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Immunity in mussels: An overview of molecular components and mechanisms with a focus on the functional defenses

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ABSTRACT

Bivalves' immunity has received much more attention in the last decade, which resulted to a valuable growth in the availability of its molecular components. Such data availability coupled with the economical importance of these organisms aimed to shift the increase in the number of immunological and stress-related studies. Unfortunately, the crowd of generated data deciphering the involved physiological processes, investigators' differential conceptualization and the aimed objectives, has complicated the *sensu stricto* outlining of immune-related mechanisms. Overall, this review tried to compiles a summary about the molecular components of the mussels' immune response, surveying an overview of the mussels' functional immunity through gathering the most recent-related topics of bivalves' immunity as apoptosis and autophagy which deserves a great attention as stress-related mechanisms, the disseminated neoplasia as outbreak transmissible disease, not only within the same specie but also among different species, the hematopoiesis as topic that still generating interesting debate in the scientific community, the mucosal immunity described as the interface where host-pathogen interactions would occurs and determinate the late immune response, and innate immune memory and transgenerational priming, which described as very recent research topic with extensive applications in shellfish farming industry.

1. Introduction

Mussels belongs to the phylum of Mollusca, the second most species-rich metazoan group with about 100,000 estimated extant species [1]. Their life style (i.e., sedentary, filter feeders) exposes them to sustained stress conditions (i.e., environmental stressors such as bacteria, viruses and chemical contaminants). The widespread exploring of mussels as sentinel organisms in eco-toxicological studies enables to survey a very well developed immune system constituted by different defense strategies (i.e., phagocytosis, antioxidant defenses and antimicrobial peptides). However, such studies helped to examine the mussels' immune response from the functional, cellular to molecular levels, therefore upgraded the knowledge about the components of the immune system and their related mechanisms. The significant advances in the collection of molecular data were achieved thanks to the increasing accessibility to high throughput technologies (e.g., Transcriptomic, proteomic and metabolomic tools). Such data have been inventoried as online databases (e.g., the MytiBase: http://mussel.cribi.unipd.it/containing clusters of *Mytilus galloprovincialis* ESTs with annotated consensus sequences) [2–6]. To date, studies reported a highly complex genome of *Mytilus galloprovincialis* with 1.6 Gb of size and 10,891 predicted putative genes [1], in addition to approximately 369,093 ESTs, 55,541 proteins [2–6]. Overall, this crowd of molecular data enabled comparative immunologists to emphasize and update the knowledge about components of the molluscan immunity (i.e., pro-inflammatory cytokines and interferon regulatory factors, complement, lysozymes, cytokines and anti-microbial peptides) [7–19]. Throughout, this review describes, in general way, the mussel immune response and its key active elements, and compiles a summary of the knowledge about the mussel immune response from the molecular and functional points of view, in addition to illustrate the state of the art about recent research topics including apoptosis, autophagy, disseminated neoplasia, hematopoiesis, mucosal immunity, innate immune memory and transgenerational priming, as processes that might interfere with immune defenses.

2. General description of the mussels' immunity

2.1. An overview of the cell-, and humoral-mediated responses

As all metazoan, mussels are protected against invading organisms or non self entities by an internal defense system [21]. Noteworthy that external/physical defense barrier are beyond the focus of this review. The immune system in all animals comprises interacted cellular and humoral elements aiming to protect an organism from potential danger. The mussels' immune response as all invertebrates only constituted by

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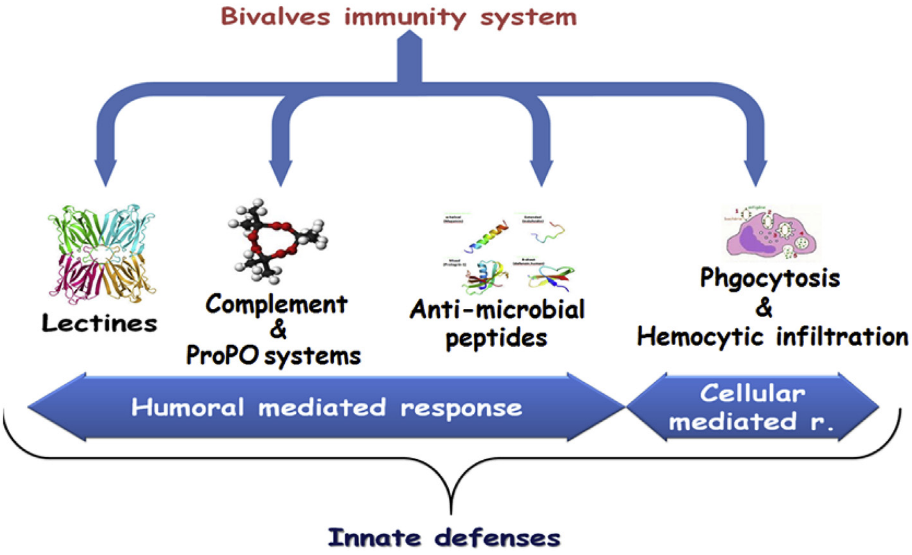

Fig. 1. Diagram representing main types and components of bivalves' immune defenses.

natural immunity (i.e., conversely to vertebrates which possesses acquired immunity [specific immunity] in addition to the innate immunity [natural immunity]). Innate immunity allows to the organism to cope with new invading foreign components (i.e., no previous exposure have been experienced with it before) and to the same repeated infectious agent (i.e., more than one previous exposure have been experienced before) with the same way [21,22]. Mussels' innate immunity compiles a coherent cascading reactions relies on the active immune cells (i.e., hemocytes of mussels) in collaboration with the relevant humoral factors (Fig. 1) aiming to activate an effective immune response. The hemolymph cells are the hemocytes responsible of mastering the general immune response and serve as the first line of defence face to foreign invaders due to their enrichments of components of innate immunity [21,22].

