CHAPTER 3

BIVALVE IMMUNITY

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Abstract: Bivalves are comprised of animals unclosed in two shell valves, such as mussels, oysters, scallops and clams. There are about 7,500 bivalve species and some of them are of commercial importance. Recently, interest in bivalve immunity has increased due to the importance in worldwide aquaculture and their role in aquatic environmental science and their position in phylogenetic research. This chapter provides a short review of bivalve immunity, including cellular and humoral immunity and the key components and the interactions involved in humoral immunity.

INTRODUCTION

The phylum Mollusca is one of the most large, various and important groups in the animal kingdom. The monophyletic Mollusca is sister to the clade that unites annelids with nemerteans, phoronids and brachiopods.[1] Together with platyhelminthes, they constitute Lophotrochozoa and further constitute Protostomia with Ecdysozoa (molting animals).[1] Bivalvia has about 7,500 species and was the second most diverse class of molluscs after Gastropoda. Many of them are sources of seafood and are important for pearl production and therefore of great commercial importance. In addition, as sedentary filter feeders, bivalves may concentrate bacteria, viruses, pesticides, industrial wastes, toxic metals and petroleum derivatives, making them important markers for biomonitoring pollution in aquatic ecosystems and ideal species for investigating the effects of environmental contaminants.[2]

In the long course of evolution, bivalves have developed an array of effective strategies to protect themselves from the attacks of various pathogens and environmental stresses. Interest in bivalve immunity has increased continuously in recent years due to

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serious diseases and mortality problems threatening the healthy development **of bivalve** aquaculture. Although pathology information has been accumulating, research on bivalve immune systems and the underlying molecular mechanisms are still at an early stage, with only some investigations in a comparatively small number of species.[3,4]

As invertebrates, bivalves rely exclusively on an innate, nonlymphoid system of immune reactions.[5] The internal defense of bivalve is mediated by both cellular and humoral components. The former includes phagocytosis or encapsulation, with subsequent pathogen destruction via enzyme activity and oxygen metabolite release, while the latter includes various reactions mediated by series of molecules.[6] Here we review our current knowledge of bivalve immunity, mainly focusing on immune recognition, signal transduction and effector synthesis involved in cellular and humoral immunity.

HEMOCYTES AND PHAGOCYTOSIS

Bivalves have an 'open' circulatory system in which the hemolymph, passing out of the open ends of arteries, bathes all the organs before returning to the heart by the way of sinuses and respiratory structures (gills). Hemocytes, the circulating cells of bivalves, are primarily responsible for the defense against pathogens.[7] Phagocytosis and encapsulation are two major mechanisms for hemocytes to eliminate nonself substances and dead cells.[8] In addition, antibacterial effectors, opsonins, nonspecific hydrolysis and toxic oxygen intermediates found in bivalve hemolymph together coordinate immune response.

The assortation of bivalve hemocytes has been controversial for a long time. Based on morphology and histochemistry research results from mussels and clams, bivalve hemocytes are generally categorized into hyalinocytes and granulocytes,[9-11] and the latter can be further subdivided into eosinophilic granular hemocytes and basophilic granular hemocytes.[6,11] There are also other hemocyte types in some species. For instance, the Type 'III' eosinophil, morula-like cells and blast-like cells were found in *Cerastoderma edule*, *Tridacna derasa* and *Scrobicularia plana* hemolymph, respectively.[6,12] Among all these cell types, granular hemocytes are the most numerous, rich in a variety of hydrolytic enzymes and the major cell, executing the process of phagocytosis.[10]

Phagocytosis is a process to recognize and ingest nonself molecules and cell debris. Bivalve hemocytes can engulf a variety of particles including bacteria, algae, yeast, foreign blood cells and latex spheres.[7] The first step of phagocytosis is the attachment of phagocyte to the targeted particle.[13] Many studies have revealed that hemocytes of many bivalve species exhibit chemotactic as well as chemokinetic reactions, type of which is dependent upon the nature of the molecules presented.[14] In the mussel *Mytilus edulis*, lipopolysaccharides (LPS) from both *Serratia marcescens* and *Escherichia coli* stimulated the migration of cells. In European flat oyster *Ostrea edulis*, hemocytes migrated from circulatory system to connective tissues after *Bonamia ostreae* infection.[15] Hemocytes of *Mercenaria mercenaria* migrated toward not only peptides or small proteins secreted by both Gram-positive and Gram-negative bacteria. After chemotactism, the phagocytes adhere to pathogens, followed by cytoskeleton modification, internalization and destruction of the engulfed target within phagosomes.[13] In *Mytilus galloprovincialis*, both hyalinocytes and granulocytes could execute phagocytosis by formation of coated vesicles and uncoated endocytic vesicles,[10] and cells of different morphology presented different levels of phagocytosis towards zymosan, latex beads and bacteria.[16]

After the nonself is phagocytosed, the phagosomes and lysosomes fuse together and the engulfed target is destroyed within phagosomes by lysosomal enzymes, reactive oxygen species (ROS), nitric oxide (NO) as well as antimicrobial factors. During phagocytosis, the release of degradative enzymes for the destruction of foreign material is accomplished by a sudden release of ROS within hemocytes, which is referred to as respiratory burst. ROS act as killing agents, either alone or in combination with lysosomal enzymes and are important in the phagocyte-mediated killing of microorganisms.[17] Studies on oyster *Crassostrea ariakensis* revealed that granulocytes were most active in spontaneous ROS production[12]. In clam *M. mercenaria*, hemocyte oxidative burst was active in response to the stimulation of zymosan and bacterial extracellular products, accompanied by the increase of ROS production in hemocytes.[18] The internalization of different phagocytic targets and the production of ROS and NO in *M. galloprovincialis* were found to be blocked by inhibition of phosphatidylinositol 3-kinase, protein kinase C and extracellular signal-regulated kinase.[16] Furthermore, many other hemolymph factors also help phagocytosis, including agglutinins (e.g., lectins) and various antimicrobial peptides. Bivalve lectins have different carbohydrate-binding specificities and are involved in nonself-recognition.[19] Antimicrobial peptides are engaged in the destruction of bacteria inside the phagocyte before being released into hemolymph to participate in systemic responses.[7] These factors will be further discussed in the following parts.

