemagglutinating activity in A. marina body fluid, most strong toward rabbit red blood cells, demonstrated in early studies (Dales, 1982). Purified originally from cell-free celomic fluid, AML-1 was immunohistochemically identified in different types of celomocytes (both floating in celomic fluid and migrating through the tissues), nephridia cells and eggs - within cytosolic granules in all cases (Vitashenkov et al., 2012). This indicates that the role of AML-1 in lugworm immunity could be more complex than just agglutination in celomic cavity, and remains to be elucidated.

Celomocytes

Body wall of polychetes consists of 4 elements: thin cuticle produced by one-layered epithelium, and layers of transversal musculature underlined by ribbons of longitudinal musculature. The later are separated from the celomic cavity by celothelium (peritoneum). In A. marina, celomic cavity is divided by segmentary dissepiments only in the most anterior and posterior parts of body, while within main body it represents continuous volume. Celomic cavity is filled by celomic fluid, where floating cells (the celomocytes) are present. Those cells might also settle onto peritoneum and migrate through tissues. There are several types of celomocytes in polychetes, such as granulocytes (syn. amebocytes), eleocytes, and hemocytes. However, the latter two are absent in Arenicola marina (rev. in Vetzicka and Sima, 2009), as well as in some other polychetes (e.g., Hermodice carunculata (Franchini et al., 2016]). Granulocytes (amebocytes) can be further subdivided into several subtypes, although relationships between them are not well understood. In Arenicola these subtypes vary in their extent of development of granular apparatus and actin fiber networks (Persinina and Chaga, 1994a, 1998; Chaga et al., 1998). Chaga et al. separated the terms amebocytes (few granules, well developed actin fibers) and granulocytes (numerous granules, thin membrane associated actin network), which corresponded to granulocytes of types I and II respectively in Dhainaut and Porchet-Henneré nomenclature (Dhainaut and Porchet-Henneré, 1986). Both cell types originate from the same cell-source - “juvenile cells” (Persinina and Chaga, 1994b).

In A. marina, bacteria inoculated into body cavity are quickly cleared by phagocytic cells in the celom and tissues (Fitzgerald and Ratcliffe, 1983). Granulocytes of both types actively phagocytize, individually or within aggregates. Individual cells loaded by engulfed bacteria migrate into the tissues; cell aggregates finally mature into “brown bodies” (Fitzgerald and Ratcliffe, 1983), which can be discarded by nephridia (Kermad, 1955). The same authors showed that there is no opsonic activity toward different bacteria in celomic fluid plasma, and that phagocytic celomocytes are able to discriminate between gram-positive and gram-negative bacteria (Fitzgerald and Ratcliffe, 1982).

Besides the absence of unambiguous interpretation of an interrelation among celomocytes subpopulations, an evident demonstration of the common hematopoietic area is also lacking. Different derivatives of celomic peritoneum, including extravasal tissue, are most often proposed as candidates (rev. in Gardiner, 1992; Vetzicka and Sima, 2009). Cells of extravasal tissue (specialized part of ventral blood vessel) possess granular cytoplasm. Along with celomocytes these cells participate in clearance of celomic cavity via phagocytosis (Braunbeck and Dales, 1984, 1985).

Melanin and ROS production

“Brown bodies” contain melanin in polychete Neris diversicolor (Porchet-Henneré and Vernet, 1992) and olygochete Eisenia fetida (Valembois et al., 1994). Melanogenesis in invertebrates is related to the activity of phenoloxidase (PO), and is accompanied by generation of a spectrum of highly toxic free radicals, including reactive oxygen species (ROS) (e.g., rev. in Nappi and Ottaviani, 2000; Cerenius and Söderhäll, 2004; Nappi and Christensen, 2005) and formation of amyloid fibrils (Falabella et al., 2012; Grimaldi et al., 2012, 2014). Appearance of “brown bodies” during clearance reaction in A. marina allows to suspect production of ROS and activation of PO during this process. Coincidence of encapsulation and PO-activation or both melanin and ROS production was described in N. diversicolor (Porchet-Henneré et al., 1987; Porchet-Henneré and Vernet, 1992) and in E. fetida (Valembois et al., 1994), respectively. Unexpectedly, the once carried testing for PO-activity in whole celomic fluid failed to reveal any in A. marina (Smith and Söderhäll, 1991). Possibly, PO and related enzymes are activated in this species only after induction. In accordance to such suggestion, both production of melanin and activation of PO in granulocytes II was demonstrated in N. diversicolor after infection (Porchet-Henneré and Vernet, 1992).