The mussels' blood cells so called hemocytes described as the unique active effector cells of the immune system. Hemocytes orchestrate the cell-mediated immune response [20,21,33]. So far, the dynamism of hemocytes as immune effectors following an initial challenge has been documented through a panel of activities initiating often cascading cell-mediated defense processes [24]. Such hemocytes activity has been explored in immunotoxicological studies due to the uses of mussels as study model. Therefore, the number of testing assays and methods using hemocytic parameters (e.g., cell viability, phagocytic and endocytic activities, adhesion capacity, total and differential circulating hemocytes counts, enzymatic release, production of oxygen free radicals, nitric oxide and phenoloxidase/peroxidase activities), have been well established and reported [24–27]. Overall, studies aiming to evaluate organism and/or environment health status after either in vitro or in vivo exposure to variety of conditions as chemical pollution, changes in environmental salinity, temperature, or food availability using hemocytes parameters lead in studying the mussels' immune components in depth [23–33].

Hemocytes also found to be the key player of mussels' defenses, as indirect-mediators of the humoral defenses. Otherwise, innate mechanisms acting through a bulk repertoire of molecules secreted by hemocytes, including the molecules of the complement system, lysozymes, cytokines and anti-microbial peptides (AMPs) and the prophenoloxidase system, which found to be released in the hemolymph of mussels, in the aim to neutralize and/or eliminate foreign invaders [26] (For further details see section 3. An overview of the molecular components of mussels' immunity). Such humoral-mediated components mostly found to enrich hemocytes and tissues at the interfaces with surrounding medium and often accompany the cellular-mediated response.

2.2. Hemocytes morphotypes and sub-populations

Hemocytes are mostly free-wandering dispersed cells in the hemolymph and extrapallial fluid [21]. Hemocytes characterization is one of the most debated topics encountered by researchers. Different clustering criteria have been followed aiming to characterize hemocyte subpopulations [24,25,27–37]. The detailed description of the different hemocytes' sub-populations, including ultra-structural characterization, within a variety of molluscan species has been achieved by the late 1990s. Morphological aspects and enzyme cytochemistry considered among the main and widespread methods used in this context [24,25,27,28,30,33–37]. Although, different terminologies were adopted depending on the studied species [25,28,30,32,33,35–37], while the most admitted ones were granulocytes and hyalinocytes describing two major morphotypes of hemocytic cells [28,29,35]. Interestingly, according to the presence/absence of granules and nucleus to cytoplasm ratio, granulocytes generally adopts a complex cytoplasm organization that contain many granules and able to spread out and produce pseudopodia, whereas hyalinocytes are small round shaped showing the characteristics of undifferentiated cells with few or no granules [24,25,28,29,31,35] (Fig. 2). Both sub-populations (hyalinocytes and granulocytes) were described in almost molluscan species, interestingly in bivalves as *Mytilus edulis* [28], *Scrobicularia plana* [30], *Pinctada imbricate* [32], *Pomacea canaliculata* [33], *Bathymodiolus ssp.*

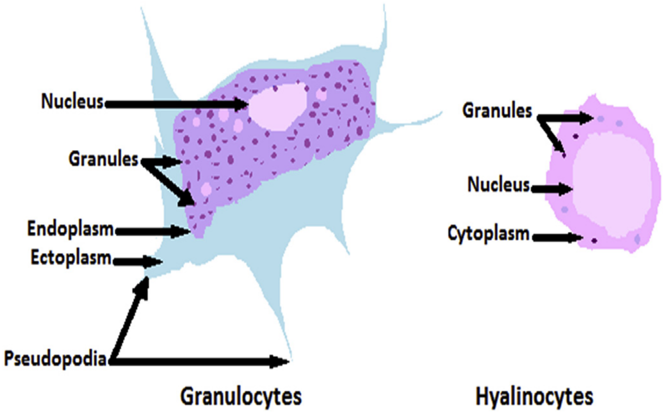

Fig. 2. Sketches of granulocytes and hyalinocytes.

[34], *M. galloprovincialis* [35] and *Cerastoderma glaucum* [36]. Divergence of further subpopulations from both main morphotypes (i.e., hyalinocytes and granulocytes) have been surveyed in some bivalves, such as small and large hyalinocytes in *C. gigas*, blast-like cells in *R. philippinarum*, basophilic granulocytes, acidophilic granulocytes or acido-basophilic granulocytes (containing a mixture of both granules) in *Mytilus galloprovincialis* [27,29,35]. Similarly, granulocytes of *R. philippinarum* were clustered into three subpopulations: basophils, acidophils and neutrophils [29].

Throughout reviewing the lineage of hemocytes, Hine [29] has revised the hypothesis suggesting that hemocyte subpopulations could be generated from two types of precursor-cells named blast-like cells. Interestingly, each cell precursor should separately and only differentiate into one subtype either granulocyte or hyalinocytes. However, Hine [29] adapted a model with only one precursor cell type that give rise to hyalinocytes which further matures into granulocytes. Specifically, basophilic granules were hypothesized to be immature granules matures to become acidophilic. According to Hine this redesigned hypothesis have been conceived due the absence of granuloblasts, therefore should the generation of different hemocytes sub-populations be occurred from only one precursor cell [29].

Different approaches like cytochemical and fluorescence staining have been coupled aiming to more emphasize the classification of hemocytes subtypes [25,32,34–37]. Ultrastructural analyses by electron microscopy allowed confirming the presence of three main hemocyte populations. Briefly, hyalinocytes show characteristics of undifferentiated cells with large central nuclei, relatively small cytoplasm occasionally with some pseudopodia and few organelles, some mitochondria, some cisternae and vesicles of rough and smooth endoplasmic reticulum and free ribosomes. The granulocytes are more differentiated and have sub-cellular, membrane-limited compartments named granules. Such granules could be small granules, large granules or both leading to distinguish different types of cells. Some granulocytes shared some hyalinocytes characteristics, but with more developed cytoplasm containing long strands of smooth endoplasmic reticulum, numerous free ribosomes and a few small granules. Granulocytes were ovoid in shape, frequently featuring thin pseudopodia and eccentric nucleus and more abundant cytoplasmic organelles as mitochondria, cisternae and vesicles of rough and smooth endoplasmic reticulum and Golgi complex. Much more details could be reviewed in Ref. [35].

The two cell categories were largely suspected to play a prominent role in defense [24,26,31]. Few reports aimed to establish functional relationships between mussel hemocytes sub-populations and immune response capacity [15,27–34]. In spite of the number of studies aiming to classify hemocytes, a common classification system still hampered by the lack of a clear described hematopoeitic site [25,32,34–37]. A summary for the main morphological criteria of the above mentioned sub-types could be presented as described below in Table 1.

