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Functional characterization of phagocytes in the Pacific oyster *Crassostrea gigas*

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Invertebrates lack canonical adaptive immunity and mainly rely on innate immune system to fight against pathogens, whose phagocytes are likely to be of great importance and have to undertake even more responsibility in immune defense. In the present study, flow cytometry combined with histological and lectin staining was employed to characterize functional features of phagocytes in Pacific oyster *Crassostrea gigas*. Based on the cell size and cellular contents, haemocytes were categorized into three cell types including granulocytes, semigranulocytes and agranulocytes. The agranulocytes with smaller cell volume and lower cytoplasmic-to-nuclear ratio did not show phagocytic activity, while the phagocytes exhibited larger cell volume and higher cytoplasmic-to-nuclear ratio, which were probably derived from both granulocytes and semigranulocytes. In addition, the granulocytes with higher internal complexity exhibited higher phagocytic activity than that of semigranulocytes. After β-integrin and lectin-like receptors were blocked by RGD tripeptide and carbohydrates respectively, the phagocytic activity of both granulocytes and semigranulocytes was significantly inhibited, indicating that β-integrin and certain lectin-like receptors were involved in phagocytosis towards microbes. Moreover, lipopolysaccharide but not peptidylglycan could enhance phagocytic activity of granulocytes and semigranulocytes towards *Vibrio splendidus* and *Staphylococcus aureus*. Lectin staining analysis revealed that *Lycopersicon esculentum* lectin (LEL) binding epitope polylactosamine was highly distributed on the extracellular cell surface of phagocytes, which could be utilized as a potential molecular marker to differentiate phagocytes from non-phagocytic haemocytes. The results collectively provided knowledge on the functional characters of oyster phagocytes, which would contribute to deep investigation of cell typing and cellular immunity in bivalves.

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ABSTRACT

Invertebrates lack canonical adaptive immunity and mainly rely on innate immune system to fight against pathogens, whose phagocytes are likely to be of great importance and have to undertake even more responsibility in immune defense. In the present study, flow cytometry combined with histological and lectin staining was employed to characterize functional features of phagocytes in Pacific oyster *Crassostrea gigas*. Based on the cell size and cellular contents, haemocytes were categorized into three cell types including granulocytes, semigranulocytes and agranulocytes. The agranulocytes with smaller cell volume and lower cytoplasmic-to-nuclear ratio did not show phagocytic activity, while the phagocytes exhibited larger cell volume and higher cytoplasmic-to-nuclear ratio, which were probably derived from both granulocytes and semigranulocytes. In addition, the granulocytes with higher internal complexity exhibited higher phagocytic activity than that of semigranulocytes. After β-integrin and lectin-like receptors were blocked by RGD tripeptide and carbohydrates respectively, the phagocytic activity of both granulocytes and semigranulocytes was significantly inhibited, indicating that β-integrin and certain lectin-like receptors were involved in phagocytosis towards microbes. Moreover, lipopolysaccharide but not peptidylglycan could enhance phagocytic activity of granulocytes and semigranulocytes towards *Vibrio splendidus* and *Staphylococcus aureus*. Lectin staining analysis revealed that *Lycopersicon esculentum* lectin (LEL) binding epitope polylactosamine was highly distributed on the extracellular cell surface of phagocytes, which could be utilized as a potential molecular marker to differentiate phagocytes from non-phagocytic haemocytes. The results collectively provided knowledge on the functional characters of oyster phagocytes, which would

contribute to deep investigation of cell typing and cellular immunity in bivalves.

KEY WORDS:

Crassostrea gigas; Phagocytosis; Flow cytometry; Lectin staining; Polylactosamine

INTRODUCTION

Phagocytosis, the uptake and digestion of exogenous particles, is an ancient, evolutionarily conserved cellular process, which plays important roles in the pathogen killing and clearance as well as the food uptake (Aderem & Underhill 1999; Greenberg & Grinstein 2002; Henneke & Golenbock 2004). In mammals, the professional phagocytes, such as macrophages and neutrophils, are particularly responsible for the killing and clearance of pathogens, and initiating signaling pathway to provoke potent immune responses (Jutras & Desjardins 2005). More importantly, some phagocytes named antigen-presenting cells (APCs) are well known not only for their potent phagocytic activity but also for the antigen presentation activity, which has been deemed as the bridge between innate immunity and adaptive immunity (Delamarre et al. 2005).

Invertebrates, which lack the canonical adaptive immunity based on B and T lymphocytes, mainly rely on the innate immune system to fight against pathogens (Kurtz & Franz 2003; Little et al. 2005). Compared with the phagocytes in higher animals, invertebrate phagocytes are likely to be of great importance and have to undertake even more responsibility in immune defense (Stuart & Ezekowitz 2008b). In the previous study, the haemocytes have been typed into different cell subpopulations in several invertebrates, and some subpopulations have been confirmed to be in charge of phagocytosis. For instance, granulocytes in *Crassostrea virginica* were found to be active in phagocytosis (Goedken & De Guise 2004). In *Mytilus galloprovincialis*, the stimulation with laminarin or yeast cells significantly promoted the phagocytosis of haemocytes (Arumugam et al. 2000). In *Drosophila melanogaster*, plasmatocytes were specifically responsible for the phagocytosis of microorganisms, while lamellocytes and crystal cells were involved in encapsulation and melanization respectively (Lemaitre & Hoffmann 2007). Recently, the capture and engulfment of bacteria by circulating or fixed phagocytes have also been reported in several invertebrates (Le Grand et al. 2011; Soderhall 2010).

Phagocytes have been proved to play vital roles in the immune defense in invertebrates. The phagocytes from sea anemones, lacking a dedicated coelomic immune system, exhibit strong reactive oxygen species (ROS) production capability in respiratory burst (Hutton & Smith 1996; Robb et al. 2014). In schyphozoans and anthozoans, the phagocytes are infiltrated and accumulated at the injured tissue to provoke an inflammatory immune response against the invaded pathogens (Olano & Bigger 2000; Reed et al. 2010). Additionally, phagocytes in *D. melanogaster* were found to play pivotal roles in the specific primed protective immune response against *Streptococcus pneumoniae* (Pham et al. 2007). Although the phagocytes have been investigated in several invertebrates, their heterogeneity of phagocytosis in bivalve molluscs requires further investigation.

The Pacific oyster *Crassostrea gigas* is an important species for physiological ecology as well as economical resource (Zhang et al. 2012). In the present study, flow cytometry combined with histological staining was employed to categorize the haemocytes based on the morphological features, and their phagocytic activities of different cell populations were also determined. The phagocytic modulation effects of β-integrin, lectin-like receptors (LLRs), lipopolysaccharide (LPS) and peptidylglycan (PGN) were investigated, and the potential glycan markers distinguishing phagocytes from non-phagocytic cells were screened in order to better understand the phagocytosis in the innate immune defense in oysters.

MATERIALS AND METHODS

Animal rearing and manipulation

The oysters with length of 10-15 cm and weight of 150-200 g were collected from a farm in Qingdao, Shandong Province, China, and 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.

Preparation of haemocytes from *C. gigas*

Haemolymph was withdrawn using a syringe equipped with a needle (0.9 × 25 mm) from the pericardial cavity of adult *C. gigas* specimens after the shells were carefully opened, 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 3-5 individuals were pooled together as one sample and stored on ice to reduce spontaneous aggregation.

May-Grunwald Giemsa (MGG) staining

For phagocytosis assay, haemocytes were incubated with *Pichia pastoris* at a ratio of 1:100 for 1 h, and washed by modified L15 medium for three times. Haemocytes were plated onto glass slides to allow cell adhesion at 18 °C for 3 h, and the glass slides were fixed with 100% methanol for 10 min. MGG was used to stain cells for another 10 min followed by PBS washing, and the cells on the slides were characterized by light microscopy according to their morphological features.