Another mechanism of invertebrate cellular immune response is encapsulation.[13] In invertebrates, encapsulation is the common immune defense reaction for foreign bodies which are too large to be phagocytosed. In general, a capsule of hemocytes encloses the foreign body (e.g., multicellular parasites) and cytotoxic products (e.g., degradative enzymes and free radicals) are released by the hemocytes in an attempt to destroy the invader. In the encapsulation response of *Crassostrea gigas* against copepods *Myicola ostreae*, the copepods were observed on the gill surface of *C. gigas* engulfed by a massive agglomerate of hemocyte-like cells encircled by a thin layer of fibroblast-like cells.[20] The clam *Dreissena polymorpha* also employed such a defense mechanism when infected with trematode *Bucephalus polymorphus*.[21] Studies on *C. edule* revealed that positively charged targets stimulated the most vigorous response and that nonspecific electrostatic forces and humoral plasma factors have a synergistic role in hemocyte attachment and the encapsulation response. Phagocytosis and encapsulation are important and complicated processes, the involved molecules and the detailed mechanism should be addressed in future studies.

IMMUNE RECOGNITION

Immune recognition is the first step in activating immune response and occupies a very important position in the immune system to discriminate nonself from self substances. The immune responses begin when specialized, soluble or cell-bound Pattern Recognition Receptors (PRRs) recognize (and bind to) the major targets, called Pathogen-Associated Molecular Patterns (PAMPs).[22,23] PAMPs are common in microorganisms but rare or absent in host animals, such as LPS or peptidoglycan (PGN) in bacterial cell walls and β-1,3-glucan on fungal cell walls.[24] Invertebrates rely only on innate immunity and develop a sophisticated system of PRRs. Seven groups of distinct PRR are identified in bivalves, including peptidoglycan recognition proteins (PGRPs), Gram-negative binding proteins (GNBPs), C-type lectins, galectins, thioester-containing proteins (TEPs), scavenger

receptors (SRs) and Toll-like receptors (TLRs). In the following, we will briefly introduce the bivalve PRRs, focusing on their binding specificity and functions.

Peptidoglycan Recognition Protein

PGRP is a member of PRRs that specifically bind to PGN, a unique cell wall component of all virtual bacteria but not present in eukaryotic cells. The knowledge of invertebrate PGRPs comes mainly from insects. These PGRPs played a central and diverse role in activating immune reactions, such as melanization cascade, phagocytosis and activating the Toll or IMD signal transduction pathways for the production of antimicrobial products to hydrolyze peptidoglycan and protect host against infection. Some bivalve PGRPs have been identified from the Pacific oyster *C. gigas* (*CgPGRP-S1S*, *-S1L*, *-S2*, *-S3* and *CgPGRP-L*),[25] bay scallop *Argopecten irradians* (*AiPGRP*)[26] and Zhikong scallop *Chlamys farreri* (*CfPGRP-S1*).[27] The identified bivalve PGRPs were all short type with a conserved amidase PGRP domain in their C-terminus. Interestingly, there was an additional goose-type (g-type) lysozyme domain in CgPGRP-L, while a defensin-like domain was present in both CgPGRP-S1S and CgPGRP-S1L.[25] The recombinant protein rCfPGRP-S1 from scallop could bind not only with PGN but also with chitin and LPS moderately. More importantly, rCfPGRP-S1 exhibited strong activities to agglutinate Gram-positive bacteria *Micrococcus luteus* and *Bacillus subtilis*, while slightly with the Gram-negative bacterium *E. coli* (unpublished data). The short type PGRP is widely present in various bivalve species and seems to be a versatile PRR not limited to the function of recognizing and binding the PAMPs and thus an indispensable component in bivalve innate immunity.

Gram-Negative Binding Protein

The GNBP family includes members that bind Gram-negative bacteria, LPS and β-1,3-glucan.[28] Research on bivalve GNBP is still limited and they have only two related reports. The LPS and β-1,3-glucan binding protein (LGBP) is one of the GNBPs with various biological functions, including the activation of the prophenoloxidase (pro-PO) system, cytolysis, bacterial aggregation and opsonic reaction. The LGBP gene was cloned from scallop *C. farreri* (*CfLGBP*) with conserved domains of the LPS-binding site and glucan-binding site. The initial up-regulation after *Vibrio anguillarum* challenge indicated that *CfLGBP* was sensitive to bacterial infection.[27] The recombinant CfLGBP could bind not only LPS and β-glucan, but also PGN and exhibited obvious agglutination activity towards Gram-negative bacteria *E. coli*, Gram-positive bacteria *B. subtilis* and fungi *Pichia pastoris* in vitro. Additionally, a beta-1,3-glucan binding protein (βGBP) was purified from the plasma of marine mussel *Perna viridis*[29] with an inherent serine protease activity. It agglutinated bakers yeast, bacteria and erythrocytes and enhanced proPO activity of the plasma. Although its gene sequence and molecular structure are still unknown, βGBP is thought to be a multifunctional molecule and functions as a recognition molecule for beta-1,3-glucan on the surface of microbial cell walls.

C-Type Lectin

C-type lectins are a superfamily of diverse proteins with one or more carbohydrate-recognition domains (CRDs) of ~130 amino acid residues. They recognize

and bind to terminal sugars on glycoproteins and glycolipids and function in nonself recognition and clearance of invaders.[30] In bivalves, lectins are undoubtedly involved in nonself recognition and also have agglutination or opsonic roles in hemocyte phagocytosis. We will quote those lectins in immune recognition of bivalve in the following and adduce their agglutination or opsonic roles later.

The forepart research on C-type lectins mainly focused on characterizing the features or detecting their activities. With the development of molecular biotechnology, much more attention was paid to their gene structure and recognition mechanism. Recent EST analysis in scallops, clams and oysters revealed a high content and variety of lectin gene homologues.[27] Some C-type lectins or proteins with similar activities have been characterized from bivalves.[31] The mRNA expression of these genes could be significantly up-regulated by bacteria or parasite, especially bacteria of *Vibrio* genus, suggesting that they were involved in the immune response against invading microbes.