Table 1
Summary of the main characteristics of hemocyte sub-populations.

Hyalinocytes	•High nucleus/cytoplasm (N/C) ratio •Small sized dimension (about 6 μM) •Round shape •Hyaline cytoplasm (no or very few granules)
Granulocytes	•Low nucleus/cytoplasm (N/C) ratio •Larger size (about 10–15 μM) •Granular cytoplasm (visually presents central granules-concentrated area named endoplasm) •Forms pseudopodia at the external hyaline area (ectoplasm)

3. An overview of the molecular components of mussels' immunity

3.1. Sensors components: receptor recognition patterns (PRRs)

Hemocytes and tissues of Mussels are enriched with molecular motifs named pathogen recognition receptors (PRRs). PRRs insure the detection step that enables the identification of potential danger, constituted by specific cascading reactions to convert the sensing phase into reaction through intracellular mediators. Such step is mandatory to activate a coordination of effective immune response (e.g., antimicrobial peptides release and cytokines production) as showed below in the mechanistic presentation of the Fig. 3.

PRRs generally constituted by three sub-families, which are extracellular, membrane-bound and cytosolic PRRs [66,68]. Extracellular PRRs (so-called secreted PRRs) is mainly constituted by two large classes of PRRs, the C1q contained domain proteins (classical pathway of complement system associated molecules) and lectins (e.g., C-type lectins, fibrinogen-related proteins and Galectins), in addition to other proteins (e.g., Gram-negative binding proteins and Apextrin-like proteins) involved in the recognition of pathogen surface structures [68,77–87]. Membrane bound PRRs constituted by two main subgroups in *M. galloprovincialis*, the Toll-like receptors and the Peptidoglycan recognition receptors (PGRPs) [66–68,88–92], while Cytosolic PRRs are the sub-group that reinforces the sensing of pathogens components inside the cell (e.g., NOD-like receptors, RIG-like receptors; sensors of viral RNA, and DEXDc helicases family and the Stimulator of interferon genes: STING; sensors of exogenous DNA) [68]. A detailed illustration of PRRs broad spectrum coverage of pathogens is presented in Table 3.

3.2. Mediators, intermediate elements and signaling pathways of mussels' immunity

3.2.1. Cytokines of mussels

Successful PRRs recognition step allows the recruitment and activation of downstream molecules aiming to translate the sensing phase into activate immune response [57]. Cytokines are major and diverse group of mediator molecules (transmitting intracellular and/or intercellular signals) that have been particularly associated with immunity [93,60,and17]]. Data about cytokines in *Mytilus spp.* has been predicted through functional activities (e.g., IL-1α, IL–8, TNF-α, platelet-derived growth factor (PDGF-AB) and transforming growth factor (TGF- β 1) using functional antibodies) [17,43,44,46,51,52,94], or by transcriptomic tools as the pro-inflammatory cytokine IL-17 (playing an important role in the clearance of extracellular bacteria, immune cells' recruitment and responsible for many receptors cross talking, in *C. gigas* and *M. galloprovincialis*) [50,52,54–56,100,101], and IL-16 like [50,54–56,66]. Cytokines as IL-2 and IL-6 have been also revealed in freshwater snails, *Planorbatius corneus* and *Viviparus ater* [17,40–42,44,45,48,49,95]. Other mediators as the macrophage migration inhibitory factor (MIF) and the Allograft inhibitory factor-1 (AIF-1) functioning as pro-inflammatory cytokine have been described in *Mytilus galloprovincialis* [53,66,96–99]. Overall, the role played by mollusks' cytokines still scarce [5,47,66].

3.2.2. Mediators and intermediate elements

An updated molecular database reported the knowledge about components of downstream processes and intermediary elements of mussels' immune response [68]. Although, many elements of TLRs intracellular signaling cascade as the Toll-interleukin-1-receptor (TIR) domain, the universal intra cytoplasm adaptor MyD88 and the resulted signaling cascade including the transcription nuclear factor-ƘB (NF-ƘB) were documented [58,59,61,66–69]. In fact, elements of both signaling pathways of TLRs leading to the production of pro-inflammatory cytokines have been reported; the MyD88-dependant pathway, and the

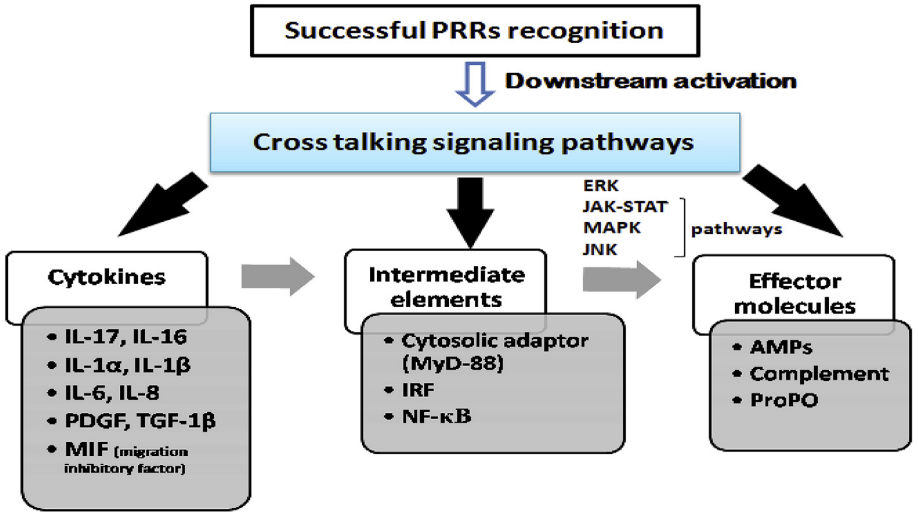

Fig. 3. Mechanistic coordination of an effective immune response: Schema showing the hypothetical interactions for a successful recognition step and post-translational modifications of sensor components (PRRs) to convert the sensing phase into defence reactions.