Preparation of FITC-labeled microbes

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

Flow cytometric analysis of haemocyte and its phagocytosis

Haemocytes were collected and analyzed on a FACS Arial II flow cytometer (Becton Dickinson Biosciences). For morphological characterization of haemocytes, forward scatter (FSC) combined with side scatter (SSC) analysis was performed to measure relative cell size and internal complexity of cells respectively. For phagocytosis analysis, FITC-labeled microbes and latex beads (Sigma-Aldrich) were incubated with haemocytes at a ratio of 100:1 at 18 °C for 1 h. The cells were then washed by modified L15 medium three times, and Trypan Blue (1.2 mg/ml) was used to quench surface-bound FITC-labeled bacteria. FSC and FL1 channel detection was immediately performed to analyze the phagocytosis of FITC-labeled particles.

RGD, carbohydrates and PAMPs treatments of haemocytes

Haemocytes were incubated with Arg-Gly-Asp (RGD) tripeptide at 0.5 mg/ml for 1 h to block β-integrin, and incubated with different carbohydrates including glucose (Glu), fucose (Fuc), mannose (Man), lactose (Lac) and N-acetylglucosamine (GlcNAc) at 100 mM for 1 h to block lectin like receptors (LLRs), respectively. For the LPS and PGN stimulations, haemocytes were incubated with LPS and PGN at 0.1 and 1 mg/ml for 1 h respectively. Cells were then washed by modified L15 medium for three times followed by incubation with FITC-labeled microbes at a ratio of 1:100. Flow cytometry was performed to analyze the phagocytic percentages of haemocytes.

Flow cytometric and confocal microscopic analysis of lectin staining

For flow cytometric analysis, haemocytes were incubated with FITC labeled microbes at a ratio of 1:100 at 18 °C for 1 h followed by extensively washing, and then incubated with phycoerythrin (PE)-labeled wheat germ agglutinin (WGA), peanut agglutinin (PNA) and *Lycopersicon esculentum* lectin (LEL) (50 μg/ml) at room temperature for 1 h. After washed with L-15 medium for three times, the haemocytes were analyzed by flow cytometry (BD FACSAria II). For microscopic analysis, haemocytes were collected and suspended in the cell culture medium at the concentration of 1×10^6 cells/ml. The cell suspension (1.5 ml) was then added in cell culture dishes and incubated for 3 h to allow cell adhesion. FITC-labeled latex beads were added at a ratio of 100:1 and incubated with haemocytes for 1 h. The haemocytes were fixed by 4% PFA at 4 °C for 15 min after washed by L-15 medium three times, and permeabilized by 0.1% Triton X-100 for 15 min. The nonspecific binding sites were blocked by adding 5% BSA and incubated at room temperature for 1 h. PE-labeled LEL (50 μg/ml) was incubated with haemocytes at room temperature for another 1 h and washed three times with PBS. The haemocytes were monitored and the fluorescent images were taken using Carl Zeiss LSM 710 confocal microscope (Jena, Germany).

Statistical analysis

The two-sample Student's *t* test was used for the comparisons between groups. Statistical analysis was performed with GraphPad Prism 5 software. The statistical significance was defined as $p < 0.05$.

RESULTS

The morphological characters of haemocytes from *C. gigas*

The haemocytes collected from *C. gigas* were gated by light-scatter characteristics using flow cytometer, and May-Grunwald-Giemsa (MGG) staining was performed to characterize the cellular morphology of each subpopulation (Fig. 1A). Based on the forward scatter (FSC) and side scatter (SSC) intensity, the haemocytes were divided into three subpopulations including agranulocytes, granulocytes and semigranulocytes. Agranulocytes were located at the lower left position on the light scatter chart with smaller size (approximate 5-8 μm), clear cytoplasm and lower cytoplasmic-to-nuclear ratio. Granulocytes were located at the upper right position with larger cell size (approximate 10-14 μm), abundant intracellular contents, and higher cytoplasmic-to-nuclear ratio. Semigranulocytes were located at the lower right position with larger cell size (approximate 11-13 μm), lower internal complexity, and higher cytoplasmic-to-nuclear ratio (Fig. 1A). In addition, most agranulocytes and granulocytes appeared approximately round shape on the glass slide, while some of semigranulocytes extended filopodia to explore the microenvironment and spread on the glass slide. A total of 10, 000 haemocytes were analyzed by flow cytometry, and the agranulocyte, granulocyte and semigranulocyte subpopulations comprised 46.2%, 31.4% and 19.6% of the total haemocytes, respectively (Fig. 1B).

Morphological identification of phagocytes from *C. gigas*

In order to gain a further observation of phagocytes, *Pichia pastoris* with large cell diameter was employed as exogenous particles to allow phagocytosis of haemocytes, and MGG staining was performed to characterize the histological features of phagocytes (Fig. 2A). Phagocytes exhibited larger cell size (approximately 9-14 μm), higher cytoplasmic-to-nuclear ratio (engulfment of 4-7 fungal cells per phagocyte), while non-phagocytic cells exhibited smaller cell size (5-9 μm), and their nucleus almost filled the cell, leaving a thin rim of cytoplasm. The morphological features of phagocytes were further characterized by flow cytometric analysis (Fig. 2B), and these cells with engulfment of FITC-labeled latex beads were featured with larger cell size (higher FSC value). The percentage of phagocytes in total haemocytes was calculated to be 8.82%. While the haemocytes with smaller cell size (lower FSC value) did not exhibit phagocytic capability towards FITC-labeled latex beads. These results suggested that phagocytes probably derived from granulocytes and semigranulocytes, which possessed larger cell size and higher cytoplasmic-to-nuclear ratio.

The involvement of β-integrin in phagocytosis

Phagocytes are in charge of phagocytizing exogenous particles, and the phagocytic capability of oyster haemocytes towards different microbes was further investigated by flow cytometric analysis. The percentages of the phagocytic haemocytes were 24.8% for *E. coli* (Fig. 3A and B), 8.2% for *V. splendidus* (Fig. 3C and D) and 14.7% for *S. aureus* (Fig. 3E and F). Moreover, granulocytes exhibited higher phagocytic percentages than that of semigranulocytes, which were 38.7% and 19.1% for *E. coli*, 9.8% and 7.4% for *V. splendidus,* and 24.1% and 10.3% for *S. aureus*, respectively. Arg-Gly-Asp (RGD) tripeptide was used to block the recognition domain of β-integrin to determine if β-integrin was participated in the phagocytosis of granulocytes and semigranulocytes. The total percentages of the phagocytic haemocytes significantly decreased 43.2% for *E. coli*, 39.6% for *V. splendidus* and 45.7% for *S. aureus* after β-integrin was blocked (Fig. 3B, D and F). Moreover, the phagocytic percentages of granulocytes and semigranulocytes decreased 37.8% and 46.4% for *E. coli*, 35.1% and 47.8% for *V. splendidus*, 40.5% and 48.4% for *S. aureus* after the blockage of β-integrin, respectively.

The involvement of lectin-like receptors in the phagocytosis of different microbes

The participation of LLRs in phagocytosis towards microbes was determined after the corresponding carbohydrate binding receptors were blocked by Glucose (Glu), fucose (Fuc), mannose (Man), lactose (Lac) and N-acetylglucosamine (GlcNAc), respectively. Fuc, Man, Lac and GlcNAc exhibited significantly inhibitory effects on the phagocytosis of haemocytes towards *V. splendidus* with 41.6%, 32.9%, 28.1% and 35.4% reduction in phagocytic percentages respectively, while Glu did not show any significant inhibition on phagocytosis towards *V. splendidus* (Fig. 4A). After the treatments with Fuc, Man, Lac and GlcNAc, the phagocytic percentages decreased to 73.4%, 80.8%, 75.5% and 72.2% in granulocytes (Fig. 4B), and 54.8%, 52.4%, 60.9% and 62.3% in semigranulocytes, respectively (Fig. 4C). In addition, Fuc, Man and GlcNAc exhibited inhibitory effects on the phagocytosis towards *S. aureus* in total haemocytes, and the phagocytic percentages significantly decreased 29.3%, 39.5% and 36.3% respectively, while the inhibitory effects of Glu and Lac on phagocytosis towards *S. aureus* were much lower (Fig. 4D). The phagocytic percentages of granulocytes and semigranulocytes towards *S. aureus* were also significantly decreased after Fuc, Man and GlcNAc treatment, resulted in 37.1%, 41.9% and 43.6% reduction in granulocytes (Fig. 4E), and 27.3%, 29.8% and 19.4% reduction in semigranulocytes, respectively (Fig. 4F).