Bivalve C-type lectins are diverse in their domain structure and organization. Besides most lectins with single CRD, there are also three and four CRDs in Cflec-3 and Cflec-4 from *C. farreri*, respectively, while most insect C-type lectins contain tandem CRDs.[32] The architecture and phylogenic analysis of these proteins together with those from *Drosophila* and *Caenorhabditis elegans* suggested that multidomain C-type lectins in different lineages did not arise from a common multidomain progenitor and these proteins served distinct functions in different animal lineages.

Bivalve C-type lectins have different carbohydrate-binding specificities and are believed to be a kind of antibodies in nonself-recognition. For instance, in clam *Ruditapes philippinarum*, MCL can bind to the surfaces of purified hypnospores and zoospores of *Perkinsus olseni* parasite by recognizing and binding the terminal GalNAc/Gal residues. Meanwhile, the hemo-agglutinating activity of MCL3 could be inhibited by GalNAc, Mannose, lactose, raffinose polysaccharides bovine mucin Type II and Candida mannan.[33] These two lectins, as well as MCL-4, contributed to the phagocytic ability to eliminate bacteria or parasite via recognition of terminal carbohydrated residues on the surface of microbes.[34] Chiletin from *Ostrea chilensis* could agglutinate sheep red blood cells through binding galactose and mannose.[35] The EPN motif in the canonical binding sites of Codakine from clam *Codakia orbicularis* proved to be important for calcium-dependence and mannose/GlcNAc-binding activity.[36] In some species, the lectins with similar carbohydrate-binding specificity may distinguish different invading microbes. For example, the recombinant C-type lectins Cflec-1, Cflec-2, Cflec-3 and Cflec-5 from *C. farreri* agglutinated *E. coli*, *Staphylococcus haemolyticus*, *Pseudomonas stutzeri* and *P. pastoris*, respectively, though they all possessed mannose-binding specificity.

The great number of C-type lectins identified from bivalves shared similar structural features and displayed binding and agglutinating activities towards a range of microbes. Since there is no antibody-mediated immunity in invertebrates, abundant lectins with diverse expression profiles and bioactivities might function as nonclonal effectors in the bivalve immune system.

Galectin

Galectins are a family of β-galactoside-binding lectins and they are probably the most conserved and ubiquitous lectin family found in multicellular organisms.[22,23,28] The bivalve galectin was purified first from oyster *Pinctada fucata martensii* in the 1980s,

but the genes were cloned and identified recently, including *CvGal* from *Crassostrea virginica*[37], *CgGal* from *C. gigas*,[31] *AiGal1* from *A. irradians*,[38] *MCGal* from clam *R. philippinarum*[39] and *Pf-galectin* from *Pinctada fucata*. Except for CgGal, which contains a single CRD, the other four galectins possess multiple CRDs. AiGal1, CvGal and Pf-galectin are quadruple-CRD galectins, which are so unique in bivalves that it has never been reported in other species. From a phylogenetic point of view, all four CRDs of AiGal1, CvGal and Pf-galectin form a single clade, suggesting that the bivalve galectin CRDs share a common ancient and the four individual CRDs of each galectin are originated by repeated duplication of a single galectin gene.

CvGal and MCGal are two major galectins for functional study. CvGal could facilitate recognition of a variety of potential microbial pathogens, unicellular algae and preferentially *Perkinsus marinus* trophozoites. Attachment and spreading of hemocytes to foreign surfaces induced localization of CvGal to the cell periphery, its secretion and binding to the plasma membrane. CvGal subsequently promoted phagocytosis for both potential infectious challenges and phytoplankton components.[37] Moreover, MCGal had an affinity towards galactose and N-acetylgalactosamine and could bind to the surface of *Perkinsus olseni* and agglutinate *Vibrio tapetis* in vitro.[39]

Thioester-Containing Protein

TEPs are a family of proteins characterized by the unique intrachain β-cysteinyl-γ-glutamyl thioester bond and a propensity for multiple conformationally sensitive binding interactions.[40] This protein family consists of complement components C3, C4, C5, protease inhibitor alpha$_2$-macroglobulin (α_2M), CD109 and a set of insect TEPs. Among them, the invertebrate C3-like molecules and insect TEPs were thought to be involved in the innate immune defense as PRRs.[28]

The bivalve TEPs were recently identified from the clam *Ruditapes decussates*[41] and scallop *C. farreri*,[42] termed as *Rd-C3* and *CfTEP*, respectively. They both contained canonical thioester motif GCGEQ, proteolytic cleavage sites and catalytic histidine residues similar to C3 molecules. However, CfTEP possessed additional features distinguished it from C3 molecules, including: (1) the absence of anaphylatoxin-like and C345C domains, (2) a distinctive cysteine signature in the C-terminus which characterized the TEP subfamily apart from complement factors and α_2M subfamilies and (3) the highly variable central region.[42] Due to these structural differences, CfTEP was phylogenetically related to the insect TEPs, while Rd-C3 was related to the invertebrate C3-like molecules. The above results supported the view that TEP and complement factors shared a common ancestor but they separated from each other at a rather early lineage.[43]

The genomic organization of *CfTEP* was similar to human and mouse C3 rather than ciona C3-1 and *Drosophila dTEP2*, indicating a complicated evolutionary history of this gene family. It was of great interest that seven different *CfTEP* transcripts were produced by alternative splicing and they displayed different expression patterns in gonads in response to different bacterial challenges, which suggested an important role of diverse *CfTEP* transcripts in the innate immune defense of scallops.[44] These results provided new insights into the role of TEPs in bivalve immune responses, as well as the evolutionary origin of this important, widespread and functionally diversified family of proteins.

Scavenger Receptor

Scavenger receptors (SRs) are a main type of endocytic receptors with multifunctions to recognize and engulf various PAMPs.[45] In contrast with other PRRs, the information about invertebrate SR is extremely limited. There are only two invertebrate SRs identified from *Drosophila* (*dSR-CI*) and *C. farreri* (*CfSR*, GQ260639.1). CfSR is structurally different from all the characterized SRs. It contains six scavenger receptor cysteine-rich (SRCR) domains absent in dSR-CI and UPAR-like and ShK toxin-like domain do not exist in any other member of SRs. CfSR shares a similar attachment site with anchor protein Sgp-2 and it was mainly detected on the outer surface of hemocytes by immunofluorescence approach, indicating that CfSR was anchored on the outer-membrane of cells. The recombinant CfSR displayed a significantly strong activity to bind not only acetylated LDL, dextran sulfate but also with various PAMPs, including LPS, PGN, zymosan particle and mannan.