MyD88-independent (so called TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent pathway) [63]. Most components of MyD88-dependant pathway in *Mytilus spp.*, were documented (e.g., as Mg-TOLLIP (ubiquitin-binding protein that interacts with several TLR elements and involved in the turnover of IL-1R-associated kinase (IRAK)), Mg-ECSIT (evolutionarily conserved signaling intermediate in Toll pathways), MgTRAF-3 and -6 (TNF receptor-associated factor 3 and 6)). Conversely, much less components of the MyD88-independent pathway have been reported (e.g., TRIF which is used by TLR3 and TLR4 (leading to activate the transcription factors IRF3 and NF-κB), in addition to several interferon regulatory factors (IRFs)) [5,60,62–67,92]. An alternative route which can be activated downstream of TLRs, is the mitogen-activated protein kinases (MAPK) pathway. The MAPK kinase cascade could act both in the TLR- and in the PGRP-mediated intracellular signaling pathways in bivalves [68]. A full repertoire of components pertaining to this pathway, including MAP kinase kinases (MKKs) and their subsequent activation elements as TAK1, in addition to the c-JUN *N*-terminal kinases (JNK) pathway could be reviewed in Ref. [68]. Furthermore, contigs of JAK 2 and STAT of the JAK/STAT pathway, in addition to two putative transcripts of the suppressor of cytokine signaling (SOCS: the negative feedback-regulator of the cytokine production) have been inventoried [66,68].

3.3. Effector molecules of the immune response

3.3.1. Complement system

The complement system is an ancient pro-inflammatory and microbial destruction system based on plasma and cell surface proteins that lead to pathogen neutralization and pro-inflammatory responses. Some core components of the mollusks' complement system have been predicted by comparative homology of proteins sequences, versus vertebrates ones [102,103]. In fact, C3-like factor, which is a central element of the three complement pathways, have been identified in *Ruditapes philippinarum* and *M. edulis* [66,68,103], in addition to C2/factor B-like sequences in *Ruditapes philippinarum* [103].

3.3.2. Antimicobial peptides AMPs

Antimicrobial peptides (AMPs) are a widespread group of heterogeneous gene-encoded molecules with antibiotic functions. AMPs are classified in different subgroups based on their structure and amino acid composition. All are characterized by multiple disulfide bridges which ensure a precisely folded, stable structure for the mature peptides that are compact, cationic and amphipathic in nature [104]. Most of AMPs families identified from *Mytilus spp.*, pertaining to the cysteine-rich subgroup, except to a recently identified category of linear/α-helical family [104]. All AMPs are characterized by ubiquitous structure presented by a cysteine-stabilized alpha-beta motif (CS-ab) invariably have a determined number of cysteine residues stabilized by arranged disulfide bridges. The number of cysteine residues and disulfide bridges vary dependently to the AMPs sub families (e.g., Defensins, mytilins and myticins are AMPs, invariably have 8 residues and 4 bridges) [68,80,105–118]. Different AMPs sub-families with their relevant properties and functional characteristics are presented in details in Table 4 (e.g., Mytimycins, mytimacins and Big Defensins, cysteine-rich peptides (CRPs), Lysozymes) [68,78,80,116,119–125]. Recently, a new family of AMPs named Myticalins potentially allocated to the structural category of linear/α-helical sub-group, their antimicrobial properties against Gram + and Gram-bacteria were in vitro confirmed, whereas, the absence of predictable secondary structure is clearly documented [104]. All Myticalins shares a peculiar feature and could be clustered into four subfamilies depending to their contents in amino acid [104]. A summary of the different types of AMPs were presented in Table 4.

Table 2
Summary of types and characteristics of mussels inflammatory response.

Inflammation type		Descriptive properties
Nodule		Small to large clusters of lamellated shape capsule of hemocytes merging into a center of essentially degenerating hemocytes (in circulation and in interstitial tissue)
Infiltrative	Focal invasion	Focal invasions of infiltrated hemocytes in injured tissue without any evidence of clustering or nodulation behavior
	Diffuse invasion	Abundant hemocytes broadly distributed over a large section of tissue with no recorded multiple centers of hemocytes infiltration. Differentiated from focal invasions,
Encapsulation (inflammatory capsule)		Hemocytes forming an enclosing structure, that visibly circumscribing too large entities for phagocytosis.

Table 3
Summary of the main PRRs identified in *Mytilus galloprovincialis*.

PRRs type	PRRs molecules	Functions	Coverage	References
Extracellular PRRs	C1q contained domain proteins	Complement associated molecules (classical pathway).	Gram +, Gram-, fungi, eukaryotic parasites	[68,79]
	Lectins: C-type lectins Fibrinogen related proteins (FREPs) Galectins R-type lectins F-type lectins	Agglutination/opsonization of parasites/pathogen Antibacterial activity Coagulation/cell adhesion role	Bacteria, *perkinsus marinus*	[68,87]
	Gram-negative binding proteins (GNBPs) Apextrin-like proteins	Anti bacterial/fungal activity	Fungi, bacteria	[68]
Membrane bound PRRs	TLRs (23 TLRs)	Sensoring/binding	PAMPs (LPS), Flagellin, Single/double stranded RNA, Fungi, Bacteria, viruses	[66–68]
	Peptidoglycan recognotion receptors (PGRPs)	Binding PGN		
Cytosolic PRRs	NOD-like receptors (NLR)	Sensoring PGN-drived molecules PAMPs		[66,68]
	RIG-like receptors	Sensor of viral 5′(ppp)-single and double stranded RNA	Viruses	
	DEXDc helicases family	Viral DNA sensors	Viruses	
	STING	Detect presence of bacteria	Bacterial/viral DNA	

3.3.3. Pro-phenoloxidase (PPO) system

The prophenoloxidase (ProPO) system, originally a melanin producer, has been suggested as an innate defence mechanism in invertebrates [126]. ProPO is cleaved by proteolysis to the enzymatically active form named PO (i.e., proteolysis could be occurred via an endogenous activator system or exogenous agents) [127]. The enzymatically active form of PO is copper-dependent and catalyses the synthesis of o-diphenols from monophenols by ortho-hydroxylation, which are then dehydrogenated into o-quinones [128]. POs appear as immune effectors involved in self/non-self recognition in hemocytes (reviewed in Ref. [129]).