ROS production in invertebrates accompanies different immunological phenomena - phagocytosis, encapsulation, and epithelial defense (e.g., rev. in Nappi and Ottaviani, 2000). Defense-related ROS production by celomocytes both in vivo and in vitro was demonstrated in the olygochete E. fetida (Valembois et al., 1994; Valembois and Lassègues, 1995). In A. marina, diverse antioxidant enzymes (superoxide dismutase, catalase, glutathione reductase, peroxiredoxin, etc.) were identified, and their activity was demonstrated in different compartments (e.g., body wall, chloragogen tissue, gills) under various stress conditions (seasonal temperature variation, pollution, etc.) (Buchner et al., 1996; Storch et al., 2001; Loumaye et al., 2008; Ramos-Gómez et al., 2011). Nevertheless, neither their involvement into immunity, nor the defensive ROS production (or ROS producing enzymes) were ever characterized in this species. So, both mechanisms of melanin production and defensive ROS functioning in A. marina remain open questions.
Antimicrobial activity

Early studies searched for antimicrobial activity in *A. marina* against Gram-positive and Gram-negative bacteria. No bacterial growth inhibitory activity was detected in celomic fluid plasma of neither intact animals nor ones induced by injection of killed bacteria into body cavity (Dales and Dixon, 1980). The same was shown 35 years later - again there was no clear antibacterial activity in plasma of *A. marina* celomic fluid, and injection of microbes into body cavity did not stimulate its appearance (Mal'tseva et al., 2014). Instead, strong antimicrobial activity was detected in acidic extracts from celomocytes, and the peptides responsible for this activity - arenicins - were identified (Ovchinnikova et al., 2004). The expression of arenicin-1 was later revealed in different compartments, though the strongest signal was found in celomocytes. This was established by different method, including immunohistochemistry, PCR (Mal'tseva et al., 2014) and MALDI-imaging mass-spectrometry (Mal'tseva et al., 2016). Expression of arenicins was constitutive in all the tissues, where it was detected (Mal'tseva et al., 2014).

Within celomocytes (both granulocytes I and II), arenicins are stored within cytosolic granules, which undergo fusion with phagosome. Arenicins functioning this way participate in inactivation of the phagocytosed pathogens, instead of being secreted into celomic cavity. Importantly, presence of arenicins was detected in granules of cells of extravascular tissue, also possessing phagocytic activity (Mal'tseva et al., 2014).

Apart from celomocytes, expression of arenicins was detected in epithelia of the body wall and gut (Mal'tseva et al., 2014). Similarly, AMPs of another annelid worm - theromcin and theromyzin from a leech *Theromyzon tessulatum* - are expressed both in intestinal and epidermal cells (Tasiemski et al., 2004). This supports the importance of arenicins as key components of both epithelial and systemic branches of host defense. Arenicins are also present in a major part of nervous system of *A. marina*, midventral neuronal cord (Mal'tseva et al., 2014). The expression of two AMPs by glia and neurons in response to injury and microbial challenge was also demonstrated in the medicinal leech *Hirudo medicinalis*. This indicates the involvement of AMPs in both defense and regeneration (Schikorski et al., 2008). The functioning of arenicins in the nervous system still remains to be elucidated although their presence in this compartment indirectly supports the multifunctional nature of AMPs in vivo.

Therapeutically perspective components

Several compounds with some potential for usage in medicine were characterized in worms of the genus *Arenicola* (*A. marina* or *A. cristata*): hemoglobin (Rousselot et al., 2006), fibrinolytic serine protease (Zhao and Ju, 2015), cell growth inhibiting and apoptosis inducing serine protease (Zhao and Ju, 2014), and arenicolesterol A (Bin et al., 2005; Wang et al., 2007). Except for evident oxygen-transporting function of hemoglobin, natural function of those compounds in lugworms remains to be elucidated. Nevertheless, up to date, arenicins represent most intensively studied *A. marina* compounds with therapeutic potential, so we will discuss their structure and activities in more details.