CfSR is one of the most primitive SR found so far in invertebrates. It displays unique structure and broader ligand binding ability. The existence of SR protein in bivalve will contribute not only to the origin and evolution of the diverse molecules, but also to the understanding of the complex mechanism of immune recognition.

Toll-Like Receptor

TLRs are an ancient family of proteins with the hallmark structure of extracellular leucine-rich repeats (LRRs), intracellular Toll/Interleukin-1 receptor (TIR) domains, which play key roles in detecting various nonself substances and then initiating and activating immune system. The reports of TLRs in mollusc are still rare although they were widely distributed in nearly all animal phyla. Only one TLR gene (*CfToll-1*) and three TLR EST fragments had been identified in *C. farreri*, *C. virginica*[46], *A. irradians*[47] and *M. mercenaria*.[48] *CfToll-1* shared the same domain architecture with *D. melanogaster* Tolls (*DmTolls*) and *Tachypleus tridentatus* Toll (*tToll*). The mRNA expression of *CfToll-1* was upregulated by LPS in a dose-dependent manner.[49] The finding suggested that *CfToll-1* might be involved in immune response against bacterial invasion. Considering their importance in innate immunity, the study of the number, assortation and the roles of bivalve TLR in immune recognition and signal transduction (discussed in other section) should be addressed in the future.

IMMUNITY SIGNALING PATHWAYS

Innate immune system is under the control of a complex network of evolutionary conserved signaling pathways, which are activated depending upon different invasions or stimuli. Several signaling pathways, such as TLR, the Janus kinase/signal transducer and activator of transcription (JAK-STAT), mitogen-activated protein kinase (MAPK) and NF-κB pathways have been extensively studied in recent years for their important roles in regulating the immune system in both vertebrates and invertebrates. In bivalves, effective immune defense systems have been developed during evolution which can protect them from infection successfully. When bivalves are challenged by a pathogen, different signaling pathways triggered by PRRs induce the systemic immune response and produce responding effectors. Although the studies are preliminary, some genes

involved in conserved signaling pathways have been identified in bivalves. In this section, we will briefly introduce what is currently known about these signal transduction pathways, which may be a crucial step for our understanding about bivalve immune system.

The Canonical NF-κB Signaling Pathway

NF-κB signaling pathway is an evolutionarily conserved process to activate NF-κB,[50] a nuclear factor which has a central role in coordinating the expression of a wide variety of genes that control immune responses.[51] In resting cells, NF-κB proteins bind to the inhibitor of κB (IκB). The NF-κB signaling pathway is activated once IκB is degraded. This degradation of IκB is catalyzed by serine kinase I kinase (IKK) which leads to the translocation of released NF-κB dimers to nucleus. The activated NF-κB proteins then bind to DNA and activate gene transcription.[50]

In recent years, the key molecules involved in bivalve NF-κB signaling pathway, such as NF-κB, IκB, IKK genes, have been identified successively, offering clues for the existence of NF-κB signaling pathway in bivalves. Now three IκB genes have been cloned from scallops *C. farreri*[27] and *A. irradians*,[47] and oyster *C. gigas*,[52] and they showed a high level of identity with insect IκB-like proteins and vertebrate IκB isoforms. The *Cg-Rel*, a gene encoding the first NF-κB homologue in bivalve, shares the structural organization with Rel/NF-κB family members. The C-terminal transactivation domain and κB binding sites were indispensable for activation of the expression of genes controlled by NF-κB pathway. The NF-κB signaling pathways were well studied in pearl oyster *P. fucata*. Three genes, *Pf-Rel*,[53] *Pf-IKK*[54] and *poIκB*[55], were cloned and they were constitutively and ubiquitously expressed in tissues of pearl oyster. LPS could transiently stimulate IκB degradation, but couldn't influence the expression level of *Pf-IKK*. Transfection experimentation in NIH3T3 cells with Pf-IKK demonstrated that Pf-IKK triggered the gene expression by activating NF-κB in an IκB-dependent manner just as mammalian counterparts do.[54] The findings above favored the hypothesis that NF-κB signaling pathway was an ancient scheme of immune gene regulation pathway, which was conserved in bivalves. Although the complex members and their detailed information of NF-κB signaling pathway are not well understood in bivalve, the accumulating evidence indicates that it bears considerable similarity with mammalian NF-κB signaling pathway.

MAPK Pathway

MAPK cascades are one of the most important signaling pathways controlling a variety of physiological processes including cell proliferation, growth, differentiation, cell death, innate immunity and development.[56] Three subfamilies of MAPKs have been well-characterized in multicellular organism. They are extracellular signal-regulated kinases (ERKs), c-Jun amino-terminal kinases (JNKs) and p38 MAPKs. The pathway represents a characteristic phosphorylation system in which a series of three protein kinases phosphorylate and activate one another.[57] MAPK pathways were proven to exist in bivalve by immunoblot or ELISA techniques.[58] The pathways are not only sensitive to various environmental stressful stimuli, but also can be activated by growth factor insulin, cytokines, hormone substances and bacterial challenge. Several ESTs homologous to the MAPK pathway components were screened from cDNA library of oyster (*Cg-MAPKK1*,

Cg-MAPKK2, *Cg-c-jun* and *Cg-phosphatase*, *Cg-focal*, *Cg-FAK*) and Manila clam (c-jun).[59] Further investigations in molecular structure and their functions are needed for the precise defining of all molecules involved in MAPK pathway of bivalves.

JAK-STAT Pathway

JAK-STAT pathway is one of the important signaling pathways, downstream cytokine receptors.[60] Although JAK-STAT pathway seems to be present throughout evolution, there is no such report regarding the existence of JAK or STAT molecule in bivalves. Nevertheless, STAT activation has been observed recently in *M. galloprovincialis* under the stimulation of cytokine and bacteria. The microbicidal activity against *E. coli* in mussel hemocytes increased significantly after the pretreatment with human recombinant IFNγ, while no effect was observed with IFNα. IFNγ induced a rapid and time dependent increase of phosphorylated STAT1-like protein, evaluated by polyclonal antibodies specific for the tyrosine phosphorylated sites of STAT1.[58] It was suspected that JAK-STAT pathway possibly existed in bivalves. More and more cytokines and their receptors found in bivalves (details in other section) provided the clue for further investigation of bivalve JAK-STAT pathway. The roles of JAK-STAT pathway and their relationship with the newly indentified cytokines from these animals may be an interesting research field which is helpful to our comprehensive understanding of bivalve immunity.