4. The functional defenses of bivalves immunity

4.1. Phagocytosis

Phagocytosis as an innate preexisting immune mechanism was characterized by the ability to induce rapid response to pathogens, and mostly react in the same way to repeated infections [21]. In mammals, phagocytosis and the related respiratory burst are fundamental defense mechanisms of professional phagocytes that often take place simultaneously, with noted complimentary roles. Phagocytosis is the physical internalization of foreign entities perceived as potential danger into cells. However, respiratory burst is a series of biochemical reactions that produce highly reactive oxygen species (ROS), through the activation of the NADPH-oxidase in the aim to digest phagocytosed components [24,26]. Phagocytosis has been reported as major defense mechanism of mussels' hemocytes against wide range of environmental stressors (i.e., marine bacteria, protist, toxins and contaminants) [35,38,39]. However, the phagocytosis capacity of mussel hemocytes and their related-production of reactive oxygen species (ROS), and nitric oxide (NO), was reported to be highly sophisticated, notwithstanding found to be regulated through intracellular signaling pathways components as Phosphatidylinositol 3-kinase (PI3-K), protein kinase C (PKC), and extracellular signal regulated kinase (ERK) [24]. Since described by Metchnikoff in the 1900s, phagocytosis has been characterized to be involved in a variety of health maintenance processes and immune functions such as clearing of damaged cells/molecules and clearance of number of pathogens as bacteria as *Vibrio tapetis* causing the brown ring disease in the clam *Ruditapes philippinarum* and *Perkinsus marinus* in *Crassostrea virginica* and quahog parasite unknown (QPX) in *Mercenaria mercenaria* [24,30,31,34,71,151,152].

4.2. Hemocytes' aggregation/infiltration and inflammatory response

Studies devoted to assess the modulation of hemocytes features-linked to the inflammatory response. Mollusks Inflammation reported as one of the well-conserved physiological processes of the innate immune response across species that can arise in any tissue in response to infectious, post-ischemic, toxic injuries [17,40–56]. Inflammation also could be enhanced as a responsive result to alterations by xenobiotics in the environment involving phagocytosis and hemocytic infiltration [57]. These processes lead to local morphological changes in injured tissues and/or immune cells [26,58–69]. Invertebrates and vertebrates inflammations share the capability to induce cellular and genomic damages promoting a sustained cellular inflammatory microenvironment. Such microenvironment, featured changes in local circulation (i.e., increased vessel permeability) accompanied by an intensive recruitment of immune cells and interstitial accumulation of exudates [15,70]. In mussels, hemocytes featured as unique inflammatory effector cells (i.e. conversely to vertebrate inflammation which recruit granulocytes, lymphocytes, and macrophages) [20,27–29,37,71]. Molluscan inflammation could be classified following morphological criteria as described below in Table 2 [15,23,72–76].

5. Apoptosis and autophagy as immune-related strategies

Cell death could be described as a fundamental response to immune stress and different forms of cellular destruction. Different forms of programmed cell death could be distinguished: apoptosis, autophagy and necrosis. The processes of apoptosis and autophagy are well controlled and extensively described pathways as immune response that might be initiated by different inducers [66,130]. Mollusks apoptosis were well characterized at the cellular level (i.e., nucleus fragmentation and translocation of phosphatidylserine to the outer membrane) [66]. Molecular components of the apoptotic cascades have been so far identified in mussels. In fact, gene sequences of p53 tumor suppressor-like protein (p53), DNA damage regulated protein (PDRP), Bcl2 protein, Bcl-2-associated X protein (Bax), Bax inhibitor-1 (BI-1) and DNA fragmentation factor 45 (Dff-A), in addition to caspases (which constitutes

Table 4
Summary of different types of AMPs identified in *M. galloprovincialis*.

AMPs subgroups	AMPs families	Structure	Activity	Examples
Cysteine-rich subgroup	Defensins	Cysteine-stabilized α-β motifs 8 cysteines residues with 4 arranged disulfide bridges	- Antibacterial - antifungal	- MGD 3, 4, and 5 - Defensine-like (6 cysteines residues)
	Mytilins	Similar to defensins with cleaved off C-terminal extension	- Antifungal - antibacterial	- Mytilins B, C, D, F and G1 - Mytilns K, N and pseudomytilins 1
	Myticins	8 cysteines like defensins and mytilins	- Highly active against Gram + bacteria	- Myticins A, B and C
	Mytimicins	Signal peptide followed by Cys-rich domain (12–14 residues) organized into 6 to 7 disulfide bridges and C-terminal EF-hand doamin	- Strict antifungal	- Mytimicins F, G and I
	Mytimacins	8 to 12 cysteins residues with 4–6 disulfide bridges	- Antimicrobial and nerve repair activities	- 5 mytimacins identified in *M. galloprovincialis*
	Big defensins	2 sepate domains: α helical hydrophobic *N*-terminal region and a C-terminal domain similar to vertebrate β-defensins	- Not specified	- 9 big defensins were identified in *M. galloprovincialis*
	Short secreted cysteine-rich peptides/Mg-CRP-I	- Lack of structured sequences and sequence similarities - Mg-CRP-I: highly conserved pre-pro-region, hypervariable mature peptide, 6 invariant cysteine residues arranged in 3 intramolecular disulfide bridges.	- No functional testing - Mg-CRP-I functional role not elucidated yet	- Myticusin: 10 cycteines residues arranged in unusual. - Mg-CRP-I 1 to 51.
	Lysozymes		- Hydrolyse 1,4-β glycosidic bonds in PGN. - Particular activity against Gram + bacteria - Digestive role	- I-type - C-type - G-type
linear/α-helical subgroup	Myticalins	- Myticalins transcripts presents the following arrangement: a signal peptide for secretion (well conserved), followed by a dibasic lysine/arginine pro-protein convertase cleavage site and a PRY arranged residues region (cationic) and the anionic C-terminal region rich in aspartic and glutamic acid residues (with variable length: 35–75 amino acids). - The absence of predictable secondary structure is clearly documented. - Extremely variable central mature peptide region (in terms of length and amino acid composition).	- Confirmed antimicrobial properties against a broad range of Gram+ and Gram- bacteria	- Four Myticalins subfamilies could be presented: 1 Rich in Arg, Pro and Tyr residues (> 10% each) 2 Containing a single member, rich in Pro, Arg and Thr residues 3 Rich in Arg, but nearly devoid of Pro residues 4 Rich in Pro Arg and Trp (or Thr) residues