The enhancement of phagocytosis after LPS treatment

LPS and PGN are important pathogen-associated molecular patterns (PAMPs) identified from Gram-negative and Gram-positive bacteria, respectively. The phagocytic percentage of oyster haemocytes towards *V. splendidus* increased 17.8% and 44.3% after 0.01 and 0.1 mg/ml LPS stimulation (Fig. 5A). Meanwhile, it increased 11.5% and 18.9% in granulocytes (Fig. 5B), 25.4% and 53.6% in semigranulocytes respectively (Fig. 5C). Similarly, LPS stimulation significantly increased the phagocytic percentages of haemocytes towards *S. aureus*. It increased 16.8% and 31.6% in total haemocytes after 0.01 and 0.1 mg/ml LPS stimulation (Fig. 5D), with 14.2% and 29.3% increment in granulocytes (Fig. 5E), 19.5% and 34.2% increment in semigranulocytes respectively (Fig. 5F). By contrast, there were no significant changes in the phagocytic percentages towards *V. splendidus* and *S. aureus* in the total haemocytes, granulocytes or semigranulocytes after 0.01 and 0.1 mg/ml PGN treatments respectively.

Lycopersicon esculentum lectin exhibited high binding specificity to phagocytes

In order to further characterize the molecular features of phagocytes, lectin staining was performed to distinguish phagocytes from non-phagocytic cells (Fig. 6A). The positive cells of *Lycopersicon esculentum* lectin (LEL) staining were in high accordance with the phagocytes from *C. gigas*. The percentages of double positive cells (PE-LEL$^+$/FITC$^+$) were approximately 23.2% for *E. coli*, 18.7% for *V. splendidus*, 24.1% for *B. subtilis* and 27.6% for *S. aureus*. While the percentages of PE-LEL$^-$/FITC$^+$ cells were no more than 3% for all the four microbes, and the percentages of PE-LEL$^+$/FITC$^-$ cells were approximately 3.2% for *E. coli*, 4.1% for *V. splendidus*, 2.7% for *B. subtilis* and 3.4% for *S. aureus*, respectively (Fig. 6B, right).

On the contrary, the positive haemocytes of wheat germ agglutinin (WGA) and peanut agglutinin (PNA) staining were not significantly associated with phagocytes. The percentages of PE-WGA$^+$/FITC$^-$ cells were even higher than that of PE-WGA$^+$/FITC$^+$ cells, indicating that WGA exhibited binding activity to both phagocytes and non-phagocytic haemocytes (Fig. 6B, left). Additionally, PE-PNA$^-$/FITC$^+$ cells exhibited higher percentages than that of PE-PNA$^+$/FITC$^+$ cells, suggesting that PNA preferred binding to non-phagocytic haemocytes rather than phagocytes (Fig. 6B, middle). The results clearly indicated that LEL positive staining cells exhibited higher accordance with phagocytes, while WGA and PNA staining exhibited much lower binding specificity towards phagocytes and non-phagocytic cells.

The distribution of polylactosamine in oyster phagocytes

The LEL binding carbohydrate epitopes, including N-linked and O-linked polylactosamines, were depicted with at least three lactosamine repeats (Fig. 7A). The phagocyte-specific distribution of polylactosamine was further confirmed by confocal microscopic analysis. Polylactosamine, as indicated by PE-labeled LEL (red color), was highly distributed on the cell membrane, and assembled to form an arc on one side of the phagocytes. It was noted that polylactosamine also concentrated as patches in cytoplasm of phagocytes (Fig. 7B). By contrast, there was no positive signal of LEL in non-phagocytic haemocytes, indicating that polylactosamine might not distribute in non-phagocytic cells.

DISCUSSION

The phagocytes were in charge of phagocytosis, encapsulation and oxidative killing, and provided the main executants to kill pathogens and sustained immune homeostasis (Pham et al. 2007). In the previous study, various criteria have been applied to the haemocyte classification in bivalve molluscs. For example, haemocytes from *C. gigas* were proposed to be divided into several groups including basophilic and eosinophilic granulocytes, three types of agranular haemocytes with or without cytoplasmic granules, blast-like haemocytes and large basophilic agranular haemocytes (Bachere et al. 1988; Hine 1999). Different haemocytes of *Crassostrea rhizophorae* were proposed to be one type of cell at different stages, which accumulated or lost granules and complexity in response to environmental or microbial challenges (Rebelo Mde et al. 2013). Moreover, a granular population composed of basophilic and eosinophilic granulocytes in oysters was reported to possess phagocytic activity (Bachere et al. 2004). In the present study, the oyster haemocytes could be divided into three cell subpopulations by flow cytometry based on cell size and intracellular contents: agranulocytes, granulocytes and semigranulocytes. Both granulocytes and semigranulocytes exhibited phagocytic activity towards FITC-labeled latex beads and different microbes, while the agranulocytes with smaller cell size did not exhibit phagocytic activity. In addition, the granulocytes exhibited higher phagocytic activity than that of semigranulocytes. It has been reported that the granules in oyster haemocytes were rich in antimicrobial peptides, which were reported to bound specifically to phagosomes and rapidly released into the phagosomes/phagolysosomes to kill the phagocytosed microbial pathogens, suggesting the vital role of granulocyte in the clearance of pathogen (Gonzalez et al. 2007; Rosa et al. 2011). The classification of phagocytes into granulocyte-derived and semigranulocyte-derived phagocytes would be helpful to further investigate the phagocytosis in the innate immune modulation and pathogen elimination of *C. gigas*.

Molecular markers have been proved to be extremely useful for cell typing in mammalian immune system, while the cell typing of haemocytes in invertebrates still needs to be elucidated (Kurucz et al. 2007; Le Foll et al. 2010). Even the invertebrate haemocytes are of great heterogeneity, the intimate relationship has been found between different molecular markers and the corresponding cellular functions. In *Mytilus edulis*, the small granules of the granulocytes were found to be *Helix pomatia* agglutinin (HPA)-positive, and the large granules of the granulocytes were of wheat germ agglutinin (WGA) positive, indicating that lectin staining could be applied in the cell typing (Pipe 1990). In *Crassostrea virginica*, haemocytes infected with *Haplosporidium nelsoni* (MSX) could be agglutinated by WGA and HPA, suggesting that haemocytes contained surface receptors resembling N-acetyl-D-glucosamine and αmethylmannopyranoside (Kanaley & Ford 1990). In *Anopheles gambiae*, only the granulocytes from individuals challenged by *Plasmodium falciparum* malaria could be stained by *Lens culinaris* agglutinin (LCA), whereas most (96%) of naive granulocytes were negative and 4% were stained weakly ($p < 0.0001$) (Rodrigues et al. 2010). In the present study, the phagocytes could be separated from non-phagocytic cells based on the differential distribution of glycans by LEL staining. LEL exhibits high binding specificity towards polylactosamines with at least three lactosamine repeats, and has been widely used to discriminate different cell types (Togayachi et al. 2007). Most of the LEL positive haemocytes from *C. gigas* were phagocytes, suggesting the abundant distribution of polylactosamine glycans in phagocytes. While the positive signals of WGA and PNA were observed in both phagocytes and non-phagocytic cells, indicating that there were no distribution differences of WGA- and PNA-binding epitopes between phagocytes and non-phagocytic cells in *C. gigas*.