Primary structure of arenicins

There are three isoforms of arenicins. They all are 21 amino acid peptides with 6 positively charged arginine residues for arenicins-1 and -2 (Ovchinnikova et al., 2004) or 4 for arenicin-3 (Sandvang et al., 2008). Arenicins-1 and -2 differ by a single amino acid residue (Val or Ile in 10th position), and contain two cysteine residues linking the only disulfide bond. Arenicin-3 is a bit more different and contains four cysteines forming two disulfide bonds.

Primary structure of arenicins displays considerable homology with previously described horseshoe crab AMPs, tachyplesins from *Tachypleus tridentatus* (Nakamura et al., 1988) and polyphemusins from *Limulus polyphemus* (Miyata et al., 1989). This is why we propose to consider all these AMPs within 'tachyplesin/arenicin family' (TAF). Other TAF members are gomesin from *Tarantula spider Acanthoscurria gomesiana* (Silva et al., 2000), androctonin from Sahara scorpion *Androctonus australis* (Mandard et al., 1999), alvinellacin from Pompeii worm *Alvinella pompejana* (Tasiemski et al., 2014) and capitellacin from *Capitella teleta* (the later one was predicted by analysis of genome database (Tasiemski et al., 2014). Thus, identified up to date TAF-related AMPs belong to two animal taxa - arthropods and chelicerates. Structures of arenicins and some other selected members of TAF are represented in Figure 1. Noteworthy, among TAF members only arenicins-1 and -2 are characterized by a single disulfide bond while all other peptides contain two disulfide bonds. Hence, the latter variant could be accepted as putative ancestral pattern.

Several AMPs unrelated to TAF were revealed in other annelid species: perinerin from Asian clamworm *Perinereis aubahiensis* (Pan et al., 2004), hedestin from sandworm *Nereis diversicolor* (Tasiemski et al., 2007), lumbricin from different olygocete and leech species, theromacin and neuromacin from leaches *Theromyzon tessulatum* and *Hirudo medicinalis*, and others (rev. in Tasiemski, 2008). There is still no evidence of presence of similar peptides in *A. marina*. Spatial structure of arenicins

As it was established by NMR spectroscopy (Ovchinnikova et al., 2007; Andrà et al., 2008), arenicins-1 and -2 in aqueous solutions adopt a conformation of β-hairpin formed by two antiparallel β-strands (Cys3-Val/Ile10 and Val13-Cys20), and stabilized by one disulfide and nine hydrogen bonds. β-Strands are connected by a type I β-turn. Reduction of disulfide bond disrupts the β-hairpin structure, at least in a case of arenicin-2. The two-stranded β-sheet in structure of both peptides has significant right-handed twist and in the case of arenicin-1 the β-sheet is bent. Right-handed twist in the β-sheet deprives the arenicin-2 molecule surface of amphipathicity (Ovchinnikova et al., 2007), while alternative study reported that arenicin-1 retains amphipathicity in aqueous solution (Andrà...
Antimicrobial activity of arenicins

Arenicins at micromolar or even lower concentrations display significant antimicrobial activity towards the wide range of Gram-positive and Gram-negative bacteria, and fungi (Ovchinnikova et al., 2004, 2007; Lee et al., 2007; Andrä et al., 2008; Sandvang et al., 2008). Several marine bacteria (Vibrio alginolyticus, Listonella anguillarum, and Planococcus citreus) were also susceptible to arenicins (Andrà et al., 2008). Among the sensitive microbes there are some antibiotic-resistant strains including clinical isolates (Sandvang et al., 2008; Cho and Lee, 2011b).

Antimicrobial activity of many cationic AMPs significantly decreases with increasing ionic strength (rev. in Bowdish et al., 2005). However, arenicins-1 and -2 display comparable antibacterial activity in both low salt and high salt conditions (Ovchinnikova et al., 2004) or retain decreased but significant activity even at high NaCl level up to 500 mM (Andrà et al., 2008). The data about fungicidal activity to Candida albicans are contradictory. Either it was retained at 150 mM NaCl for arenicin-1 (Park and Lee, 2009) or decreased 4 - 5 fold at 100 mM NaCl for arenicin-1 and -2 (Ovchinnikova et al., 2004). The peptide NZ17074, which is an artificial derivative of arenicin-3, demonstrated fungicidal activity moderately impaired at high salt conditions (Wang et al., 2014). Resistance to enhanced salt concentrations is considered to be evolutionary selected feature of arenicins essential for AMPs of marine animals (Andrà et al., 2008, 2009).