the core proteins of apoptotic machinery) associated with the mitochondrial (intrinsic) apoptotic pathway have been characterized in *M. galloprovincialis* [130]. Romero et al. [86] characterized a number of novel initiator and executioner caspases in *M. galloprovincialis*. Two sequences were included into the initiator caspases group designated as caspase-2 and caspase-8. Four sequences were included into the executioner caspases, were caspase-3/7–1, −3/7–2, −3/7–3 and −3/7-4 according to their identity with the caspase-3/7 group. In contrast to apoptosis, autophagy does not necessarily lead to cell death. Interestingly, unwanted particles and organelles in the cell would be enclosed by double membrane vesicles so called autophagosomes, which will fuse with lysosomes for enzymatic degradation of the enclosed contents. mTOR (target of rapamycin) signaling system found to be a key regulator of autophagy (its inhibition induce autophagy). A large group of autophagy-related genes as ATGs, Beclin 1, BCL-related genes and mTOR transcripts responsible for autophagy induction, beside different genes involved in the endo-lysosomal trafficking pathways in different bivalves such as mussels have been identified and inventoried which highlights a well developed machinery of autophagy in bivalves [1,66,68]. In *Mytilus spp*., autophagic processes have been mainly investigated in a context of oxidative stress scenarios and ecotoxicologic studies [131–134]). In this context, Balbi et al. [135] addressed the autophagic processes (e.g., the classical mammalian autophagy inhibitor Wortmannin, rapamycin, MDC and LC3-II) in *Mytilus galloprovincialis*. However, the autophagic machinery has been investigated as an innate immune mechanism against microbial infection by *Vibrio tapetis*. Overall, the protective role of autophagy towards bacterial challenge has been evidenced for the first time, in bivalve hemocytes [135]. Both processes have been extensively investigated in context of eco-toxicological studies aiming to evaluate the effect of environmental conditions and stress on affecting bivalves health status. In fact, many studies demonstrated the ability of environmental contaminants as heavy metals, hydrocarbons, pharmaceuticals and nanoparticles to interfere in stimulating extrinsic/and or extrinsic pathway-apoptosis and endolysosomal-autophagy as response face such contaminations [7–9,38,75,131–135,155]. Recently, it has been proved the capability of *Perkinsus marinus* to alter the immune response of *C. virginica* through inhibiting its ability to activate apoptotic cell death in infected hemocytes aiming to insure its own survival [168].

6. Hematopoiesis: preservative process of immunity through hemocytes' replenishment

It has been so far reported that the immune response of bivalves, interestingly of mussels, is an active phenomenon that showed effectiveness in early phase of life cycle [136,137]. Studies, reported the differential intensity of the immune response dependently to the development stage [137–139]. However, during the fulfillment of their role of defense, hemocytes the immune response effectors, found to be deeply affected which could hamper the fulfillment of such role (i.e., depletion of circulating hemocytes once being involved in wound healing, pathogen killing and/or stressors elimination through phagocytosis, encapsulation and production of cytotoxic molecules) [139]. Stressed animals showed to possess a highly dynamic mechanism of generation and recruitment of new cells, functionally active when needed. In this context, many authors aimed to emphasize the hematopoiesis as mandatory mechanism of hemocytes replenishment to orchestrate the effectiveness of the immune response in mussels, thereafter reaching the steady state equilibrium (homeostasis) through the physiological roles that hemocytes might assures. Despite that there is no described mechanism detailing the hematopoietic process in any molluscan group, many authors reported that the location where the hematopoiesis could occurs varies greatly in mollusks dependently to species [33,45,136,137,139,140]. In gastropods, hemocytes productions take place in the anterior pericardial wall [45] or in the external surface of ctenidial and renal veins in the pericardial cavity [33]. In bivalves, the irregularly folded structure (IFS) of the gills has been suggested as hematopoietic site for the oysters (*C. gigas*) [140]. Firstly, it has been proved through cytochemical analysis and transcriptomic tools that cells attached to the endothelium of blood vessels expressed factors described as important in vertebrate embryonic hematopoiesis [139]. Recently, Jemaa et al. [140] demonstrated that only one specialized structure from the gills is the progenitor of oysters' adult hemocytes, which are the IFS. While in the clam (*T. philippinarum*), it was suggested that newly formed hemocytes might be originated from the division of the precursor circulating cells called hemoblasts [36]. Dyachuk [136] reviewed the hematopoiesis in bivalves and documented that the development of the immune system reaches full maturation early in bivalves development stages, thus immune cells appear early, and are functionally active by this stage, which leads to hypothesize that the development of the immune system inextricably linked with the formation of the digestive system. Despite that it was already affirmed that hemocytes are developmentally close to the endothelia and the mesothelia, the lack of circulatory system at early larval stages made the generation of hemocytes possible from precursor cells from the digestive system (mesothelia), which showed to be formed earlier at the trocophore larval stage (due to the mandatory transition of the organism life style to be benthic and filter feeding on microalgae) [136]. Therefore it seems more logic that immune cells formation being coupled with the larvae ability to access to marine environment, which increase the potential of infections to happen [141]. Such formation, proposed to use as precursor cells from the digestive system in the absence of any formation of circulatory system at the trocophore stage. This hypothesis was later proved by immunostaining tools that showed integrins-like protein-positive cells identified in the digestive cell mass of the early phase of trocophore, while only hemocytes and tissue membranes (i.e., where hemocytes found to be free wandering cells) were marked with same antibody in adults. Such demonstration corroborates the possibility to identify marked cells in larvae as immature dividing hemocytes.