Co-evolutionary arms races between pathogens and hosts, and the competitions are considered to be of immense importance in the evolution of living organisms (Akira et al. 2001; Akira et al. 2006). Integrins are required for the correct formation of phagosomes, and they play important roles in the phagocytosis (Oliva et al. 2008; Stuart & Ezekowitz 2008a; Wang et al. 2008). In the present study, the blockage of β-integrin by RGD inhibited phagocytosis towards both Gram-negative and Gram-positive bacteria in granulocytes and semigranulocytes, indicating that β-integrin was extensively involved in the phagocytosis of oyster haemocytes. RGD-containing peptides were reported to induce haemocyte apoptosis in *C. gigas* at the concentration of 3 mM (Terahara et al. 2003; Terahara et al. 2005), which was much higher than that used in the present study, suggesting that lower concentration of RGD peptide could inhibit phagocytosis, while higher concentration of RGD peptide could induce cell apoptosis.

Lectin, as a typical pattern recognition receptor (PRR), is involved in the pathogen recognition and phagocytosis. For example, a C-type lectin (CfLec-3) from *Chlamys farreri* with three carbohydrate-recognition domains (CRDs) could modulate haemocyte phagocytosis via binding to different PAMPs and microbes (Yang et al. 2015). The native lectin FcLec4 could bind to β-integrin to promote haemocytic phagocytosis in *Fenneropenaeus chinensis* (Wang et al. 2014). Genes encoding lectin-like receptors (LLRs) are highly over-represented in oyster genome (p < 0.0001) (Zhang et al. 2012). In the present study, the blockage of LLRs by Fuc, Man and GlcNAc exhibited an inhibitory effect on phagocytosis towards *V. splendidus* and *S. aureus* in granulocytes and semigranulocytes, indicating that LLRs were involved in the recognition of different microbes and modulation of phagocytosis. In addition, Lac showed phagocytic inhibitory activity towards *V. splendidus* but not *S. aureus*, indicating that the Lac specific LLRs was involved in the phagocytosis towards *V. splendidus*. However, Glu exhibited little inhibitory effect on the phagocytosis towards *V. splendidus* or *S. aureus*, suggesting that the Glu binding LLRs might not participate in the phagocytosis of the two bacteria.

PAMPs are important stimuli which play vital roles in the activation of immune responses (Iliev et al. 2005). LPS has been proved to act as an extremely strong stimulator of innate immunity in mammals (Alexander & Rietschel 2001; Kawai & Akira 2010). The extracellular membrane receptors, such as Toll-like receptor 4 (TLR4), could recognize LPS and initiate the rapid immune-activation through an intracellular signaling pathway (Chu & Mazmanian 2013;

Shenoy et al. 2012; West et al. 2011). Moreover, various forms of β-glucans have been proved to possess the potential utilization value in shrimp and fish aquaculture, which could increase the numbers of circulating haemocytes, promote long-term activation of haemocytes and enhance the haemocytic aggregation (Anderson et al. 2011). In the present study, LPS stimulation substantially increased the phagocytic activity of both granulocytes and semigranulocytes towards *V. splendidus* and *S. aureus*, while PGN stimulation had no effect on the phagocytosis. It is noteworthy that a number of Gram-negative bacteria, including *V. splendidus*, have been identified to be important aquaculture pathogens, which could cause massive mortalities of oysters (Garnier et al. 2008; Richards et al. 2015). The enhancement of phagocytic activity towards microbial pathogens under LPS stimulation contributed to better understand the modulation of phagocytosis in oysters, and suggested the potential application in oyster aquaculture.

In conclusion, the present study showed that *C. gigas* haemocytes were categorized into three cell types including granulocytes, semigranulocytes and agranulocytes. The phagocytic capacity of granulocytes and semigranulocytes towards different microbes was determined, and the β-integrin and certain LLRs were found to play important roles in the phagocytosis of granulocytes and semigranulocytes. In addition, LPS but not PGN could significantly enhance the phagocytic activities. Moreover, LEL binding epitope polylactosamine was highly distributed on the extracellular cell surface of phagocytes, which could be utilized as a potential molecular marker to differentiate phagocytes from non-phagocytic haemocytes. Collectively, the present study investigated in the phagocytosis of haemocyte subpopulations towards different microbes, which help to better understand the phagocytosis in the innate immune defense of oysters.

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ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Shuai Jiang conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.

- Zhihao Jia performed the experiments, analyzed the data, and contributed reagents/materials/analysis tools.
- Tao Zhang conceived and designed the experiments, and contributed reagents/materials/analysis tools.
- Lingling Wang conceived and designed the experiments, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Jinsheng Sun and Limei Qiu conceived and designed the experiments, and analyzed the data.
- Linsheng Song conceived and designed the experiments, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.

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Figure legends

Fig. 1. Flow cytometric analysis and May-Grunwald-Giemsa (MGG) staining of haemocytes from *C. gigas*. (A) Haemocytes were categorized into different subpopulations by flow cytometry followed by MGG staining analysis. Bar: 5 μm. (B) The percentages of each subpopulation were calculated with statistically analysis. Results are means ± S.E.M. (n = 6).

Fig. 2. Morphological identification of phagocytes from *C. gigas*. (A) Haemocytes were incubated with fungal cells *Pichia pastoris* to allow phagocytosis, followed by MGG staining and microscopic analysis. The fungal cells were indicated with asterisks, N stands for cell nucleus. Bar: 5 μm. (B) Haemocytes pre-incubated with FITC-labeled latex beads (2 μm diameter) were analyzed by flow cytometry.

Fig. 3. The involvement of β-integrin in phagocytosis towards different microbes. Haemocytes were treated with or without RGD tripeptide, and the phagocytic activities towards *E. coli*, *V. splendidus* and *S. aureus* were determined by flow cytometry (A, C and E). The statistical results of phagocytic percentages were shown respectively (B, D and F). Results are means ± S.D. (n = 6), *$p < 0.05$, **$p < 0.01$.

Fig. 4. The involvement of lectin-like receptors (LLRs) in the phagocytosis of different microbes. The haemocytes were pre-incubated with different carbohydrates, and the phagocytic inhibitory activity towards *V. splendidus* (A, B, C) and *S. aureus* (D, E, F) were determined. Results are means ± S.D. (n = 6),*$p < 0.05$, **$p < 0.01$.

Fig. 5. The enhancement of phagocytosis after LPS treatment. The haemocytes were pre-treated with LPS and PGN with different concentrations, and the phagocytic activities towards *V.*

splendidus (A, B, C) and *S. aureus* (D, E, F) were determined. Results are means ± S.D. (n = 6), **p* < 0.05, ***p* < 0.01.

Fig. 6. Lectin staining analysis of the phagocytes from *C. gigas*. (A) Haemocytes were incubated with FITC-labeled *E. coli*, *V. splendidus*, *S. aureus* and *B. subtilis*, and PE-labeled WGA, PNA and LEL were used to stain haemocytes respectively. The correlation between lectin staining and phagocytes was analyzed by flow cytometry. (B) The percentages of haemocytes gated on $PE^+/FITC^+$, $PE^+/FITC^-$ and $PE^-/FITC^+$ were calculated (n = 5).

Fig. 7. The distribution of polylactosamine in oyster phagocytes revealed by confocal microscopy. (A) LEL binding epitopes polylactosamine are indicated by dotted rectangles, and the predicted carbohydrate structures are represented in *N*-linked and *O*-linked glycans. (B) Haemocytes were incubated with FITC-labeled latex beads, and then fixed and permeabilized, followed by PE-labeled LEL staining. The representative phagocyte and non-phagocyte were shown.

Figure 1 (on next page)

Figure1

Flow cytometric analysis and May-Grunwald-Giemsa (MGG) staining of haemocytes from *C. gigas*. (A) Haemocytes were categorized into different subpopulations by flow cytometry followed by MGG staining analysis. Bar: 5 μm. (B) The percentages of each subpopulation were calculated with statistically analysis. Results are means ± S.E.M. (n = 6).

