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The echinoderm innate humoral immune response

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Abstract
Multicellular organisms have an immune system, which is essential for the survival of living beings. Interest in the immune system has been expanded since common characteristics of innate immunity between *Drosophila melanogaster* (Meigen, 1830) and mammals were discovered in the 1980. Since then, immunology has mainly focused on the adaptive immune system that seems to be restricted to vertebrates. Unlike the innate immunity, the adaptive one is acquired after exposure to a specific antigen (Ag) and includes: antigen-presenting cells such as macrophages, proliferation of B and T lymphocytes, Ag-specific antibody/cytokine production and immunological memory. Innate immunity is instead a process of cellular defense at low specificity, which is designed to prevent and combat infectious agents that penetrate at the tissue level, and may be the only form of immunity present in invertebrates such as sea urchins. The immune system of invertebrates acts through (i) cellular components (cell-mediated immunity) in which the effectors of defense reactions are represented by immune cells; (ii) soluble factors (humoral immunity), secreted by the immune cells, such as lectins, agglutinins, lysins, antimicrobial peptides and the prophenoloxidase (proPO) activating system, which act in parallel with the immune cells to fight pathogens and other foreign substances. Here we aim to deepen the study on humoral immunity of invertebrates, especially referring to the phylum Echinodermata because of its features shared with protostomes and other deuterostomes, and suggesting a key step during evolution.

Keywords: *Defense molecules, evolution, immunity, invertebrates*

Introduction

The immune system is responsible for recognition and destruction of pathogens, due to particular microbial surface molecules, which are identified as non-self. These molecules are known as pathogen-associated molecular patterns (PAMPs), and their receptors, present on immune cells and in body fluid as soluble factors, are called pattern-recognition receptors (PRR), and possess a variability lower than those of adaptive immunity (Gay & Gangloff 2007). Among the most common PAMP molecules there are various components of the bacterial cell wall such as lipopolysaccharide (LPS), peptidoglycan (PGN) and lipopeptides, as well as flagellin, DNA and double-stranded RNA (dsRNA; reviewed by Mogensen 2009). In addition to PAMPs, exclusively expressed by microbes, the receptors also recognise the danger-associated molecular patterns (DAMPs), which are intracellular proteins, such as heat shock proteins, and protein fragments of the extracellular matrix released by necrotic or dying cells (reviewed by Jounai et al. 2013). Regarding PRRs, studies of the genome of *Strongylocentrotus purpuratus* (Stimpson, 1857) have shown that an abundance of genes are related to immune receptors, such as more than 200 toll-like receptors (TLRs), scavenger receptor cysteine-rich (SRCR) domain proteins, and nucleotide-binding oligomerisation domain (NOD)-like receptors (NLR) (Buckley & Rast 2012). The response to an infection put in place by the immune system is shown in Figure 1.

As shown in Figure 1, the vertebrate immune system has three main degrees of defense, each with increasing specificity: the chemical/physical barrier,

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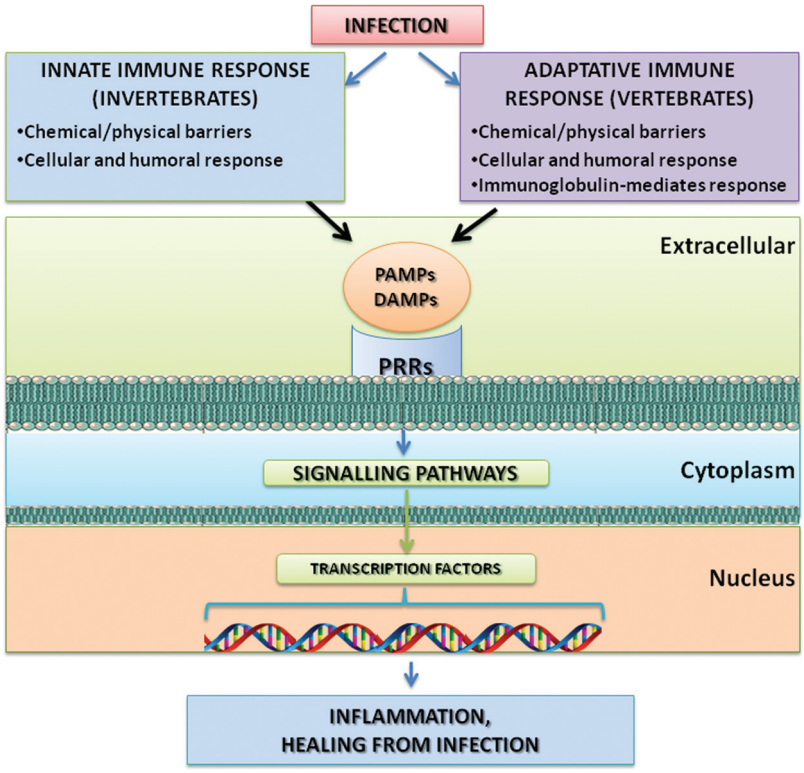