The Toll-Like Receptors Signal Pathway

The characterization of TLR and its signaling pathway is one of the greatest propellers to acquaint with the immune system in the last decades. The receptors, adaptors and transducers in this pathway, such as TLR, myeloid differentiation factor88 (Myd88), tumor necrosis factor receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase (IRAK) displayed striking similarity from *Drosophila* to mammals, while the function of this pathway is different between human and fly. TLR pathway in mammals is a skillful system that detects invasion of various pathogens and plays a key role in bridging innate and adaptive immunity. Toll pathway in *Drosophila* is indispensable for both development and antifungal/anti Gram-positive bacteria immunity. It is worth noting that the absence of Myd88 orthologue and their very low similarity of TRAF and IRAK homologues strongly imply the evolutionary 'loss' of Toll signaling pathway in nematodes.[61] Moreover, *tol-1* in *C. elegans* is not involved in immune response but only in development. More information about TLR pathway in other invertebrates is required for better understanding of its function and evolution.

Fortunately, the key components involved in TLR pathway, including TLR,[49] Myd88,[62,63] TRAF6,[64] TRAF3,[65] and IRAK[46] have also been identified in bivalves. And the expressions of *CfToll-1*,[46] *CfMyd88*,[62] *CfTRAF6*[64] in scallops were up-regulated by stimulation of LPS or PGN. Furthermore, when the *CfToll-1* was "knock down" by RNAi technique, the scallop was more susceptable to pathogen and the expression of downstream genes was also down-regulated. Undoubtedly, the canonical TLR signaling pathway existed in bivalve. Considering scallop is a relatively primitive animal, the presence of TLR signaling pathway is epochmaking to understanding its origin, evolution and crucial roles in innate immunity.

Complement Pathway

The complement system is a major component of vertebrate innate immunity and also an essential bridge between innate and adaptive immunity. It can be activated mainly through four separate pathways, referred to as the classical, alternative, lectin[66] and coagulation[67] pathways. Recent investigation on the evolution of complement system has demonstrated the origins of complement system could be traced to near to the beginnings of multi-cellular animal life and the components of complement system should have some evolutionary traces in inferior species. However, there are very few reports on complement system in invertebrates and even less in bivalves. To our knowledge, only two complementary factors, C3 (*Rd-C3*) and B factor-like (*Rd-Bf-like*) molecules have been identified as representatives of the alternative pathway from carpet-shell clam (*Ruditapes decussatus*). The Rd-C3 shares distinctive structural characteristics with complement proteins (C3/C4/C5). Rd-Bf-like is composed of two complement control protein modules (CCP domains) and shares about 30% similarity with other known Bf proteins.[68] Additionally, three complement-like factors, including *CfTEP*[42] (details in other section), *CfC1qDC*[69] and *AiFREP*[70] were identified from scallops. TEP reported in scallop suggested that it might be an ancient equivalent component of the key factor, C3, and there was likely to be a primitive, simple complement-like system in bivalves. CfC1qDC is a novel C1q-domain-containing protein with LPS binding activity and is suspected to be a candidate of classical pathway in scallop. AiFREP, a member of protein with fibrinogen-like (FBG) domain at the C-terminus, exhibited a very high similarity with FBG domain of mammalian ficolin (at 61% approximately with *Rattus norvegicus*) and other invertebrate FREP proteins. *AiFREP*, the EST fragment of MBL and ficolin from oyster and kinds of lectin in bivalves indicated the high existent probability of lectin pathway. The results place us in a puzzle about the bivalve complement system and its activation.

IMMUNE EFFECTOR

Innate immunity encompasses a complex array of defense reactions, in which immune effectors are fundamental molecules and utilized as executor for the incapacitation and elimination of invaders. The immunity of bivalves relies upon the production of immune effectors that are active against a large range of pathogens or sensitive for the environmental stress. The importance of those molecules is underlined by increasing outbreak of a variety of diseases and summer mortalities in bivalve aquaculture. The following details recent knowledge of immune effectors, such as antimicrobial peptides (AMPs), cytokines, complement components, antioxidant enzymes, acute phase proteins and draws their repertoire in bivalve immune response.

Antimicrobial Peptides

AMPs have been characterized as one of the key immune effectors in innate immunity and are widespread in plants and animals. All the known AMPs are classified into four structure groups according to their amino acid sequences, secondary structures and functional similarities: (1) the linear basic peptides forming amphipathic α-helices

conformation and deprived of cysteine residues; (2) peptides containing cysteine residues with one to six intra-molecular disulphide bonds; (3) peptides rich in regular amino acids like proline with a variable structure; (4) the peptides produced by the hydrolysis of large inactive or proteins with little activity. Approximately 20 AMPs have been identified from bivalves, with 10 from *M. edulis* and *M. galloprovincialis*, which were organized into four distinct groups according to their antimicrobial action based on their hydrophobic and cationic properties and the amphipathic structure: MGD (defensin), mytilin, myticin and mytimycin.[71,72]

MGDs (MGD1 and MGD2) characterized from *M. galloprovincialis* with eight cysteines showed high similarities to the arthropod defensin family, which contains 6 cysteins. They were believed to be the original members of the arthropod defensin family because of the presence of two extra cysteines.[73] MGD1 and MGD2 share significant homology with the high conserved cysteines, hydrophilic and hydrophobic residues,[72] and they are essentially active against Gram-positive bacteria, including some pathogens for marine invertebrates. There are five kinds of mytilin isoforms in the bivalve mussels. Mytilin A and B were isolated from *M. edulis* plasma and mytilin B, C, D and G_1 were isolated from *M. galloprovincialia* hemocytes. Mytilin A, B, C, D exhibited significant activities against both Gram-positive and Gram-negative strains, while mytilin G1 was only active on Gram-positive bacteria. Mytilin B and D were also active against the filamentous fungus *Fusarium oxysporum.* Mytilin C was tested against the protozoan parasite, *P. marinus.*