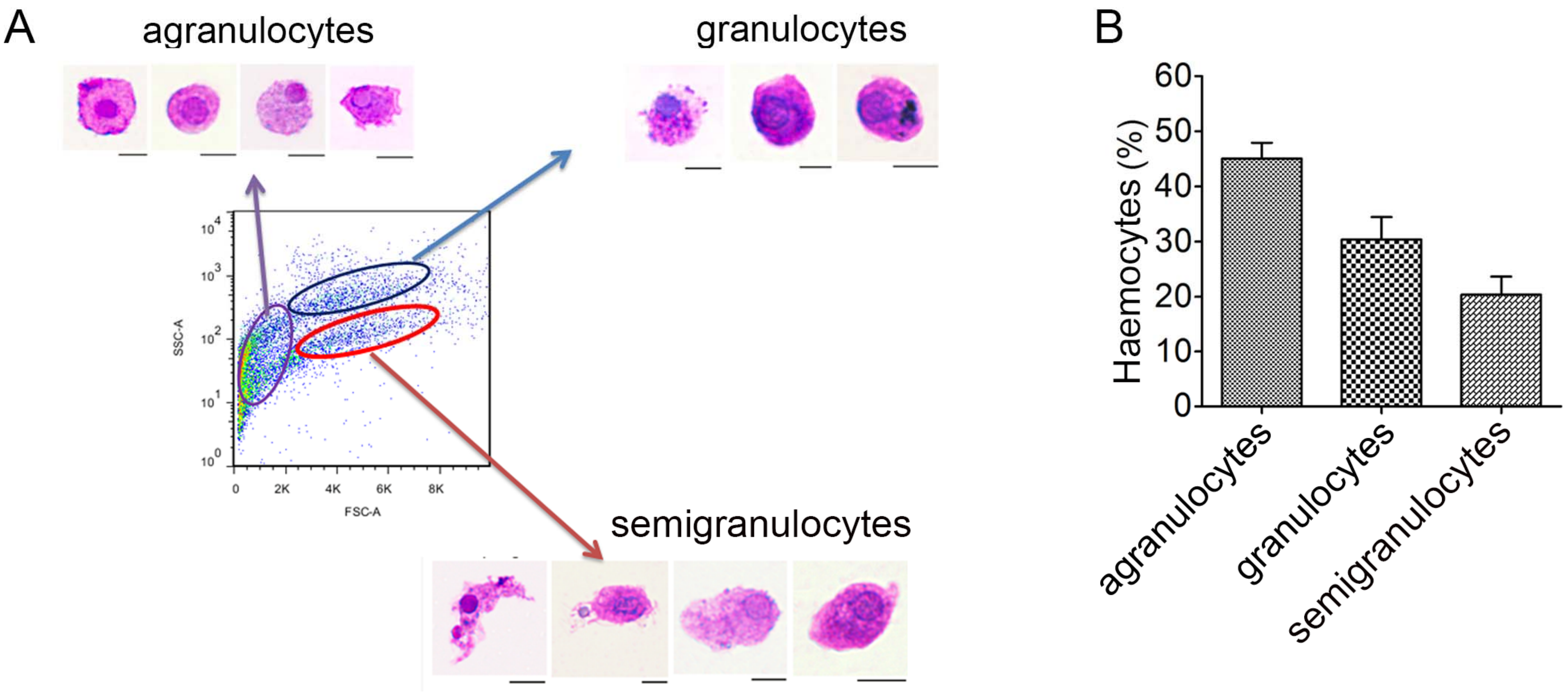

A
agranulocytes
granulocytes
semigranulocytes
SSC-A
FSC-A
0
2K
4K
6K
8K
B
Haemocytes (%)
60
50
40
30
20
10
0
agranulocytes
granulocytes
semigranulocytes

Figure 2(on next page)

Figure 2

Morphological identification of phagocytes from *C. gigas*. (A) Haemocytes were incubated with fungal cells *Pichia pastoris* to allow phagocytosis, followed by MGG staining and microscopic analysis. The fungal cells were indicated with asterisks, N stands for cell nucleus. Bar: 5 μm. (B) Haemocytes pre-incubated with FITC-labeled latex beads (2 μm diameter) were analyzed by flow cytometry.

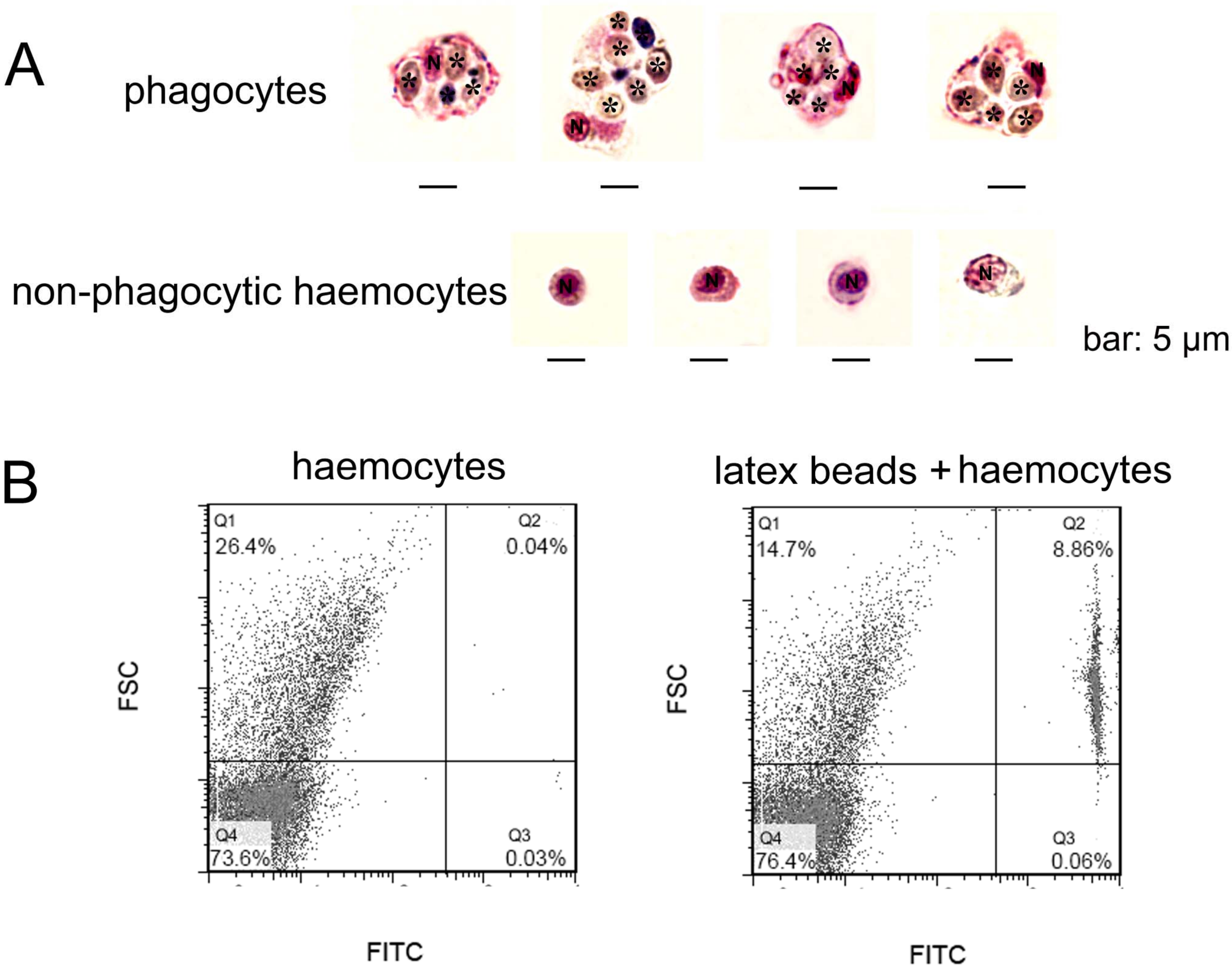

A
phagocytes
N
non-phagocytic haemocytes
bar: 5 μm
B
haemocytes
latex beads + haemocytes
Q1
26.4%
Q2
0.04%
Q4
73.6%
Q3
0.03%
Q1
14.7%
Q2
8.86%
Q4
76.4%
Q3
0.06%
FSC
FITC

Figure 3 (on next page)

Figure3

The involvement of β-integrin in phagocytosis towards different microbes. Haemocytes were treated with or without RGD tripeptide, and the phagocytic activities towards *E. coli*, *V. splendidus* and *S. aureus* were determined by flow cytometry (A, C and E). The statistical results of phagocytic percentages were shown respectively (B, D and F). Results are means ± S.D. (n = 6), *$p < 0.05$, **$p < 0.01$.