Figure 1. Process of the response to an infection (virus, bacteria, fungi) put in place by the immune system in invertebrates (innate response) and vertebrates (adaptative response). Pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) link to pattern recognition receptors (PRRs), triggering a series of cascade events of the signaling pathways, that in turn activate the transcription of specific genes able to combat infections.

the cellular/humoral defence and the immunoglobulin-mediated response. In contrast, invertebrates have only two lines of defense. The first defense, common to invertebrates and vertebrates, is the physical barriers that prevent pathogens from entering the organism, such as the epidermis or cuticle. As an example, invertebrates have developed some chemical and physical barriers to detect and respond to antigens like lipopolysaccharides (LPS), lipoproteins, peptidoglycan (PGN) and (1-3)-β-D-glucans (Begum et al. 2000). An interesting example of a chemical barrier is present in invertebrate animals such as cnidarians, annelids and molluscs, where mucous secretions containing lysozyme have antibacterial activity. The lysozymes are widely distributed throughout the animal kingdom, and catalyse the hydrolysis of the peptidoglycan of bacterial cell walls and act as non-specific molecules against the invasion of pathogenic bacteria (Goven et al. 1994; Stabili & Pagliara 1994).

If these barriers are overcome, the potential invaders come into contact with a series of cells and humoral factors capable of recognising them as non-self, then activating a more appropriate response: they stimulate the second step of internal defense, which includes cellular and humoral responses such as the activation of phagocytic cells, the release of antimicrobial molecules, and the inflammatory response (McCaughey & Bodnar 2012).

As usually understood, only vertebrates, with the third level of defense that is the adaptive immune response, show an immunological memory able to destroy the same pathogen more rapidly when they meet it again. Both the adaptive and innate immunity require the capacity to distinguish between self and non-self molecules, through the high specificity of membrane protein receptors. Immune receptors are present in many types of cells, and they favour communication between humoral events and cellular events of immune response. Cellular events (such as phagocytosis, opsonisation, encapsulation, etc.) and humoral events (such as antimicrobial peptides, lectins, cytokines, etc.) are outlined in Figure 2. In the background, a toll-like receptor is drawn as an example of a receptor able to link the two events.

Nevertheless, in insects, a mechanism called "immune priming" was discovered. Briefly, the samples re-exposed to homologous PAMPs had