Myticins were identified as novel cysteine-rich peptides. At present, three classes of myticin, A, B and C have been reported. Only one isoform has been described for myticin A and B and a total of 74 different isoforms have been reported for myticin C so far.[74] Myticin A and B were purified from the hemocytes and the former was also from the plasma of *M. galloprovincialis.* The mature peptides of myticin A and B comprise 40 residues with four intra-molecular disulfide bridges and a cysteine array in the primary structure different to that of the previously characterized cysteine-rich AMPs. The two myticins had marked activity against the gram-positive strains, *M. luteus, Bacillus megaterium* and *Aerococcus viridans.* Myticin B was also active against the filamentous fungus, *F. oxysporum* and moderately against the Gram-negative, *E. coli D31*. Myticin C had a high variability on the nucleotide sequence. It was a ubiquitous peptide expressed early in the development and associated with mussel survival. Different myticin C isoforms were observed along their life when mussel touched with new potential pathogens or nonself molecules in general. Therefore, myticin C could deal with a huge range of potential pathogens present in the marine ecosystem. However, the mechanisms to generate diverse isoforms and its implication in the fight against pathogens should be further studied.[74]

There is only one mytimycin identified from *M. edulis*[75] with molecular weight of 6.2 kD. The search in the peptide sequence data bases did not yield any homology with known peptides. There are twelve cysteines engaged in the formation of six intra-molecular disulfide bridges. Its antimicrobial activity was strictly antifungal.

In addition to the AMPs found in *M. galloprovincialis*, defensins have been identified from other bivalves, including *Cg-Def*, *Cg-Defh1* and *Cg-Defh2* from *C. gigas*,[76] and a big defensin (*AiBD*) from *A. irradians*.[77] Cg-Defh1 and Cg-Defh2 identified from hemocyte were almost same and shared around 80% identity with mantle Cg-Def.[76] Recombinant Cg-Def was active in vitro against Gram-positive bacteria but displayed

no or limited activities against Gram-negative bacteria and fungi. *AiBD* was the first bivalve big defensin gene cloned from bay scallop *A. irradians.* It consisted of 531 nucleotides with a canonical polyadenylation signal sequence AATAAA and a poly(A) tail, encoding a polypeptide of 122 amino acids. Recombinant AiBD showed activities against both Gram-positive and Gram-negative bacteria and some fungi.

As the most important effector in bivalve immunity, more AMPs will be found and well characterized, which will provide a better understanding of the immune defense mechanisms of bivalve and new insights into health management and disease control in aquaculture.

Lysozymes

Lysozyme is a ubiquitous enzyme existing in numerous phylogenetically diverse organisms such as bacteria, bacteriophages, fungi, plants and animals, which catalyzes the hydrolysis of β-1,4-glycosidic linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine of PGN and causes bacterial cell lysis.[78] It has been widely accepted that lysozyme functions as a crucial effector molecule in innate immunity. In general, the lysozymes are classified into six types according to the organisms where they are first identified, chicken-type (c-type), goose-type (g-type), invertebrate-type (i-type), phage, bacterial and plant lysozyme.

Several lysozymes and their activities have been characterized in bivalves. All previous reported bivalve lysozymes are i-type.[79] But recently, two g-type lysozymes (*CFLysG*, *AILysG*) were identified in scallops and no such thing has been reported before. It demonstrated that g-type lysozyme was not vertebrate-specific and its origin should precede the divergence of invertebrate and vertebrate.[80] Chlamysin from *Chlamys icelandic*,[81] lysozymes from *Tapes japonica*[82] and *C. virginica*[83] have been verified as i-type lysozymes with remarkable antibacterial activity against both Gram-positive and Gram-negative bacteria. The g-type lysozyme CFLysG possessed all conserved features critical for the fundamental structure and function of g-type lysozymes, such as three catalytic residues (Glu 82, Asp 97, Asp 108) and showed more potent inhibitive activities against Gram-positive bacteria. The self-defense activity of lysozyme have been detected widely in the body fluid and various tissues of bivalves, including the genus of *Mytilus*, *Bathymodiolus*, *Calyptogena* and *Chlamys*. A recent study has demonstrated that lysozyme could augment the activity of AMPs through a synergistic mechanism.[84] The lysozyme can also serve as a digestive enzyme in the digestive organs,[85] which has been further verified by lysozyme activities in hepatopancreas of several bivalves.

Lectins

The function of lectins includes not only self/nonself recognition but also engaging associated effector mechanisms, such as complement-mediated opsonization and killing of potential pathogens. Diverse lectins have been reported in oysters,[31,35] scallops and clams,[86,87] and they are involved in the immune response against pathogens. The recombinant C-type lectins Cflec-1, Cflec-2, Cflec-3 and Cflec-5 from *C. farreri* agglutinated *E. coli*, *S. haemolyticus*, *P. stutzeri* and *P. pastoris*, respectively. The lectin from *C. virginica* agglutinates a wide variety of bacteria.[88] The sialic acid-binding lectin of horse mussel, *Modiolus modiolus*, has strong antibacterial activity against *Vibrio* strains.[89] A calcium independent lectin isolated from the foot muscle of marine

bivalve *Macoma birmanica,* named MBA, could interact with both Gram-positive and Gram-negative bacteria.[90] There is increasing evidence to support the suggestion that the presence of isoforms and diverse roles of lectins provide bivalves with a functional diversity to the innate immune response.

Cytokines

Cytokines comprise a large number of regulatory molecules, interleukins (IL), IFN, TNF and chemokines and many of them function vitally in the vertebrate immune system.[91] In view of the significant functions, the studies of cytokine in bivalves have been pursued since the early 1990s. In the first publication, oyster cells were found to respond to IL-1 and TNF in a manner similar to that of human granulocytes.[92] Subsequently, the information about the effect of cytokines on immunity such as cell motility, chemotaxis, phagocytosis and cytotoxicity has been accumulating in bivalves.[93] However, the previous knowledge about bivalve cytokine is from immunological experiments with human antibodies, which lacks molecular evidence.[94] Recently, cytokine homologs and their receptors have been validated at molecular level.