7. Disseminated neoplasia: a pathological process that affect mussels' immune cells

Hemocytes, as key effectors of the immune response (e.g., hemocytosis and phagocytosis) [21], could be deeply affected by pathological processes as neoplasia [142,143]. Neoplasia, have been reported in different species of marine mollusks including *Mytilus galloprovincialis* [142]. Two types of neoplasia have been reported in bivalves; gonadal neoplasia and disseminated neoplasia (so called haemic neoplasia "HN" and leukemia) [23]. Carella et al. [23] reported that the disseminated neoplasia consists of a proliferation of atypical hemocytes. Due to the intimate relationships between haemic neoplasia, and hemocytes, the mussels' immune cells, this type of neoplasia have attracted the attention of many researchers in the field. In fact, many researchers have reported that neoplastic cells in mussels are abnormal circulating cells of unknown origin, but most probably with hemocytic origin due to their shared morphological characteristics with hemocytes, in addition to that they have been firstly observed in the circulatory system [142–147]. Neoplastic cells affected by DN have been described as large cells with a rounded or oval shape and a high nucleus to cytoplasm ratio, in which the patent nuclei are round or pleomorphic coupled with high frequency of mitotic figures [16,142]. To date, at least five types of atypical hemocytes was reported in *M. galloprovincialis* [16,23,142], including two main types of neoplastic cells (A and B) firstly described in *M. edulis* by Lowe and Moore [144]. A-type cells featured a predominance in early stages of the disease, which described as cytoplasmic pattern, while the B-type cells showed to be featured in more advanced phases and have been described as nuclear pattern in mussels [16,23]. Carella et al. [16] described the A-type cells as ovoid shaped cells marked with pleomorphism, vesicular nuclei and evident nucleoli, while the B-type cells exhibited a rounded shape with dense chromatin pattern nuclei. The other morphological atypical cells (indented/cleaved cells, binucleated cells and multinucleated giant cells) have been affiliated as A or B cell subtypes [16]. Muttray and Vassilenko [147], documented that the advancement of the disease would certainly be coupled by a progressive replacement of normal hemocytes by neoplastic cells. Such cells, exhibiting a significantly high densities and limited immune activity compared to normal cells, which will lead to the loose of the normal hemocytes functionality [147]. Studies about the etiology of DN showed that many genes and pathways of cancer related genes as the p53 superfamily members (i.e., p53, mdm, ras, p63/73) and the heat-shock family members proteins (e.g., Hsp70 and Hsp90) are evolutionarily conserved in molluscs (also used as markers of the disease) [23,142]. Overall, many authors have illustrated in depth, through studying the molecular mechanisms of oncogenesis, the role of such evolutionarily conserved molecular components in molluscan cancers [7–14,16,18–20]. In fact, the infectious etiology of the DN has been demonstrated and its capability to debilitating the hemocytes, therefore overcoming the innate immune response was documented as well [147]. Although, Metzger et al. [148,149], have hypothesized that DN derived from a single original mussel (or clam) that experienced de novo retroviral infection, which have overlapped the innate p53-mediated apoptotic response that normally should be triggered by the viral infection. Later, the disease has the ability to spreads as a clonal transmissible cell between individuals from the same species or between species without needing de novo viral infection as result of the curtailed immune functions of hemocytes [148,149]. In this context, it would be noteworthy to highlight that etiology of neoplastic transformations suggested genetic alterations, virus, retrotranspons as *Steamer* RNA and contaminants as potential causes [142,147]. Furthermore, horizontal transmission of DN as contagious cancer cells as cause of DN spreading between animals has been recorded [142,147–149]. However, Metzger et al., [149] demonstrated that DN in *Mytilus trossulus*, *Cerastoderma edule* and *Polititapes aureus* are attributable to independent transmissible cancer lineages, noting that for the two first species cancer lineages are derived from their respective host species, but cancer cells from the last specie are all derived from third specie (*Venerupis corrugate*) living in the same geographical area [149]. Overall, horizontal transmission of disseminated neoplasia in the marine environment is common in multiple species with noted spreading within the same origin species, but cross-species transmission of contagious cancer cell could occur as well [147,149].

8. Mucosal immunity: an emerging topic to be considered for a proper exploring of the immune response

To date, bivalves' immunity still shed the light as very promising and prolific field of research. Lately but not lastly, the mucosal immunity has been emerged as new important, yet understudied, topic [150]. Several studies conducted by Allam and coworkers [151], have clearly evoked but not fully elucidated this topic in details [152]. In fact, it has been reported that, in a context of studying host-pathogen interactions, that mucosal surfaces, which have been described to be present in all the interfaces with the external environment (e.g., epithelia of the respiratory and gastrointestinal tracts) [22], played a key role in activating the systemic immune response [151]. Interestingly, the physiological role of mucus has been primarily described as cargo for feeding particles, but also as eliminating agent of foreign invaders (i.e., as pseudo-feces) [153–155]. Furthermore, many studies highlighted the role of mucus in host-microbe interactions as intriguing central role in animal health with an important feedback mechanism for the maintenance of mucosal homeostasis, therefore the whole organism homeostasis [152]. In this context, mucus has been described to be composed by a network of glycolproteins coupled with various cells (e.g., mucocytes, epithelial cells and hemocytes), and bioactive molecules (e.g., c-type lectins) that aims to trap foreign invaders from reaching soft tissues (reviewed in Ref. [152]). Besides, mucus has been demonstrated to be involved in the interactions with waterborne pathogens [169]. In fact, several investigations proved the ability of pathogens as *Perkinsus marinus* to determinate its virulence to its host through interfering in the expression of bioactive molecules of mucus secretions [169,170]. According to Allam and coworkers [152], whom showed through a high throughput transcriptomic investigations that peripheral and/or focal immune response (i.e., mucosal immunity, focal inflammation) greatly contrast systemic immune response [151], which definitely should affect data for a proper evaluation of the immune response. In this context, it was highlighted that the early initiation of the immune response (at the mucosal interfaces) will determinate the late (i.e., systemic) response. More interestingly, Pales-Espinosa et al. [170] demonstrated that pallial mucus from oysters increased the pathogenicity of *P. marinus*, which is not the case of digestive extracts, which corroborate the hypothesis of the heterogeneity of the immune response (systemic response versus peripheral response [170]. Overall, a major consciousness should be followed in consideration of the spatial heterogeneity (i.e., peripheral/mucosal immunity and systemic immunity) of immunomarkers to the better understanding of immune response' evaluation while outlining outputs. Such immunomarkers presented close similarity in the virtue of immune components, but considerably recorded a differential regulation (mainly in quantitative feature) against foreign invaders. In this context, it might be interesting that hemocytes from different body fluids (hemolymph, mantle, gills and body wall) of *Crassostrea virginica*, despite showing common general characteristics, revealed differences in their cell surface epitopes (as clusters of differentiation and lectins) as well as functional assays as phagocytosis [168]. Similarly, molecular components of both responses (systemic and mucosal immunity) described to be constituted, in both responses, mostly by immune recognition proteins such as lectins and C1q-domain containing proteins, hydrolytic enzymes such as lysozyme and other factors of effectors of the immune response.