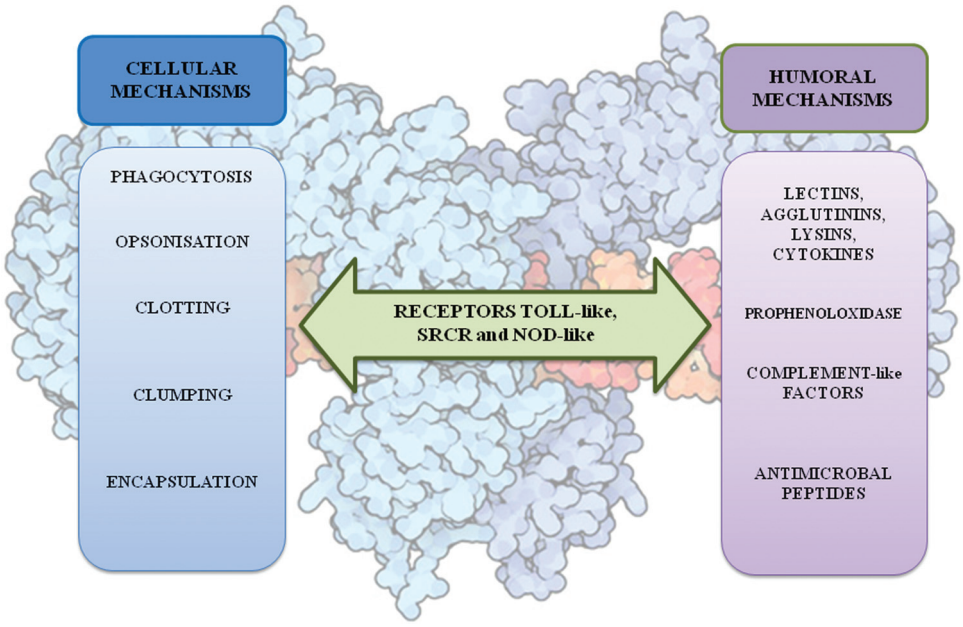

Figure 2. The schematic drawing shows the main mechanisms of innate immune response in Echinodermata, divided into: cellular (phagocytosis, opsonisation, clotting, clumping, encapsulation) and humoral (lectins, agglutinins, lysins, cytokines, prophenoloxidase, complement-like factors, antimicrobial peptides) events. All of these mechanisms are mediated by receptors. In the background can be seen the figure of a toll-like receptor. SRCR, Scavenger Receptor Cysteine-rich; NOD, Nucleotide-binding Oligomerisation Domain receptors.

significantly higher survival rates than the ones exposed to heterologous PAMPs (Pham et al. 2007). Also, in the sea urchin genome Ig-like genes and recombination activating genes (RAG) were annotated, opening the question on a putative adaptative immune response in invertebrates, even if no functional evidence was reported (Hibino et al. 2006). However, as the question on adaptive immunity in invertebrates remains open, and cellular response in echinoderms was widely studied (Arizza et al. 2007; reviewed by Smith 2012; Pinsino & Matranga 2015), here we report a general view on innate immunity, but we focus our attention on the humoral mechanisms of immune response in the deuterostome Echinodermata phylum.

Echinoderm immune system

Echinodermata is a wide and differentiated phylum of animals that have spread all over the marine ecosystem, and consists of five classes: Asteroidea (sea stars), Crinoidea (feather stars), Ophiuroidea (brittle stars), Echinoidea (sea urchins) and Holothuroidea (sea cucumbers).

Many species of this phylum have been used as model systems for studies on immunology as well as cell and developmental biology. In this scenario, sea urchins are particularly utilised (Matranga et al. 2005; Hibino et al. 2006; Rast et al. 2006; Smith et al. 2006).

The echinoderm immune system is structurally and physiologically specialised to perform a range of functions, including immune defense. It consists of the coelomic cavity where the coelomic fluid flows, which contains seawater with traces of nitrogen and non-nitrogen proteins (urea or ammonia), potassium ions, small amounts of lipids and also a population of freely circulating cells, namely the immune cells, once called coelomocytes (Endean 1966). The immune cells are a heterogeneous population of cells, at both morphological and functional levels. The profiles of immune cells may vary among species in terms of morphology, abundance, size, role and physiology. Four subpopulations of immune cells in *S. purpuratus* are described: phagocytes, vibratile cells, and colour-less and red spherule cells (reviewed by Smith et al. 2006). Indeed, three main cell types of circulating immune cells have been described in *Paracentrotus lividus* (Lamarck, 1816): amoebocytes, vibratile cells and phagocytes including different subtypes (Matranga et al. 2005; Pinsino & Matranga 2015). Moreover, Ramírez-Gómez and García-Arrarás (2010) summarise all the immune cell types reported for echinoderm classes, and, considering the morphological criterion, describe nine classes of immune cells, seven of which are present in Echinoidea.

The immune cells mediate the immune response through phagocytosis (Ito et al. 1992), cytotoxicity,

antibacterial activity, transplant rejection (Hildemann & Dix 1972; Karp & Hildemann 1976), encapsulation and opsonisation (Clow et al. 2004). Moreover, females possess a significantly higher number of immunocytes (phagocytes and uncoloured spherulocytes) than males, in *P. lividus* sea urchin, and so the degree of immune responses differs according to sex (Arizza et al. 2013).

Besides, the coelomic fluid consists of humoral factors such as lysins, agglutinins, lectins, molecules with antimicrobial, lytic or opsonic activities (Ryoyama 1973; Parrinello et al. 1979; Canicattì 1990; Pagliara & Canicattì 1993). The humoral defense topic will be mainly discussed in this paper.

Cellular mechanisms in echinoderms

A first line of cellular defense is represented by phagocytosis, carried out by phagocytes responsible for other activities, such as encapsulation, opsonisation, graft rejection and bacterial clearance. Phagocytes follow some steps similar to vertebrates: chemotaxis, adhesion, ingestion (formating pseudopodia wrap) and killing. The amebocytes, especially in the petaloid form, are those cells specifically responsible for the activity of phagocytosis, while the filopodial cells are responsible for clotting (reviewed by Smith et al. 2006; Pinsino & Matranga 2015). In vitro studies of phagocytosis in sea urchin *Strongylocentrotus nudus* (A. Agassiz, 1864) have revealed that phagocytes share some functional features with vertebrate macrophages and granulocytes (Ito et al. 1992).