Mechanism of antimicrobial action of arenicins

It is accepted that the most common mechanism of toxic action of cationic AMPs is performed via permeabilization of the cytoplasmic membrane of the target cell (Yeaman and Yount, 2003). Accordingly, many studies aimed to describe the "behavior" of arenicins in lipophilic media such as natural membranes, liposomes, micelles, lipid mono- and bilayers. Detailed data, obtained for arenicin-2, allowed to outline a model of its action on bacterial membrane. Binding of the peptide to membrane due to electrostatic interactions with anionic phospholipid heads leads to conformational changes of the peptide molecule. Partial unwinding of twisted β-hairpin occurs making it more planar and resulting in disclosure of amphipathicity of the arenicin-2 molecule (Ovchinnikova, et al., 2007; Sainikov et al., 2011). The next step is the formation of peptide dimers via parallel association of N-terminal β-strands (CN↑↑NC type of association) (Ovchinnikova et al., 2008; Shenkarev et al., 2011). Finally, the peptide dimers turn into the transmembrane orientation establishing ion-conducting pores within the membrane constituted of 2-4 arenicins’ dimers in a complex with anionic lipids head groups (Ovchinnikova et al., 2008; Shenkarev et al., 2011, 2014; Sychev, 2015). This mechanism is consistent with the toroidal pore model implying the formation of pores by both peptides and lipids with overall torus-like geometry (Matsuzaki, 1999). Experimental data concerning arenicin-1 imply a similar mechanism, though it may differ in some details (Andrà et al., 2008, 2009; Shenkarev et al., 2011).

Cellular membrane permeabilization also occurs during fungicidal action of arenicin-1 (Park and Lee, 2009; Cho and Lee, 2011a) and of arenicin-3-related peptide NZ17074 (Wang et al., 2014). However, the downstream events are important for killing of fungal cell. The action of arenicins on fungi is energy-dependent, since it is blocked by metabolic inhibitor (sodium azide) as well as by low-temperature conditions (+4 °C) (Park and Lee, 2009; Wang et al., 2014). The latter is true only for fungi, but not for bacteria, which are equally
susceptible to arenicin-1 action at both +37 °C and +4 °C (Andrä et al., 2008). Within a fungal cell, arenicins attack mitochondria leading to increased generation of hydroxyl radicals and other ROS and activation of apoptosis, presumably via ROS-sensitive metacaspases (Cho and Lee, 2011a; Wang et al., 2014). According to Choi and Lee, increased intracellular ROS production accompanies killing of bacterial cells by arenicin-1 as well (Choi and Lee, 2012).

**Structure-function interrelation**

Significance of the particular amino acid residues in the structure of arenicins for their antimicrobial activity was intensively investigated. Arenicin-1, devoid of disulfide bond due to substitution of both cysteines by serines, was 2-4-fold less active than the natural peptide (Lee et al., 2007; Andrä et al., 2009, 2011). Substitution of cationic Arg11 by neutral Ala11 or deletion of N-terminal dipeptide Arg-Trp decreased the activity of arenicin-1 towards Gram-positive and Gram-negative bacteria, and fungi by 2-3 times (Cho and Lee, 2011b; Park et al., 2011). Similarly, arenicin-1 analog with Trp2 replacement retained only a quarter of antimicrobial activity compared with prototype peptide (Panteleev et al., 2015b). Another tryptophan residue (Trp21) is also critical for antimicrobial activity of arenicin-1, as its replacement causes approximately 2-fold increase of minimal inhibitory concentration (Panteleev et al., 2015b). The same is observed after replacement of all arginine residues by lysines (Andrä et al., 2009, 2011). Among other residues, Ala6, Val4 and Val10 are also essential for antimicrobial activity of arenicin-1 (Panteleev et al., 2015b).

**Interaction with mammalian cells and molecules**

The degree of toxicity towards mammalian cells is critical for potential therapeutic agents. Arenicins-1 and -2 poorly discriminate between microbial and animal cells. These peptides exhibit high hemolytic activity against human erythrocytes (Lee et al., 2007); and nucleus-possessing mammalian cells can also be damaged by arenicins. Arenicin-1 was toxic to both cancer (Jurkat) and normal (embryonic fibroblasts, astrocytes) human cells in vitro (Andrä et al., 2008; Panteleev et al., 2016). In vivo experiments revealed that arenicin-2 should be referred to as a Class III toxicity (20 > LD50 > 700 mg/kg) for CD-1 mice (Dyachenko et al., 2012). At the same time, arenicin-3 seems to be non-toxic for mammalian cells, and displays very low hemolytic activity (Hoegenhaug et al., 2011).