9. Memory of the innate immunity and transgenerational priming

The innate immune memory in invertebrates recently has been described to present adaptive characteristics [156]. This emerging topic also referred to as immune priming, still yet fully uncovered and controversial due to lack of detailed mechanistic description of the process at cellular and molecular levels [157,158]. In vertebrates, the innate immune memory reported as a stimulus-induced reprogramming phenomenon of innate immune functions that shall results either, to some sort of tolerance/decreased reactivity, or to potentiating the immune response to enhance the responsiveness during a secondary challenge [159]. Milutinovic and Kurtz [160] suggested a definition for such phenomenological topic that separates the strict sense of the process from mechanisms that provide memory. In fact, it was then suggested to describe immune memory to the ability of the immune system to store and re-use the information about a previously encountered invader at time of secondary challenge [160]. Such re-use of the “already available” data for the immune system lead to acquire some specificity measures to the response. However, such specificity which described as mandatory aspect of memory enabled the differentiation between different antigens [160]. The phenomenon of innate immune memory have been firstly described, since almost half a century ago, through histological analyses of pre-exposed snails to a manipulated digeneans by Lie and Heyneman [161]. To date, such topic still controversial and not fully elucidated because the considerable variety in experimental design [162]. Unfortunately, different methodologies have been conceptualized, either to demonstrate the specificity measurements that the innate memory would presents, or to demonstrate the way with achieving priming itself [159,160]. In this context, it's important to report that different priming strategies have been documented such as direct exposure (via administration route) to live or heat-killed parasites, or by septic priming via picking the animal by bacteriacontaminated needle or by injection of bacteria, or also by the transfer of immune soluble factors in the hemolymph through cell free hemolymph transfer (i.e., PAMS or synthetic virus-associated molecular patterns like poly(I:C)) [159,160,163,164]. Furthermore, different immune priming models, in virtue of priming agent combinations (i.e., parasites), have been followed (e.g., homogenous priming for the same parasite used for priming and re-challenge and heterologous priming for different parasites used for priming and re-challenge) (reviewed by Refs. [159,160,164]). Whatever the priming design was, the innate immune memory, which is described as multidimensional phenomenon, definitely could not skip the extinction step that constitutes the key step of immune response re-activation and memory [159]. Overall, the memory constituently could be a: 1) recall to the immune response which would be at this time, within the second meddling, faster and more powerful than the first one, 2) a shift from type to type response (i.e., from cellular-to humoral-mediated response), 3) sustained unique response due to long-lasting upregulation of immune effectors protecting from second infection [157–159]. Different mechanistic models have been suggested to develop immune priming since the histological description conducted by Lie and Heyneman, [161]. Such mechanisms implicate the involvement of cellular and humoral factors. Most assessments focusing on immune memory used to evaluate such specificity through evaluating phagocytosis/phagocytic index/ability of hemocytes to phagocyte, hemocytosis and changes in circulating hemocytes [159,160,165–167]. However, no relevant study has addressed the mechanistic process of the immune memory at molecular level until recently. In fact, Pinaud et al. [158] evidenced, through integrative study using the snail *Biomphalaria glabrata* and combining histological, transcriptomic and proteomic investigations, the shift from cellular-to humoral-mediated immune response during procure of immune memory (noteworthy to highlight that vertebrate immune response exhibit a shift from cellular response, innate granulocytes-based response, to humoral-antibody-based response supported by a cellular memory B and T cells [157]). Also, it has been proved, for the first time, that molecular basis of innate immune memory demonstrating the involvement of diverse repertoire of FREPs and other PRRs as collaborative humoral factors (processed as homologous or heterologous multimers to increase the pathogens PRRs repertoire and acquire a genotype-dependant innate immune memory) [158,162]. In this same context, FREPs have been described as complex pattern recognition proteins undergoing somatic rearrangements and intriguingly present a dual role as receptor and effector just like vertebrate antibodies [157]. To the best of our knowledge, the topic of innate immune memory has been addressed only snails (*Biomphalaria glabrata*, *Gastropoda*) and bivalves (*Crassostrea gigas* and *Chlamys farreri*, *Ostreoida*) from the phylum *Mollusca*. Milutinovic and Kurtz [160] suggested that memory might be explicable by the conserved components of the immune system such as Toll pathway, which has been fully elucidated in *Mytilus galloprovincialis* [92]. Toll pathway showed to have the ability to discriminate and be activated by different set of pathogens, which based on a set of diversified PRRs that in turn will lead to elaborate more discrimination ability for PAMPs underlying high measures of specificity [160]. Overall, it was evident that immune priming, which could be achieved through different mechanisms is involved in acquiring innate immune memory (specificity), and generally could be characterized by the scale of the secondary response (that must: neutralize more entities, represent greater numbers of molecular effectors (humoral and/or cellular) and recognition receptors with higher affinities), and the speed of the response which normally would be shorter in latent period of initiation [162]. As perspectives of such topic, the transgenerational immune priming showed to be a consistently reproducible (e.g., *C. gigas* proved the capability to acquire immune plasticity to OsHV-1 through heterologous priming using a synthetic virus-associated molecular pattern, which could be long-lasting persisting for at least 5 months thereafter transferred to offspring generating improved survival of parents-stimulated larvae), and highly promising strategy to be used in reducing mortalities in hatchery production of relevant aquaculture species like *C. gigas*, thus the possibility to adapt therapies for aquaculture [164].

10. Conclusion

Considering the available data on bivalves' immunity, scientists should bring in mind the ensemble of immune mechanisms in addition to the emerging topics while evaluating the whole immune response to the better understanding of immune mechanisms interactions with biotic and abiotic factors aiming to promote the exploring of bivalves immunity for a sustainable aquaculture industry and for further extensive applications as biomedical research.

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