The first identified cytokine is oyster IL-17 homologue (*CgIL-17*). Bacteria challenge induced a large and rapid elevation of *CgIL-17* transcript in oyster.[91] This is the solely IL identified in bivalve. Although other cytokines are not available in bivalves, two TNF receptor genes were cloned from Zhikong scallop, which was homologous respectively with p75 neurotrophin receptor (*p75NTR*) and osteoprotegerin.[95] Another receptor for the transforming growth factor beta (*TGF*β) has been identified from oyster *C. gigas*.[96] The phylogenetic and structural analysis as well as the expression pattern during early development suggested that Cg-TGFβRI belonged to the TGFβs.s./activin type I receptor. The existence of different receptors suggested the corresponding cytokines should be present in bivalves.

It is exciting that a bivalve TNF (*RpTNF*) gene was cloned from clam *R. philippinarum*. The typical structure indicated that RpTNF was a true Type II (i.e., intracellular N terminus and extracellular C terminus) transmembrane protein. The recombinant RpTNF induced the death of tumour cells just like its mammalian homologs (unpublished data). Considering the broad immune response of cytokines, a multiplex cytokine-receptor system must exist and mediate bivalve immunity in a specific "ligand-receptor" manner, which may take important roles in regulating the bivalve immunity.

Complement Components

The complement system is one of the major effector arms of immune response in vertebrates and a necessary complement for antibodies to play a role in cytolytic. Its effector functions include opsonization leading to enhanced phagocytosis and lysis of microbes. In the evolutionary progress, it appears earlier than the acquired immunity. The previous research on sea urchins, tunicates and horseshoe crabs also revealed a simple opsonic complement defense system in invertebrates.[97] However, there are few reports on its role in bivalves.

The recent identification of C3 and B factor-like (details in other section) from carpet-shell clam provides insight into their conserved characters critical for function. Three other molecules with the hallmark domain structure of complement factors

have also been cloned from scallops (details in other section). A C1qDC protein has been identified from *C. farreri* to have LPS binding activity.[69] The *CfTEP* transcripts were mainly detected in the tissues of hepatopancreas and gonad and remarkably up-regulated by microbial challenge.[42] Its expression was complicatedly mediated by alternative splicing mechanism. Recombinent AiFREP agglutinated chicken and human A, B, O-type erythrocytes. The agglutinating activities were calcium-dependent and could be inhibited by acetyl group-containing carbohydrates. It also agglutinated Gram-negative bacteria *E. coli* JM109, *V. anguillarum* and Gram-positive bacteria *M. luteus* in the presence of calcium ions. The attractive studies collectively favored that those complement factors play significant role in bivalve immune responses and complement system should be an intriguing driver for understanding the characteristic of innate immune system in bivalves.

The Antioxidant Enzymes

ROS are free radicals that contain the oxygen atom, constantly generated when the organism is attacked by invaders or contaminant exposures.[98] Low concentrations of ROS, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) may be beneficial or even indispensable in processes including intracellular signaling and defense against micro-organisms. Excessive production of ROS may, however, lead to oxidative stress, loss of cell function and ultimately apoptosis or necrosis.[99] As with chemical antioxidants, cells are protected against oxidative stress by an interacting network of antioxidant enzymes. In bivalves, a large number of antioxidant enzymes have been identified over the last decades and their response against microbe challenge and environmental stress have been studied. The knowledge of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) is summarized briefly in this section.

Superoxide Dismutase

SODs are a class of antioxidant enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. According to their metal content, SODs are classified into four distinct groups: iron SOD (FeSOD), manganese SOD (MnSOD), copper/zinc SOD (Cu/ZnSOD) and nickel SOD (NiSOD). However, there were only Cu/ZnSOD and MnSOD identified presently in bivalves.[100] Most of SODs were constitutive proteins that could play a crucial role in bivalve defense. Many studies have indicated the importance of SODs to the immune response as well as the role in protecting cells against various challenges.[100] For example, the SOD genes have been identified from scallop *C. farreri* and *Nodipecten subnodosus* and a rapid elevation of SOD activities were observed after microbe infection.[101,102] The SOD activity in gills and mantle of *M. galloprovincialis* increased obviously at the toxic chemical pollutants, especially heavy metals.[103] While in *Chamelea gallina*, the SOD activity decreased in haemocytes with increasing temperature or exposure to benzo[a]pyrene.[104,105] A higher-level of mRNA expression of MnSOD was detected in gill and mantle of *A. irradians* after being challenged with *V. anguillarum* and the expression level in gill was even higher, indicating that MnSOD was necessary in the immune responses against *V. anguillarum* infection. The responses of bivalve SOD to environmental stress were quite variable depending upon isoforms

and tissues. When exposed to heavy metals, SOD is predominantly detected in the cytosolic fractions of gill and digestive gland, with the highest amount in the gill. The mitochondrial Mn-SOD activity in the gill and digestive gland is lower than cytosolic SOD.[106] The whole set of results make them potential target biomarkers aiming at the given environment factors in monitoring strategy.

Catalase

CAT is one of the central enzymes involved in scavenging the high level of ROS. It catalyzes the decomposition of hydrogen peroxide to gaseous oxygen and water molecules.[107] This enzyme is ubiquitous and present in archaea, prokaryotes and eukaryotes. In mammals, the functional catalase is a tetramer of four identical subunits with a molecular weight of approximate 240 kD.[108] Recently, more and more proteins with catalase activities and the genes have been reported in bivalves, such as *M. edulis*,[109] *D. polymorpha*,[110] *C. gigas*[111] and *C. farreri*.[112] The expression of CAT from *C. farreri* increased gradually after-*Vibrio* infection. *M. galloprovincialis* CAT activity was increased 2-3 times at the polluted coastal areas, with high activity in winter and spring.[103] This rise of *M. galloprovincialis* catalase activity was significant with temperature, salinity and light duration. The importance of these enzymes in regulating oxidative stress is recognized. CAT is considered as an important and sensitive biomarker of environmental stress, used to reveal the biological effect on the redox status of bivalve organisms.