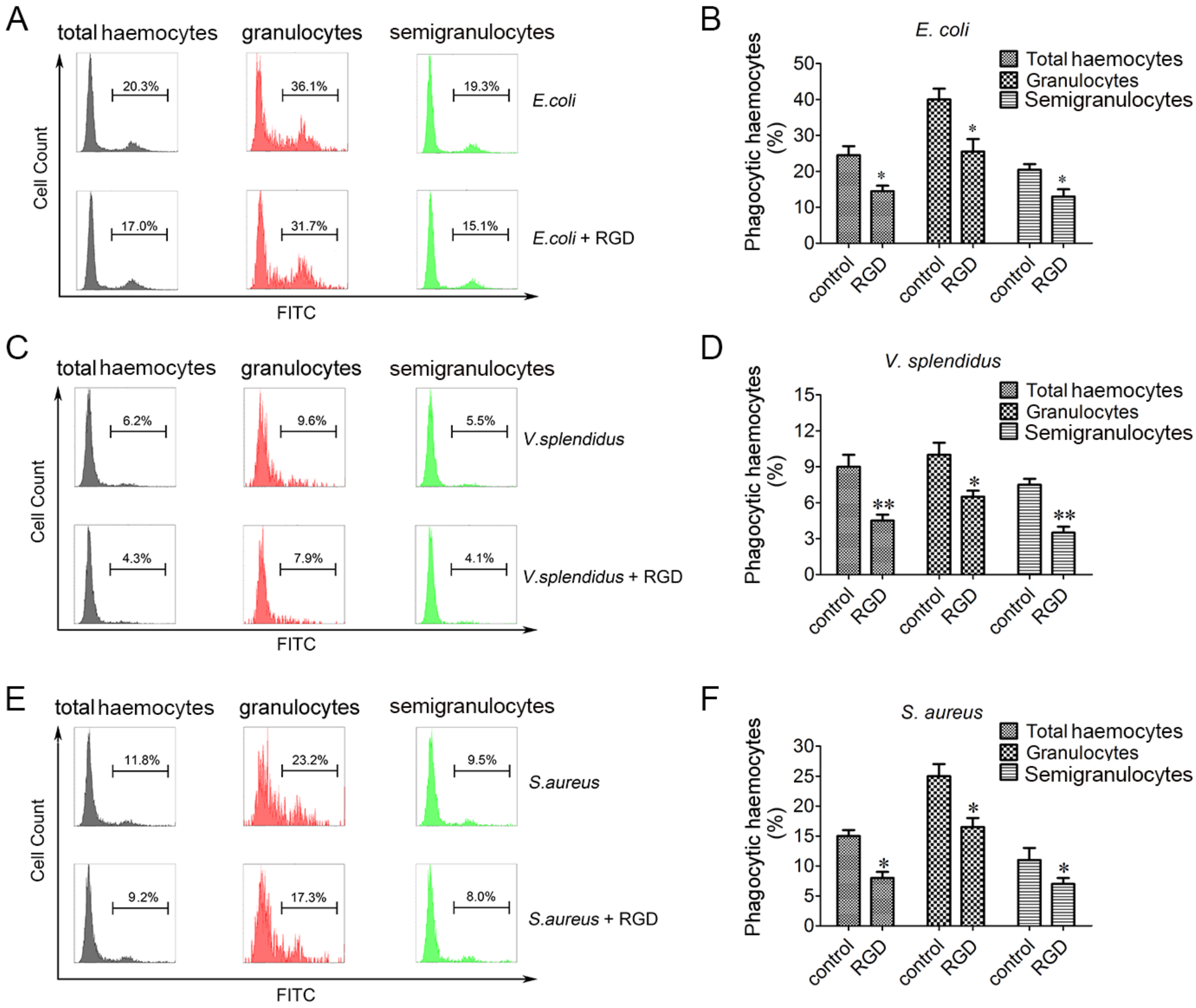

A
total haemocytes
granulocytes
semigranulocytes
20.3%
36.1%
19.3%
E.coli
17.0%
31.7%
15.1%
E.coli + RGD
Cell Count
FITC
B
E. coli
Phagocytic haemocytes (%)
Total haemocytes
Granulocytes
Semigranulocytes
control
RGD
C
6.2%
9.6%
5.5%
V.splendidus
4.3%
7.9%
4.1%
V.splendidus + RGD
D
V. splendidus
E
11.8%
23.2%
9.5%
S.aureus
9.2%
17.3%
8.0%
S.aureus + RGD
F
S. aureus

Figure 4 (on next page)

Figure4

The involvement of lectin-like receptors (LLRs) in the phagocytosis of different microbes. The haemocytes were pre-incubated with different carbohydrates, and the phagocytic inhibitory activity towards *V. splendidus* (A, B, C) and *S. aureus* (D, E, F) were determined. Results are means ± S.D. (n = 6),*$p < 0.05$, **$p < 0.01$.

