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The cytochemical and ultrastructural characteristics of phagocytes in the Pacific oyster *Crassostrea gigas*

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ABSTRACT

Phagocytes have been proved to play vital roles in the innate immune response. However, the cellular characteristics of phagocytes in invertebrates, especially in molluscs, remain largely unknown. In the present study, fluorescence activated cell sorting (FACS) was employed to sort the phagocytes from the non-phagocytic haemocytes of the Pacific oyster *Crassostrea gigas*. The cytochemical staining analysis revealed that phagocytes were positive staining for α-naphthyl acetate esterase and myeloperoxidase, while negative staining for toluidine blue and periodic acid-Schiff. The non-phagocytic haemocytes exhibited positive staining for periodic acid-Schiff, weak positive staining for toluidine blue, but negative staining for α-naphthyl acetate esterase and myeloperoxidase. In addition, phagocytes exhibited ultrastructural cellular features similar to those of macrophages, with large cell diameter, rough cell membrane and extended pseudopodia revealed by the scanning electron microscopy, while the non-phagocytic haemocytes exhibited small cell diameter, smooth cell surface and round spherical shape. Transmission electron microscopy further demonstrated that phagocytes were abundant of cytoplasmic bodies and mitochondria, while non-phagocytic haemocytes were characterized as the comparatively large cell nucleus with contorted and condensed heterochromatin adherent to the nuclear envelope. Moreover, compared with non-phagocytic haemocytes, phagocytes exhibited significantly higher levels of intracellular cytokines, including tumor necrosis factor, interferon-like protein and interleukin-17, and significantly higher abundance of lysosome and reactive oxygen species, which were of great importance to the activation of immune response and pathogen clearance. Taken together, these findings revealed the different cytochemical and ultrastructural features between phagocytes and non-phagocytic haemocytes in *C. gigas*, which would provide an important clue to investigate the mechanism of phagocytosis underlying the innate immune response.

1. Introduction

Phagocytosis is an evolutionarily conserved process, which plays essential roles in pathogen clearance, immune surveillance, embryonic development and metabolic homeostasis [1,2]. In higher vertebrates, such as mammals, the specialized phagocytic leukocytes (e.g. neutrophils, eosinophils and monocytes) are particularly responsible for the killing and clearance of invasive pathogens, as well as activation of innate and adaptive immune responses [3,4]. And in invertebrates, the circulating haemocytes, especially phagocytes, play crucial roles in the innate immune responses [5]. The plasmatocytes in *Drosophila melanogaster* are found to be specifically responsible for the phagocytosis of microorganisms with the typical function of macrophages [6]. Haemocytes in *Crassostrea gigas* were found to possess potent recognition and phagocytic capability towards parasites/pathogens, which possibly contributed to the higher immune resistance than that of *Ostrea edulis* [7,8]. Although the functions of phagocytes have been analyzed in several invertebrate species, the cellular characteristics of phagocytes in invertebrates still remain elusive.

The cellular features of phagocytes have been extensively

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investigated in mammals [9]. For instance, the lysosomal enzyme α-naphthyl acetate esterase (NAE) was abundant in the cytoplasmic vacuoles of macrophages [10], and the intracellular myeloperoxidase (MPX) was also highly expressed in macrophages to induce the oxidation of phophatidylserine and subsequently played an essential role in macrophage-dependent phagocytosis [11]. Moreover, the macrophages exhibited distinctive ultrastructural features, such as large cell size of 14–20 μm cell diameter, which allowed the engulfment of massive microbial pathogens as well as apoptotic cell corpse [12]. The plasma membrane of macrophage was featured by large, round, and hemispherical craters, as well as long and thin pseudopodia extending from the cell body. These structures were involved in the capture of exogenous particles and consequently ingestion into the cytoplasm [13,14]. In addition, the expressions of immunological factors were up-regulated in phagocytes via the interaction of pattern recognition receptors (PRRs) with pathogen-associated molecular patterns (PAMPs) [15,16]. And in the phagocytes of mice, zymosan could activate Dectin-1 signaling pathway promote phagocytosis, and induce the synthesis of cytokines and reactive oxygen species (ROS) [17]. CpG dinucleotides from the bacterial DNA was recognized by intracellular Toll like receptor 9 (TLR9) of macrophages, and induced the production of tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and interleukin-12 (IL-12) cytokines [18].

The Pacific oyster *Crassostrea gigas* is a keystone mollusc species of coastal and estuarine ecology [19]. As sessile marine invertebrate animals living in estuarine and intertidal regions, oysters have to cope with extraordinary abundant microbial pathogen challenges (more than 10^{23} infections occur every second) from the surrounding oceanic environment [20]. The phagocytes have been proved to play vital roles in the innate immune responses against microbial pathogens, the study of phagocytes is therefore conducive to the investigation of anti-microbial innate immunity. In the present study, the phagocytes from *C. gigas* were sorted by fluorescence activated cell sorting, and further characterized by various cytochemical staining as well as ultrastructural analysis. Moreover, the intracellular cytokines in phagocytes, including tumor necrosis factor (TNF), interferon-like protein (IFNLP) and interleukin-17 (IL-17), were examined by immunofluorescent intracellular staining, and the abundance of intracellular lysosome and reactive oxygen species (ROS) were analyzed by flow cytometry to reveal the cellular characteristics of phagocytes.

