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Functional characterization of hemocytes from Chinese mitten crab *Eriocheir sinensis* by flow cytometry

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ABSTRACT

Hemocytes comprise a diversity of cell types with functional and structural heterogeneity, and they play key roles in the host defense of invertebrates. In the present study, the hemocytes from Chinese mitten crab *Eriocheir sinensis* were directly separated into two groups by flow cytometry. The hemocytes in P1 group were full of round and abundant granules with deeply staining cytoplasm, while P2 hemocytes were more diverse with a wide range of sizes and less granularity. Both P1 and P2 hemocytes exhibited phagocytic ability, but the phagocytic rate of P1 hemocytes increased which was significantly higher than that of P2 hemocytes after LPS stimulations. The levels of ROS production and intracellular Calcium as well as lysosome content were higher in P1 hemocytes than that in P2 hemocytes under both normal and immune-activated situations. The genes involved in phagocytosis, antimicrobial and antioxidant activities were mainly expressed in P1 hemocytes, while the genes involved in proPO activation system were highly expressed in P2 hemocytes. These results collectively suggested that P1 hemocytes were the main immunocompetent hemocytes in Chinese mitten crab and P2 hemocytes mainly participated in proPO activation system.

1. Introduction

The immune system is commonly divided into two major branches: innate and adaptive immunity [1]. Innate immunity is the first line of defense which is comprised of multiple immune cells and molecules while adaptive immunity is mostly referred as specific immunity or immune memory [2,3]. For the lack of the complexity of the adaptive immune system, invertebrates have to rely solely on innate immunity to maintain a highly efficient defense system against infections [4,5]. The innate immune system is further divided into humoral and cellular defense responses. The humoral immune response refers to the reaction cascades of immune recognition, signal transduction and production of immune effectors. The cell-mediated immune defense in invertebrate is mainly carried out by circulating hemocytes which are continuously produced from self-renewing hematopoietic stem cells in the adult animals [6–8]. Hemocyte are involved in the recognition and elimination of the invading pathogens by phagocytosis, encapsulation, melanization, nodule formation and cell agglutination and play key roles in the defense mechanism of invertebrates [9,10].

There are several highly specialized cell types in vertebrates, which are involved in gas transport (red blood cells, or erythrocytes), blood clotting (thrombocytes), and immune response/tissue repair (white blood cells, or leukocytes) [11,12]. In invertebrates, the circulating hemocytes with functional and structural heterogeneity are considered to be the counterpart of vertebrate leukocytes [13] and play different roles in host defense reactions [14–17]. The classification of invertebrate hemocytes has been investigated for a long period, but the function features of different sub-populations are still far from well understood. In the previous studies, hemocytes were characterized mainly by morphological and cytochemical criteria, such as the size, nucleo-cytoplasmic (N:C) ratio, cytoplasmic complexity and enzyme content [18–20]. In

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Drosophila, circulating hemocytes were classified as plasmatocytes, crystal cells and lamellocytes based on their shape [10,21]. In crustacean, such as *Carcinus maenas* and *Pacifastacus leniusculus*, hyalinocytes, semi-granulocytes (SGC) and granulocytes (GC) were initially identified by morphologic observation and isolated by density gradient centrifugation [22,23]. Hyalinocytes are small with very few or no granules, and most of them act as phagocytes [24]. SGC is the most abundant cell type, which makes up approximately 65% of the total circulating hemocytes. There are a variable number of small eosinophilic granules in SGC, and SGC is involved in early recognition, coagulation and encapsulation of microorganisms. SGCs are usually participate in melanization after encapsulation reactions [25]. GCs are densely packed with large eosinophilic granules, which contains various antimicrobial peptides [26], and proteinase inhibitors [27]. After activation, GCs can release their active components by exocytosis.

Flow cytometry is a technology for fast qualitation, quantitative analysis and separation of sing cells or other biological particles. It provides rapid and accurate measurements for individual particle and allows the discrimination between cells and detritus or suspended sediments. The size and granularity of the particle can be measured by using the side scatter (SSC) and forward scatter (FSC) [6,28]. Flow cytometry can also be used for the analyses of apoptosis [29], lymphocyte division [30], phagocytosis [31], cell cycle [30]. It can separate different cell types by using surface markers or intracellular probes correlated with the cell functions. For example, the phagocytic ability of trout B lymphocytes could be examined by cell sorting with flow cytometry using monoclonal antibody (mAb) specifically for IgM and fluorescent beads [32]. The dendritic cells were enriched from zebrafish whole kidney marrow by flow cytometry with biotinylated peanut agglutinin [33]. Fluorescence activated cell sorting was also performed by flow cytometry to separate phagocytes and non-phagocytic hemocytes from *Crassostrea gigas* [34]. The flow cytometry was also employed to determine the intracellular ROS level in *Eriocheir sinensis* [35].