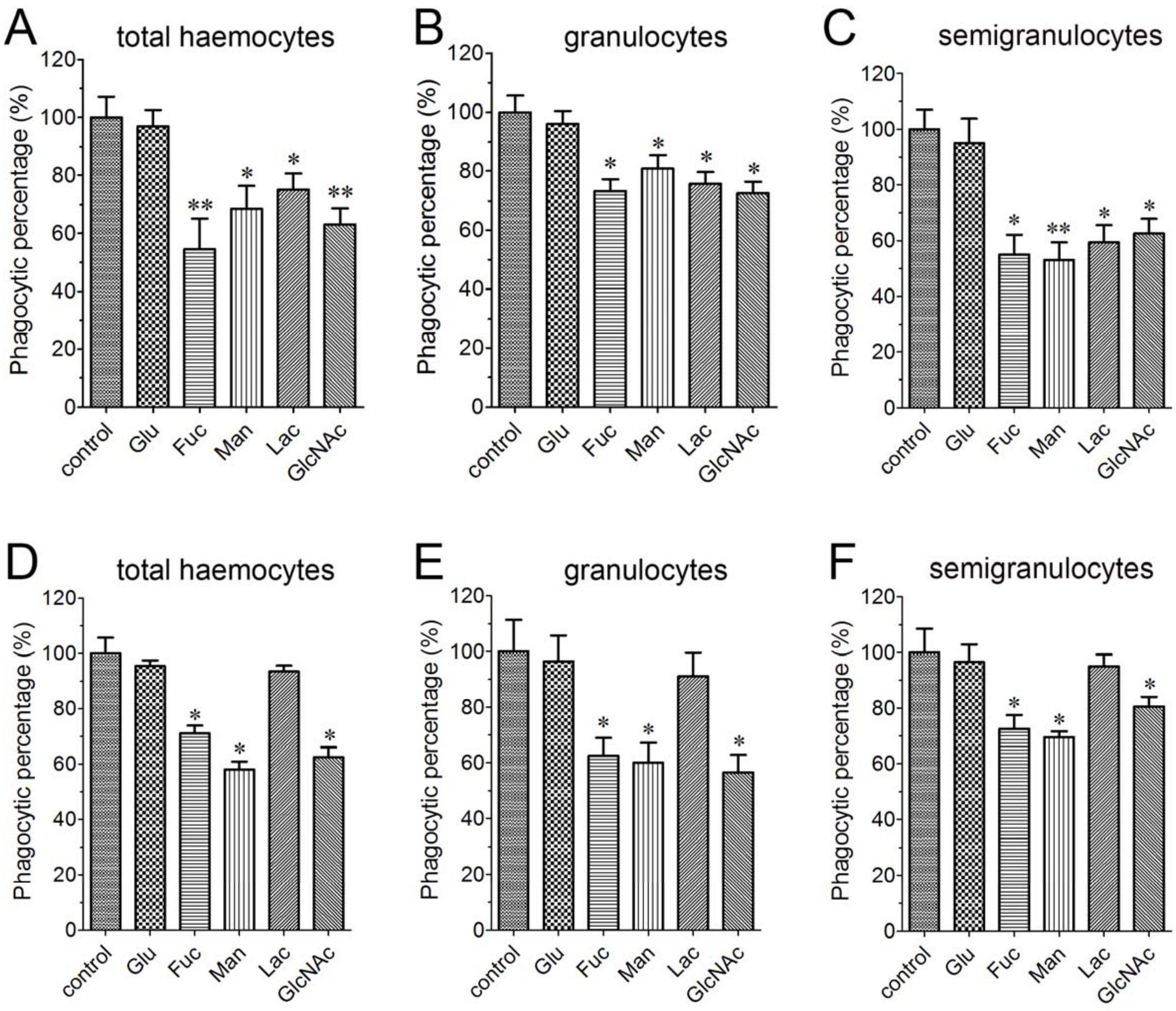

A
total haemocytes
B
granulocytes
C
semigranulocytes
D
total haemocytes
E
granulocytes
F
semigranulocytes
Phagocytic percentage (%)
0
20
40
60
80
100
120
control
Glu
Fuc
Man
Lac
GlcNAc
*
**

Figure 5(on next page)

Figure5

The enhancement of phagocytosis after LPS treatment. The haemocytes were pre-treated with LPS and PGN with different concentrations, and the phagocytic activities towards *V. splendidus* (A, B, C) and *S. aureus* (D, E, F) were determined. Results are means ± S.D. (n = 6), $*p < 0.05$, $**p < 0.01$.

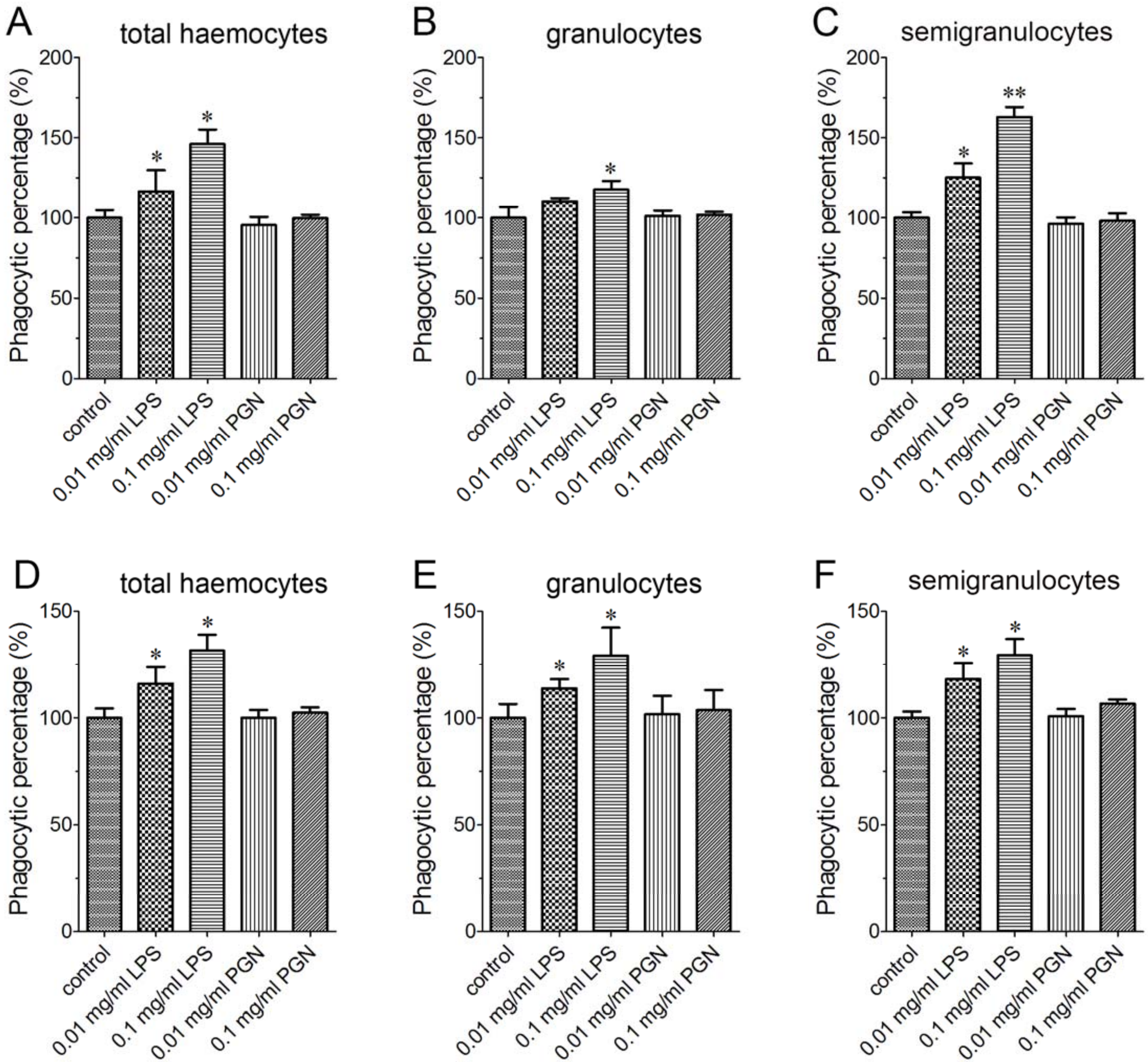

A
total haemocytes
B
granulocytes
C
semigranulocytes
D
total haemocytes
E
granulocytes
F
semigranulocytes
Phagocytic percentage (%)
0
50
100
150
200
*
**
control
0.01 mg/ml LPS
0.1 mg/ml LPS
0.01 mg/ml PGN
0.1 mg/ml PGN

Figure 6(on next page)

Figure6

Lectin staining analysis of the phagocytes from *C. gigas*. (A) Haemocytes were incubated with FITC-labeled *E. coli*, *V. splendidus*, *S. aureus* and *B. subtilis*, and PE-labeled WGA, PNA and LEL were used to stain haemocytes respectively. The correlation between lectin staining and phagocytes was analyzed by flow cytometry. (B) The percentages of haemocytes gated on $PE^+/FITC^+$, $PE^+/FITC^-$ and $PE^-/FITC^+$ were calculated (n = 5).