Glutathione Peroxidase

GPx is the general name of an enzyme family with peroxidase activity whose main biological role is to protect the organism from oxidative damage. It reduces lipid hydroperoxides to their corresponding alcohols and reduces free hydrogen peroxide to water. Two isoforms of GPx had been identified, selenium-dependent GPx (Se-GPx) and selenium-independent GPx. Se-GPx catalyses the reduction of both organic and inorganic peroxides like hydrogen peroxide (H_2O_2) while selenium-independent GPx reduces only organic peroxide. The detection of GPx activity and identification of GPx molecules collectively suggest that GPx may respond to bacterial infection and hydrogen peroxide exposure and be involved in protection against oxidative stress and immune defense in bivalves.[2,113-115] To date, the activity of GPx has been detected in bivalves *Corbicula fluminea*,[113] *Pinna nobilis*,[114] *M. galloprovincialis*[115] and *Ruditapes decussatus*.[2] There have also been several reports on the GPx genes cloned from bivalves, such as *D. polymorpha*, *Unio tumidus* and *C. gigas*.

Glutathione S-Transferase

GSTs are comprised of classes of dimeric enzymatic proteins that catalyse the conjugation of glutathione to a wide variety of hydrophobic compounds through the formation of a thioether bond with their electrophilic centre. Most mammalian GSTs are cytosolic enzymes with molecular masses of 23 to 28 kD as homodimers or heterodimers. The cytosolic GSTs have been classified into at least 12 different classes based on their N-terminal amino acid sequence, substrate specificity, antibody cross-reactivity

and sensitivity to inhibitors. To date, many novel classes of GST sequences have been identified and classified from nonmammalian organisms. A few GSTs have been studied in bivalves, including *Atactodea striata*, *R. decussates*, *M. edulis*, *C. fluminea,* etc.

GSTs are major Phase II detoxication enzymes found mainly in cytosol and function as a substrate of antioxidant enzymes to eliminate the reactive oxygen induced by xenobiotic compounds,[116] providing protection against electrophiles and products of oxidative stress. Most GST studies in bivalves focused on purification and/or biochemical measurement of total GST or different GST isoforms. GST has been used as a biomarker in environmental assess in bivalves for a number of years. The level of GST in bivalves was responsive, at some extent, to the potential environmental contamination exposure. Mussel population affected by nontreated wastewaters exhibited significantly higher GST activities as a result of an increase in conjugating activities.[117] A rapid increase of GST activities was observed in primary cultured digestive gland acini of *Pecten maximus* treated with Tributyltin, ethylmethane sulfonate and the water-soluble fraction of crude oil, in a time and dose dependent manner.[118]

Acute Phase Proteins

In addition to the discription above, there are other effectors, such as acute phase proteins, to protect bivalve against the toxic effects of contaminants or bacteria challenge. Although nonspecific, acute phase response serves as a core of the innate immune response involving physical and molecular barriers and responses that serve to prevent infection, clear potential pathogens, initiate inflammatory processes and contribute to resolution and the healing process. Acute phase proteins, an integral part of the acute phase response, have been identified in many bivalve species. Among these, heat shock protein and metallothioneins (MT) are two families of acute phase proteins recently studied in bivalves.

Heat shock proteins (HSPs) are ubiquitous and highly conserved stress proteins, not only playing important roles in response to potentially deleterious stress conditions, but also preventing cell toxicity and cell death to protect cells and tissues against damage. More recently, it has also been suggested that HSPs could function as potent activators of the innate immune system.[119] The principal HSPs range in molecular mass from 15 to 110 kD. According to apparent molecular mass, they are classified into several families. Members of HSP22, HSP60, HSP70 and HSP90 have been identified from invertebrate and most of them were found in bivalves. Recent studies in different species of bivalves have confirmed the relevant physiological roles of HSP expression in thermal tolerance and multiple stress response.[120] The different forms of HSPs might be highly affected by temperature and salinity, as well as by a great variety of chemical stressors that might be often found in seawater or in the sediment.[120,121] Meanwhile, the response of some HSPs against bacteria challenge has also been reported in bivalves. After *Vibro* stimulation, a clearly time-dependent expression pattern of HSP22, HSP70 and HSP90 was observed in scallops. HSPs might serve as powerful biomarkers of marine pollution and be helpful for health management of bivalve aquaculture.

Metallothionein (MT) is a superfamily of cysteine-rich proteins with low molecular weight, no aromatic amino acids and high metal binding affinity and widely found in a large variety of organisms.[122] In the past years, multiple functions have been attributed

to MT proteins, such as homeostasis of essential metals, detoxification of toxic metals, protecting against ionizing radiation and oxidative stress, the scavenging of free radicals and response to estrogenic compounds. Bivalves are known to accumulate high concentrations of heavy metals in their tissue and are widely used as bioindicators for pollution in marine and freshwater environments. There has been considerable research dedicated to the diversity of metal-inducibility and expression in different MT isoforms from bivalves *C. virginica*, *M. galloprovincialis* and *D. polymorpha*.[123,124] The mRNA expression of MT from scallop was increased drastically to hundred fold post*Vibrio* challenge respectively.[125] The sensitivity of MT from scallop to bacteria challenge offers us a hint of its regulation in scallop immune defense. The progress opens research perspectives for the use of this marker to assess the effect of various pollutants in the aquatic environment and a better knowledge of its functional multiplicity in the bivalve immune system.

CONCLUSION

Bivalves are always challenged by their environment with high bacterial and viral loads, pollutants and they have evolved a multiplicity of efficient defense strategies to defend against microbial attack. In recent years considerable progress has been made in our understanding of the bivalve immunity. As described in this chapter, bivalve immune system is effective, multifaceted and incorporates cellular and humoral components. It is well accepted that the immune system in bivalves lacks the components of adaptive immunity present in the vertebrates, but there is also a complex mechanism involving an array of different molecules and various multi-step cascade processes. Many aspects of bivalve immunity are not well understood. Even some of the molecules and immune response mechanisms are found to be structurally and functionally similar to that in vertebrate animals. The research progress about the bivalve immunity has been hampered by absence of genome, tools for genetic manipulation and mutants and stable long-term cell lines for in vitro studies. Thus, there is no doubt that the collaborative efforts among immunologists, cell biologists, physiologists and geneticists are necessary for the study of bivalve immunity. It is still an essential task for us to fully characterize the molecules, responses, cascade pathways involved in bivalve immunity. The analysis of the precise underlying molecular mechanism of bivalve immunity will assist in understanding the nature and evolution of immune system and the connections between immune defense in invertebrates and vertebrates.

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