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Review

Hematopoiesis in Bivalvia larvae: Cellular origin, differentiation of hemocytes, and neoplasia

Vyacheslav A. Dyachuk [a, b, *]

[a] A. V. Zhirmunsky Institute of Marine Biology, Far Eastern Branch of the Russian Academy of Sciences, Palchevsky Str. 17, 690041, Vladivostok, Russia
[b] Far Eastern Federal University, 690950, Vladivostok, Russia

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ABSTRACT

Hemocytes play vital roles in the immune response. Despite progress in the characterization of molluscan hemocytes and immune cells, including their cellular receptors and signal transduction pathways, the processes that lead to their differentiation in bivalve larvae remain unknown. Furthermore, the molecular mechanisms of that decide hemocyte stem cell fate and self-renewal during development remain poorly characterized. Similar to adult mollusks, the larvae are filter feeders and are highly susceptible to pathogens and biotoxins; therefore, it is important to understand the development and function of their immune system. This review summarizes the current data on the appearance of elements of the immune system in bivalve larvae. I have discussed why the immune cells emerge before the circular blood vessel system, which differentiates at the late stages of development. I also discuss how molluscan hemocytes are involved in the development of disseminated neoplasia.

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1. Morphological heterogeneity of hemocytes and hematopoietic organ of adult mollusks

The immune system of mollusks consists of blood cells (hemocytes), which are the main cellular components of the hemolymph ("blood") that covers all soft tissue. Hemocytes can be found in all taxa of the animal kingdom, even in those without a body cavity (Van de Vyver, 1981). Molluscan hemocytes are classified as granulocytes or hyalinocytes (agranulocytes) based on their cytoplasmic consistency (Cheng, 1981, 1984; Hine, 1999). In mytilids, 3 subpopulations of hemocytes, i.e., hyalinocytes and acidophilic or basophilic granulocytes, have been identified (Carballal et al., 1997; Bayne et al., 1979), although this classification system is controversial (Pila et al., 2016). Although both types of hemocytes have phagocytic capability, granulocytes are the primary cells involved in the cellular response to infection (Chu, 2000). In the giant clam, *Tridacna crocea*, morula-like cells, in addition to granulocytes and agranulocytes, have been identified (Nakayama et al., 1997). Various techniques, including morpho-functional approaches (Ottaviani et al., 1998), immunocytochemistry (Dyrynda et al., 1997), flow cytometry and transmission electron microscopy (de Freitas Rebelo et al., 2013), have been used to classify hemocytes

* A.V. Zhirmunsky Institute of Marine Biology, FEB RAS, Vladivostok 17 Palchevsky Str., Vladivostok, 690041, Russia.
E-mail address: slavad83@gmail.com.

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of several bivalve molluscan species (Allam et al., 2002); however, a consensus has not been reached on the most appropriate classification scheme for molluscan hemocytes.

Although the hematopoietic organs of various molluscan taxa have been described, e.g., the amebocyte-producing organ in gastropods (Jeong et al., 1983) and white glands in cephalopods (Cowden and Curtis, 1981), the hematopoietic organs and/or tissues of adult bivalves still have not been identified in the adult and during development (Vogt, 2012). Matozzo and co-authors suggested that in the Manila clam, *Tapes philippinarum*, at least some of the newly formed hemocytes originate from the division of precursor cells called hemoblasts, which possess stem cell-like features (Matozzo et al., 2008). Recently, hemocytes were proposed to differentiate from a population of adult somatic cells that reside in an irregularly folded structure in the gill of the adult oyster, *Crassostrea gigas* (Jemaà et al., 2014). These cells possess high proliferative capacity supporting their stemness and ability to differentiate into hemocytes. Thus, adult bivalves likely do not produce hemocyte precursors or mature hemocytes from a centralized organ, as occurs in other molluscan taxa. Multiple or ubiquitous sites of hematopoiesis may exist, comprising a system in which stem-like cells receive determining signals from neighboring specialized cells or tissues. However, the question of when and how the immune system developed is yet unanswered.