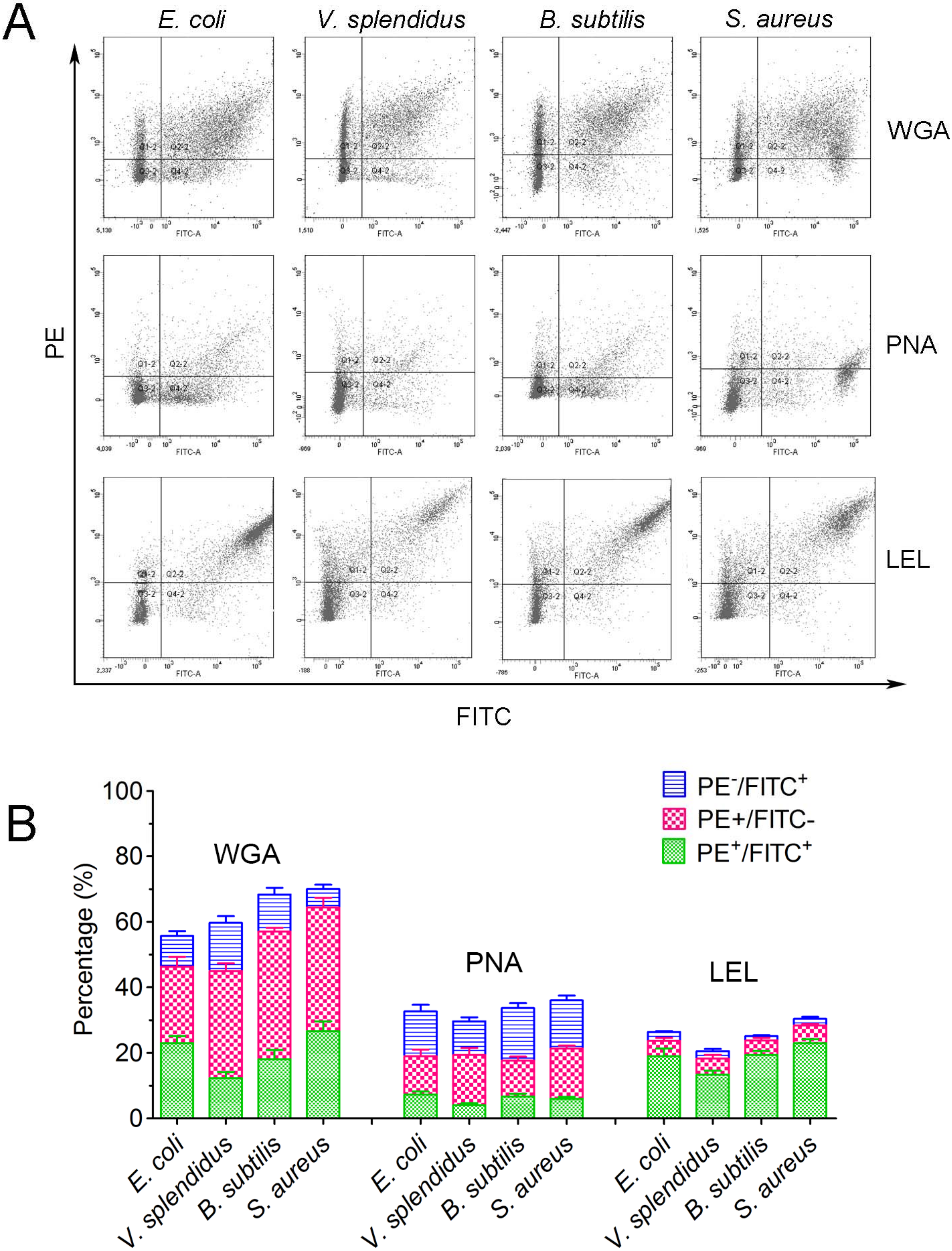
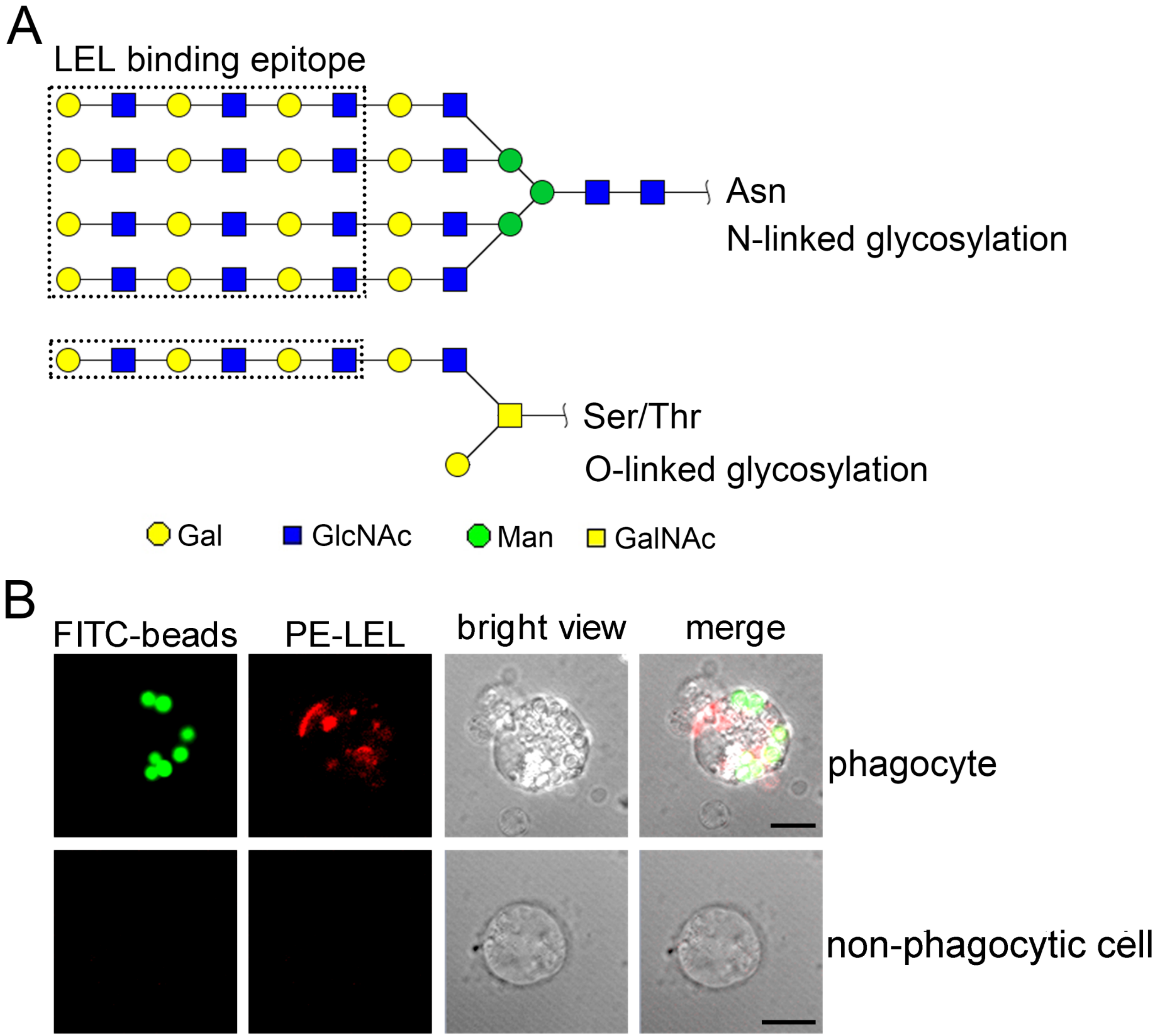

A
E. coli
V. splendidus
B. subtilis
S. aureus
WGA
PNA
LEL
PE
FITC
B
Percentage (%)
100
80
60
40
20
0
WGA
PNA
LEL
PE-/FITC+
PE+/FITC-
PE+/FITC+
E. coli
V. splendidus
B. subtilis
S. aureus

Figure 7(on next page)

Figure7

The distribution of polylactosamine in oyster phagocytes revealed by confocal microscopy. (A) LEL binding epitopes polylactosamine are indicated by dotted rectangles, and the predicted carbohydrate structures are represented in *N*-linked and *O*-linked glycans. (B) Haemocytes were incubated with FITC-labeled latex beads, and then fixed and permeabilized, followed by PE-labeled LEL staining. The representative phagocyte and non-phagocyte were shown.


A
LEL binding epitope
Asn
N-linked glycosylation
Ser/Thr
O-linked glycosylation
Gal
GlcNAc
Man
GalNAc
B
FITC-beads
PE-LEL
bright view
merge
phagocyte
non-phagocytic cell