Deletion of N-terminal dipeptide Arg-Trp from the arenicin-1 sequence resulted in dramatic decay in hemolytic activity (Cho and Lee, 2011b). In non-hemolytic arenicin-3 there is no Arg-Trp dipeptide. Thus, hydrophobic Trp2 residue is essential for the interaction with erythrocyte membrane, however, its replacement with any of three different residues only moderately affects hemolytic activity of arenicin-1.
Other hydrophobic residues are also important for toxic activity of arenicin-1 towards erythrocytes. One of five valine residues at positions 4, 8, 10, 13, and 15 or alanine at 6th position can be replaced with more hydrophilic amino acid with significant decrease in hemolytic activity of arenicin-1. An analog of arenicin-1 with Arg substituted for Val8 retains the high antimicrobial activity with dramatically decreased hemolytic properties (Panteleev et al., 2015b). Similar activity shift was observed for shortened analog of arenicin-1 with deletion of two dipeptide fragments: Tyr7-Val8 and Val13-Leu14 (Panteleev et al., 2016). The toxicity of those analogs towards nuclear human cells was significantly lower compared to the natural peptide. These analogs can be considered as a perspective base for the development of therapeutic variants of arenicin. Another possibility to produce a novel therapeutics is related with non-hemolytic arenicin-3. Its analog NZ17074 with Asn and His substituted for Tyr5 and Tyr17 respectively is currently at the preclinical stage as an agent against multiresistant Gram-negative bacteria (Fox, 2013).

Other than antimicrobial activities of arenicin-1 in mammalian system in respect of possible medical application are also highly important. The ability of arenicin-1 to form a complex with human complement protein C1q resistant to high salt concentration was demonstrated recently (Berlov et al., 2015). Previously similar data were shown for homologous peptide tachyplesin, which is able to interact with human C1q and activate complement cascade on the surface of TSU tumor cells (Chen et al., 2005). Thus, possible therapeutic application of arenicins may concern not only their antimicrobial action but also some immunomodulatory activities. There are still no direct data about particular site of C1q molecule responsible for interaction with arenicin. However, available reports on other β-structural AMPs (defensins and tachyplesin) point at the collagenous domain of C1q as the site involved in the interaction with peptides (van den Berg et al., 1998; Chen et al., 2005). Possibly, ability to interact with C1q reflects a more general feature of arenicin that may be functionally important in the organism of A. marina.

Conclusion

Immunity of the lugworm A. marina is still comparatively poorly studied. Basically it relies on both systemic and epithelial branches of immune responses. Several types of celomocytes were described. These cells are active phagocytes, able to aggregate under stress conditions, and capable to discriminate between gram-positive and gram-negative bacteria. Though, no PRRs on celomocytes surface were identified up to date. Among immune effectors, antimicrobial peptides arenicins are most well characterized. These are constitutively expressed in a wide range of tissues including celomocytes and various epithelia. In celomocytes, arenicins participate in phagocytosis during fusion of cytosolic granules with lysosomes without being exocytized into celomic fluid plasma. In the epithelia of body wall and gut, arenicins most probably are secreted into cuticle and intestinal cavity, respectively. Involvement of the same AMPs into both systemic and epithelial immunity is rather uncommon, as usually epithelia and phagocytes produce their own set of AMPs (e.g., in humans - α-defensins 1-4 in neutrophils, LL-37 in monocytes, α-defensins 5 - 6 in intestine and different β-defensins in skin and other borders (rev. in De Smet and Contreras, 2005)).

Arenicins are very potent antimicrobials that retain their activity in presence of salt, which is critical for marine organisms. β-Hairpin structure relates them to members of tachyplesin/polyphemusin family of AMPs and is essential for realization their toxic action. Although native arenicin-1 and -2 are highly toxic to different mammalian cells (a feature limiting their application in medicine), several modified analogs with great therapeutic potential were designed based on their structure.

Yet, many aspects of A. marina immunity, important from both fundamental and practical point of view, still wait for elucidation.

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