In sea star *Asterias forbesi*, (Desor, 1848) an alternative reaction to phagocytosis in the body cavities is clumping, i.e. the formation of multicellular aggregates called nodules or capsules, which incorporate foreign particles (Kanungo 1982).

The formation of multinucleated giant cells is interpreted as the behaviour of those phagocytes designated to wrap a large foreign body. This feature of fusogenic phagocytes has been reported in vitro in all studies on echinoderms, and the configuration of syncytia varies from a voluminous mass of cytoplasm, in the case of a sea cucumber, to a fine structure in the lamellipodiale of the sea urchins (Bertheussen & Seljelid 1978; Dan-Sohkawa et al. 1993).

The vibratile cells are associated with clotting and movement of coelomic fluid (Bertheussen & Seljelid 1978; Smith et al. 2010). Cytotoxic cells present in echinoderms are capable of destroying foreign cells, even if their mechanism of action is still not known (Lin et al. 2001). In *P. lividus*, in vitro cytotoxic activity against rabbit erythrocytes was assayed, and this activity seems to be carried out by colourless spherulocytes in cooperation with the amebocytes (Arizza et al. 2007). The cytotoxic activity following the release of lysins by colourless spherulocytes appears to be amplified by soluble factors released by amoebocytes. Previous studies showed calcium-dependent cytolytic activity, against rabbit erythrocytes, which would seem to be attributed to the content of the granules of the amoebocytes (Pagliara & Canicattì 1993).

Studies on the rejection of transplants in echinoderms have shown that phagocyte and lymphocyte-like cells infiltrate the transplanted tissue, destroying its organisation. The rejection of the transplant is manifested as a hyperplasia of dark colour along the zone of contact between the dermis of the host and of the transplanted donor, the loss of pigmentation, the contraction of the transplanted tissue and the growth of the recipient tissue (Hildemann & Dix 1972; Karp & Hildemann 1976). In the sea urchin *Lytechinus pictus* (Verrill, 1867), the red spherulocytes characterise the first major response to allografts, migrating from the underlying tissue and remaining present during the time of rejection (Coffaro & Hinegardner 1977). However, the exact mechanism by which it accomplishes transplant rejection in echinoderms is still unknown. Red spherule cells contain naphthoquinone pigments, like echinochrome A, which shows antibacterial activity (Service & Wardlaw 1984).

Humoral mechanisms in echinoderms

The humoral defense consists of many soluble factors such as lysins, agglutinins, cytokines, lectins, molecules with antimicrobial (AMP), and lytic or opsonic activities.

Lysins or hemolysins are lytic molecules that have been observed in many species of echinoderms such as sea stars, sea cucumbers and sea urchins (Ryoyama 1973; Canicattì & Parrinello 1985; Canicattì 1991; Canicattì & Rizzo 1991). These molecules, synthesised by immune cells, are released as a result of a coagulation process or when in the presence of foreign substances. They are generally calcium-dependent and heat-labile and interact with sugars or glycolipids on the target cell membranes, causing circular lesions and producing cellular lysis (Canicattì 1990). The cytolytic effect is due to the formation of transmembrane holes of different sizes (5–20 nm). The membranes of rabbit erythrocytes, lysed by hemolysins of *P. lividus*, when observed under the electron microscope, show circular holes that resemble the membrane attack complex (MAC) present in vertebrates (Rosado et al. 2007). On the basis of some analogies with vertebrate lytic

molecules, it is conceivable that the hemolysins have evolved from a common ancestral gene.

Through the use of a wide range of target vertebrate erythrocytes, the reactivity of the invertebrate lysins was examined, for example in *Spirographis spallanzani* (Viviani, 1805). The lytic *S. spallanzani* hemolysin is a non-enzymatic, calcium-dependent factor that occurs naturally in the coelomic fluid (Canicattì & Roch 1993).

In the Echinoidea *Strongylocentrotus droebachiensis* (O. F. Müller, 1776) (Bertheussen 1983) and *S. nudus* (Ito et al. 1992), lysins have a role as opsonins as well as in *Holothuria polii* (Delle Chiaje, 1823); in fact, when sheep erythrocytes were injected into the coelomic cavity of *H. polii*, a decrease of hemolysis was observed, possibly due to the involvement of the hemolysins in clearance mechanisms. The hemolysins behaved like opsonins, causing the formation of rosettes and consequently stimulating phagocytosis (reviewed by Canicattì 1988).