2. Larval bivalve immune system

Bivalve mollusks have a biphasic life cycle wherein pelagic larvae (blastula, trochophore, and veliger) undergo settlement (pediveliger larva) and metamorphosis (juvenile stage). Mollusks at larval stages are highly sensitive to bacterial, viral, and fungal infections, resulting in high mortality during larval development and a significant decrease in the benthic population. Whether larvae can resist microbial infections in water ecosystems and whether they possess cells or organs of the immune system remain poorly characterized. Phagocytic cells were first found in *Ostrea edulis* oyster larvae (Yonge, 1926). Yonge demonstrated that phagocytes in larval *O. edulis* absorbed and concentrated particulate material in the connective tissue surrounding the digestive gland. The similar observation in this study also suggests that phagocytes may play a role in the distribution and processing of particulate nutrients in the visceral cavity. Erdmann (1935) also observed 'free, mesenchymal cells' in *O. edulis* larvae, which are likely to be the phagocytes referred to by Yonge (1926). Later, Cole (1938) observed invasion of phagocytes into the velum, foot and also present in the connective tissue under the eyespots in pediveliger larvae of *O. edulis*. Cole (1938) initially supported the hypothesis that the pediveliger eyespots acted mainly as hemopoietic tissue from which blood cells migrated into the circulation at metamorphosis, but subsequently suggested that phagocytes migrated around the body without blood cell generation from the eyespots. Early studies identified phagocytic cells with bacterial fragments (named coelomocytes) in the visceral cavity of veliger larvae of *Crassostrea virginica* and *Crassostrea gigas* and the extrusion of redundant phagocytes through the velum of oyster larvae (Elston and Leibovitz, 1980). Elston (1980) claimed that SER (smooth endoplasmic reticulum, non-phagocytic) cells and phagocytes represent two functionally distinct and differentiated cell types in oyster larva that are able to protect the larvae from the environment. Moreover, both the mantle tissue and visceral cavity (presumptive coelom) of oyster larvae are places where unattached immune precursor cells might possess a high mitotic index (Elston, 1980). More recent data have shown that some elements of the immune system described for adult mollusks are also present in trochophore and veliger larvae (Dyrynda et al., 1995); larval cells are positive for phenoloxidase and arylsulphatase, and these cells able to phagocytose *E. coli*.

With the development of molecular biological tools, researchers are increasingly using modern techniques for identification of gene and protein expression related to immune cells. Tirapé et al. (2007) characterized the gene expression of 18 immune-related genes during ontogenesis in the oyster, *Crassostrea gigas*. The expression patterns of 4 genes (*Cg-timp*, *Cg-tal*, *Cg-EcSOD*, and *Drac3*) suggest that hemocytes appear in the gastrula and persist up to the trochophore stage. The localization of *Cg-tal* expression suggests that hematopoietic cells are derived from endothelial cells in the blood vessels and/or the artery. Moreover, bacterial challenge was found to affect the expression of these immune-related genes. The reduced capacity of larvae to respond to bacterial infections could also be a result of their anatomical and physiological differences (Tirapé et al., 2007). Flow cytometry and fluorescence microscopy studies have revealed the activation of phagocytosis by hemocytes at the early stages, i.e., 24 h post-fertilization (hpf), in the mussel, *Mytilus galloprovincialis*. Moreover, the expression of immune-related genes was found to increase throughout the different developmental stages of the mussel (trochophore, veliger, metamorphosis, post-settlement, and spat), switching on during metamorphosis and increasing during the transition from the trochophore to the spat stage (Balseiro et al., 2013). Balseiro and co-authors also suggested that upregulation of immune-related genes during metamorphosis might reflect active innate immunity at this stage, maturation of the innate immune system, and/or reorganization of larval tissues. Recently, the immunological capacity of the Pacific oyster, *Crassostrea gigas,* during ontogenesis was characterized (Song et al., 2016). Phagocytosis appeared in the early D-veliger larval (17 hpf) stage, especially in the velum, suggesting the presence of functional hemocytes. Whole-mount immunofluorescence analysis of three pattern-recognition receptors (integrin β-1, caspase-3, and C-type lectin 3) and one immune effector gene (IL17-5) suggested the velum and digestive gland as potential immune organs in veliger larvae, supporting our in vivo data (Dyachuk et al., 2015). Based on the low activity of antioxidant and hydrolytic enzymes as well as the low expression of 12 immune genes at the early stages (9 hpf, hatching blastula), Song et al. concluded that the innate immunity of oyster *C. gigas* larvae was intrinsically hypoactive. However, various immune molecules (mRNAs or proteins) could be maternally transferred to provide sufficient protection during embryonic development. In oyster larvae, immune system development is initiated during the trochophore stage (15 hpf) and the immune system reaches full maturation at the early umbo larval stage (120 hpf). Thus, cells of the immune system appear at early stages in mollusk development and are functional by the early umbo larval stage (Song et al., 2016). Similarly, the immune system of the scallop, *Chlamys farreri,* is reported to first appear in the mid-ventral region of the prototroph in the trochophore and mature after the late D-hinged larval stage based on immunofluorescence studies (Yue et al., 2013). Using a specific monoclonal antibody against granulocytes of *Chlamys farreri,* Xing et al. found that granulocytes were distributed in the velum, digestive gland, and esophagus of D-shaped veligers (Xing et al., 2014). The TFs, Runx1, CBFb, and GATA1/2/3, were detected in the scallop, *Chlamys farreri,* at the 32-cell embryo, morula and trochophore, and their expression peaked at the 32-cell embryo, gastrula stage, D-shaped veliger and, pediveliger larva. However, it is still unclear whether these transcriptional factors are expressed in hemocytes, epithelial cells or other cell types in scallop larvae, as well as in larvae of other bivalves. These factors likely control hematopoiesis and the development of hemocyte-associated digestive organs in larval bivalves at the trochophore and veliger stages.

I hypothesize that the development of the immune system is