Chinese mitten crab *E. sinensis* is one of the most economically important aquaculture species in East Asia [36], and the knowledge about its immune defense mechanism is of vital importance for the sustainable development of aquaculture. Increasing studies have been focused on the characterization of multiple immune molecules and immune responses [37–39], as well as hematopoietic tissue [6]. However, the information about the hemocytes sub-populations is still very limited. In the present study, flow cytometry was employed to characterize the sub-populations of hemocytes with the main objectives (1) to record the features of hemocytes by side scatter (SSC) and forward scatter (FSC) at normal and immune challenged condition, (2) to separate hemocytes sub-populations by cell sorting, (3) to compare the immunological activities of hemocytes sub-populations, and (4) to provide new histological and cytological evidences for further understanding of crustacean cellular immunity.

2. Methods and materials

2.1. Crabs and sample collection

Adult Chinese mitten crabs *E. sinensis*, approximately 30 g in weight, were collected from a commercial farm in Lianyungang, China and cultured at 20 ± 1 °C in tanks for 10 days before processing.

The hemolymph was isolated following previous description (Sun et al., 2015). In brief, 2.5 mL syringe was used to collect the hemolymph from the last walking legs of crabs with half volume of pre-cooled anticoagulant solution (510 mmoL/L NaCl, 100 mmoL/L glucose, 200 mmoL/L citric acid, 30 mmoL/L sodium citrate, 10 mM EDTA·2Na, pH 7.3). The hemocytes was harvested by centrifugation of the hemolymph at 1000×g, 4 °C for 10 min, and the hemocyte pellets were resuspended with resuspension solution (450 mmoL/L NaCl, 5.6 mmoL/L KCl, 2.4 mmoL/L Na_2HPO_4, 12.9 mmoL/L $MgSO_4$, 6.7 mmoL/L $CaCl_2$, 2.0 mmoL/L L-Glutamine, containing 1 g/L Heparin, pH 7.3) for subsequent experiments.

One hundred and eighty crabs were randomly divided into three groups, two stimulation group and one control group. Crab received an injection of 100 μL lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 (Sigma-Aldrich, 0.5 mg/mL in saline) or 100 μL *Aeromonas hydrophila* (10^7 CFU/mL) were employed as stimulation group. The rest crabs received an injection of same volume of sterile saline were employed as control group. Three individuals were randomly collected from each group at 0, 3, 6, 12 and 24 h after injection, respectively. Hemocytes were isolated for subsequent ROS, phagocytosis, lysosome and calcium detection. 1 mL hemolymph with about 5×10^6 hemocytes were got from each individual.

2.2. Detection of ROS, phagocytosis, lysosome and calcium in hemocytes

The peroxide-sensitive fluorescent probe DCFH-DA (Beyotime S0033, China) was used as substrate to detect the intracellular ROS level following the previous report with some modification [40]. Hemocytes were washed twice with resuspension solution, resuspended in 500 μL resuspension solution, and then incubated with 5 μL of 10 μmoL/L DCFH-DA (in resuspension solution) at room temperature for 20 min with rotation in dark. After three times of washing, the supernatant was removed and the pellet was resuspended with resuspension solution. The relative fluorescence intensity was detected by FACS Arial II flow cytometer (Becton, Dickinson and Company). The laser channel was FITC and the wavelength of exciting light was 488 nm.

Phagocytosis assay was performed following the previous description [31]. Briefly, hemocytes were incubated with Latex beads (2 μm, Sigma) at room temperature for 1 h with rotation, and the phagocytic rate was analyzed using FACS Arial II flow cytometer (Becton, Dickinson and Company). The laser channel was FITC and the wavelength of exciting light was 488 nm.

Intracellular Ca^{2+} concentration was measured using the Ca^{2+}-sensitive fluorescent probe Fura-2 (Beyotime S1052, China) as substrate. In brief, hemocytes were incubated with 500 μL of 10 μmoL/L flo-2 (in resuspension solution) at 37 °C for 45 min with rotation. After three times of washing with resuspension solution, the relative fluorescence intensity was determined by FACS Arial II flow cytometer (Becton, Dickinson and Company). The laser channel was Texas Red and the wavelength of exciting light was 595 nm.

Relative content of intracellular lysosome were detected using lysosome-sensitive probe Lyso-Tracker Red (Beyotime C1046, China) as substrate. In short, the hemocytes were collected with the same method as above, and incubated with 500 μL of 10 μmoL/L Lyso-Tracker Red (in resuspension solution) at 37 °C for 30 min with rotation. The lysosome content measured by relative fluorescence intensity was detected by FACS Arial II flow cytometer (Becton, Dickinson and Company). The laser channel was Texas Red and the wavelength of exciting light was 595 nm.

2.3. Cell sorting

FACS Arial II flow cytometer (Becton, Dickinson and Company) was used to separate the hemocytes following manufacturer's protocol. In brief, 6 mL hemolymph were collected from six normal crabs and immediately centrifuged at 1000×g, 4 °C for 10 min to harvest the hemocytes, and the single-cell suspensions were prepared using the resuspension solution. The viability of hemocytes