Agglutinins are a group of adhesion molecules that play roles in cell aggregation, blood clotting, wound healing and encapsulation. They have been found in the coelomic fluid of echinoderms such as sea urchin (Ryoyama 1973; Canicattì et al. 1992), sea star (Kamiya et al. 1992) and sea cucumber (Canicattì & Parrinello 1985); they are generally heat-stable, are calcium-dependent, have a strong ability to agglutinate red blood cells and also act as opsonins (Finstad et al. 1972; Bertheussen 1983). Agglutinins increase the adhesive properties of circulating cells in *P. lividus* (Canicattì et al. 1992), and *in vitro* they agglutinate sea cucumber coelomocytes, probably having a role in clotting events (Canicattì & Rizzo 1991).

A group of proteins called lectins are able to bind to cellular sugar molecules and free glycoconjugates, making the cells sticky and causing clumping, thus enhancing phagocytosis. Lectins are ubiquitous and were isolated from all organisms, vertebrate and invertebrate (reviewed by Malagoli et al. 2010). Lectins isolated from the sea urchin (Bulgakov et al. 2013) are related to a family of vertebrate proteins called collectins. In humans, collectins (collagen-containing C-type lectins) are a family of collagenous calcium-dependent lectins, and are soluble PRRs; their function is to bind to oligosaccharides or lipids which are on the surface of microorganisms. Their binding triggers many mechanisms such as complement activation, opsonisation and phagocytosis aggregation reactions, which in turn cause the elimination of microbes (Thiel et al. 1997; van de Wetering et al. 2004; Gupta & Avadhesha 2007). Similarly to vertebrates, several lectins have been identified in the celomic fluid of echinoderms, where they play important roles in opsonisation, lytic cytotoxicity, clot formation and wound repair (Hatakeyama et al. 1999; Gross et al. 1999, 2000; Kouzuma et al. 2003). Different lectins with specific recognition abilities have been found in asteroids (Kamiya et al. 1992) and holothuroids (Gowda et al. 2008a, 2008b).

Two important features of the vertebrate immune defense, the complement system and the cytokine-mediated processes, seem to be absent in invertebrates, even if analogous mechanisms have been identified in invertebrates.

The vertebrate complement system contains many serine proteases involved in the proteolytic cascades, which are responsible for activation of the central component C3 which has a thioester bond capable of reacting with the microbe surfaces, stimulating the opsonisation mechanism (i.e. the removal of non-self by phagocytosis) and the induction of inflammation. Vertebrate proteases involved in the complement activation cascades are classified into two families, the complement factor B (Bf) family and the mannose-binding lectin-associated serine protease (MASP) family. All three of these families of genes have been identified from all invertebrate deuterostomes analysed so far, except for MASP which is absent in sea urchin (Al-Sharif et al. 1998; Smith et al. 1998; Materna & Cameron 2008). By contrast, only C3 and Bf were identified from protostomes (Zhu et al. 2005). C3, Bf and MASP were also identified from cnidarian sea anemone and coral (reviewed by Cerenius et al. 2010).

The initial evidence of a complement system in echinoderms was obtained from sea urchins, sea cucumbers and sea stars (Parrinello et al. 1979; Bertheussen & Seljelid 1982). Smith et al. (1998) identified the first echinoderm homologue of the C3 and factor B component. Mogilenko et al. (2010) reported the finding of a C3 complement homologue in the sea star *Asterias rubens* (O.F. Müller, 1776), whose expression is induced by stimulation with LPS. Additional components of the system have been identified from the sea urchin genome (Sodergren et al. 2006), suggesting that echinoderms possess a complement pathway that plays a key role in opsonisation (Hibino et al. 2006).