2. Materials and methods

2.1. Animal rearing and manipulation

The oysters *C. gigas* used in the present study were marine cultured animals with length of 10–15 cm and weight of 150–200 g. They were acclimated in aerated seawater at 18 °C for two weeks prior to use. All the experiments were conducted according to the regulations of local and central government. The animal experiments were approved by the local animal care and use committee.

2.2. Preparation of haemocytes from C. gigas

Hemolymph was collected from the blood sinusoid of adult *C. gigas* with a syringe, and mixed immediately with prechilled anticoagulant ACD-A (0.1 mol/L trisodium citrate, 0.11 mol/L dextrose and 71 mmol/L citric acid monohydrate) at a ratio of 7:1. The haemocytes were pelleted at 800 g, 4 °C for 10 min, and washed twice with modified Leibovitz L15 medium (supplemented with 0.54 g/L KCl, 0.6 g/L $CaCl_2$, 1 g/L $MgSO_4$, 3.9 g/L $MgCl_2$, 20.2 g/L NaCl, 100 units/mL penicillin G, 40 μg/L gentamycin, 100 μg/mL streptomycin, 0.1 μg/mL amphotericin B and 10% fetal bovine serum). The haemocytes from 3 to 5 individuals were pooled together as one sample, and at least three replicates were performed in the following each assay.

2.3. Preparation of FITC-labeled microbes

Vibrio splendidus, *Staphylococcus aureus* and *Pichia pastoris* were grown in 2216E media at 28 °C, 220 rpm for 12 h, LB media at 37 °C, 220 rpm for 8 h, and YPD media at 30 °C, 220 rpm for 24 h, respectively. The cells were cultured to mid-log phase and harvested by centrifugation at 6000 *g* for 15 min. After fixed with 4% Paraformaldehyde (PFA) for 10 min and washed with 0.1 mol/L $NaHCO_3$ (pH 9.0) for three times, the microbes were labeled by mixing them with 1 mg/mL FITC (Sigma-Aldrich) in 0.1 mol/L $NaHCO_3$ (pH 9.0) buffer with continuous gentle stirring at room temperature overnight. The FITC-labeled microbes were washed with PBS for three times to eliminate free FITC molecules.

2.4. Fluorescence activated cell sorting of phagocytes from non-phagocytic haemocytes

Haemocytes were collected and incubated with FITC-labeled Latex beads (2 μm) in a ratio of 1:100 at 18 °C for 3 h. Trypan blue (1.2 mg/mL) was used to quench surface-bound FITC-labeled beads. Cells were analyzed on a FACS Arial II flow cytometer (Becton Dickinson Biosciences), and the FITC-positive and -negative cells were gated and sorted based on the FITC fluorescence intensity. The sorted cells were re-analyzed by flow cytometry to confirm the purity. Flow cytometry data were analyzed with BD FACSDiva (Becton Dickinson Biosciences).

2.5. Cytochemical staining analysis

The sorted phagocytes and non-phagocytic haemocytes were collected and plated onto the glass slide to allow cell adhesion at 18 °C for 3 h. Haemocytes staining was performed as previously described [21]. Briefly, for Wright-Giemsa (WG) and Hematoxylin-eosin (HE) staining, haemocytes were fixed by 4% PFA and stained with WG and HE for 10 min, respectively. For α-naphthyl acetate esterase (NAE) staining, haemocytes were fixed by 25% glutaraldehyde and stained with NAE for 1 h. Toluidine blue (TB) staining was conducted by fixing the haemocytes with 4% PFA and stained with TB for 2 h. For periodic acid-Schiff (PAS) staining, haemocytes were fixed by 5% ethanol and oxidized in 0.5% periodic acid solution for 5 min, followed by staining with PAS for 30 min. For myeloperoxidase (MPX) staining, haemocytes were fixed by 10% formal-ethanol and stained with MPX for 10 min. The morphological characteristics of haemocytes were observed by light microscopy (Olympus DP72, Japan).

2.6. Scanning electron microscopy and transmission electron microscopy analysis

Phagocytes and non-phagocytic haemocytes were sorted and incubated on the cover glass for 3 h to allow cell adhesion. For scanning electron microscopy (SEM), the samples were fixed in 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer (pH 7.4) for 1 h, followed by further fixation in osmium tetroxide solution (1% OsO_4, 1.5% $K_3Fe(CN)_6$, 0.1 mol/L sodium cacodylate, pH 7.4) at room temperature for 1 h. The samples were washed, and dehydrated by sequential incubation in 30%, 50%, 70%, 90%, 95% ethanol at 4 °C (15 min each), and finally incubated in 100% ethanol at room temperature for three times (15 min each) followed by critical point drying. The specimens were mounted onto metal stub and coated