In place of a complement, several invertebrates, including various insects, crabs and worms, exhibit a similar response, called the prophenoloxidase (proPO) system. It comprises a complex system of pattern recognition proteins as well as proteinases involved in the synthesis of melanin at the site of infection or injury, with the aim of facilitating wound healing. The active form of the enzyme phenoloxidase (PO) is synthesised as an inactive zymogen named proPO (PPO), which is cleaved

after the proteolytic cascade activation (reviewed by Cerenius & Söderhäll 2004). The PO catalyses the oxidation of phenols to quinones and finally synthesises melanin, which participates in the encapsulation of microbes. In fact, the deposition of melanin on microbes forms a physical barrier able to localise the toxic compounds involved in the resolution of infection. The *Drosophila* genome encodes three PPOs. Two of them, PPO1 and PPO2, are found in the crystal cells and in the hemolymph at the larval stage (Binggeli et al. 2014). PPO genes were cloned by many other arthropods of the genera *Aedes* and *Anopheles*, as well as in crustaceans (reviewed by Cerenius & Söderhäll 2004). The PO process bears functional resemblance to the complement system, although the final melanisation is different in arthropods and insects (reviewed by Cerenius et al. 2010). proPO was found in the coelomic fluid of the crustaceans and ascidians, and at low levels in the starfish *Asterias rubens* and in the sea urchin *Diadema antillarum* (Philippi, 1845) (Smith & Söderhäll 1991).

Some very interesting inducible factors, responsible for bacterial clearance, are the antimicrobial peptides (AMPs); they play a central role in humoral defences, though they have not been studied in depth in marine organisms.

AMPs are amphipathic and positively charged small proteins, containing less than 100 amino acids. They are important immune effector molecules in invertebrates, such as insects, molluscs, crustaceans and tunicates, that have a broad spectrum of antimicrobial activity against bacteria, viruses and fungi (Hancock & Sahl 2006; Mastore et al. 2014). Their hydrophobicity allows their interaction with the hydrophobic lipid bilayer of the microbial membrane. AMPs, because of their cationic nature, are prone to attach to bacterial membranes with a negative charge. The mechanism by which AMPs interact with the structures of the cell wall of Gram-negative and Gram-positive bacteria is still poorly known. It is known that, in invertebrates, high concentrations of peptides compromise the integrity of the membrane, even if the formation of pores is not the only mechanism of antimicrobial activity and the intracellular targets of antimicrobial peptides are nucleic acids or enzymes (Brogden 2005).

Previous studies have highlighted the presence of antibacterial activity in Holothuroidea, Asteroidea and Echinoids (Leonard et al. 1990; Stabili et al. 1996; Haug et al. 2002). Many AMPs, more than 2200, have been identified in marine invertebrates, such as myticin and mytilin in mussels, and clavanin and styelin in ascidians (reviewed by Tincu & Taylor 2004). In Echinoidea, strongylocines are a new family of AMPs rich in cysteines, isolated from *S. droebachiensis* sea urchin coelomocytes. Another sea urchin family of AMP are the centrocins (Li et al. 2008, 2010). Recently, authors have found that transcripts of strongylocins were localised in phagocytes, vibratile cells and/or colourless spherule cells, while centrocins were localised in phagocytes. Moreover, transcripts of these AMPs were present in pluteus larvae (Li et al. 2014). Fragments of larger proteins, such as beta-timosine, have been identified in the antimicrobial activities of echinoderms (Stoeva et al. 2008). In the coelomic fluid of *P. lividus*, a variety of AMPs with inhibitory capacity against Gram-positive and Gram-negative bacteria and fungi have been identified (Schillaci et al. 2010, 2012, 2014).

In addition to these humoral activities, echinochrome A is worthy of mention; it is a naphthoquinone with antibacterial activity, released by red spherule cells (Service & Wardlaw 1984).

Finally, the discovery of humoral cytokines and growth factors in invertebrates deserves attention too. In fact, since the 1990s, IL-1 and IL-6 molecules have been isolated from the starfish *A. forbesi* (Beck & Habicht 1991, 1996; Beck et al. 1993). Later, when the sea urchin genome was sequenced, genes such as IL-1receptor, IL-17like, IL-17receptor, Rag1/2, members of the tumor necrosis factor (TNF) superfamily and interferons were found, indicating a perspective of latent adaptive immunity in the Echinodermata phylum (Hibino et al. 2006).

Conclusions

This review summarises the status of echinoderm humoral innate immune systems, by focusing on the molecular functions and structures of defense components identified in invertebrate hemolymph and tissues. After an infection, the relations between humoral and cellular immunity are narrow and almost form a network (see Figure 2). In fact, the signals that trigger immune responses are consequent to recognition mediated by receptors; the immune cells react with the non-self molecules, triggering a cellular response; some humoral molecules are already present in immune fluids, but others are secreted by immune cells. The phylum Echinodermata provides interesting models for understanding the evolution of immune systems. The importance of echinoderms in immunology is given by their key position in the evolutionary tree as they belong to the

deuterostomes, and that places them immediately before vertebrates, sharing with them many features.

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