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# Changes in holothurian coelomocyte populations following immune stimulation with different molecular patterns

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ABSTRACT

Echinoderms possess a variety of cells populating the coelomic fluid; these cells are responsible for mounting defense against foreign agents. In the sea cucumber *Holothuria glaberrima*, four different coelomocyte types were readily distinguished using morphological, histochemical and physiological (phagocytic activity) parameters: lymphocytes, phagocytes, spherulocytes and "giant" cells (listed in order of abundance). Monoclonal antibodies generated against sea cucumber tissues and one polyclonal against sea urchin mayor yolk protein (MYP) were also used to characterize these cell populations. The effects of several pathogen-associated molecular patterns (PAMPs): Lipopolysaccharides from *Escherichia. coli* (LPS), heat-killed *Staphylococcus aureus* (SA) and a synthetic dsRNA were studied on coelomocyte cell populations. PAMPs increased the phagocytic activity of the holothurian coelomocytes, and were able to induce selective immune responses in several of these populations, demonstrating the ability of the sea cucumber to respond to a different variety of immune challenges. Overall, these results show the variety of cells that populate the coelomic fluid of the holothurian and demonstrate their involvement in immune reactions. These animals represent an untapped resource for new findings into the evolution and development of the immune response not only in invertebrates but also in phylogenetically shared reactions with vertebrates.

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## 1. Introduction

The study of echinoderm immunity has a long history, extending from the discovery of cellular immunity in starfish [1] to the groundbreaking work of sequencing the genome of the sea urchin *Strongylocentrotus purpuratus* [2–4]. In the late 19th century, the Russian Nobel laureate Ilya Metchnikoff, discovered the presence of phagocytic cells, when he inserted a foreign body (a rose prickle) into the larva of the starfish *Astropecten pentacanthus* and observed how cells tried to engulf it [1]. Later studies showed the ability of several echinoderm species to differentiate self from non-self tissues through allograft rejection experiments [5,6]. These observations along with studies in other sea urchins, defined the basics of echinoderm immunity, showing the importance of the immune cells and the different roles they play in immunological defense [7,8].

The effector cells of the echinoderm immune system are the coelomocytes; they are the primary mediators of allograft rejection [5,7], response to injury or infection, and the clearance of foreign substances and bacteria [9–13]. In general, morphologically distinguishable cell types have been described in the coelomic fluid of echinoderms [14]. However, not all types are necessarily present in every echinoderm species [15]. Moreover, their classification can be sometimes confusing when comparing different species. Five cellular types have been reported to be present in holothurians, i.e. hemocytes, phagocytes, spherule cells, lymphocytes and crystal cells, as normal components of the coelomic fluid. The first four are thought to be the ones involved in immune reactions [16]. These cell types have been identified and characterized using mainly classical histological methods. Nowadays, the availability of new techniques (e.g. immunofluorescence) represents an important tool to gain insight into the characterization of these cells.

Echinoderm coelomocytes possess different roles, for example, phagocytes as their name implies act as an efficient clearance mechanism due to their recognition, ingestion and efficient degradation of ingested particles [12,17]. They also can produce reactive oxygen species (ROS) inducible by stimulation with non-self materials [18]. During wound healing, they accumulate at the injury site and engulf cellular debris [19]. Both phagocytes and spherule cells appear to be involved in cell clumping and the formation of capsules around ingested particles. It is possible that spherule cells release bactericidal substances, including, lipase,

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peroxidase and serine proteinase [20] that cause the breakdown of phagocytized material [21]. The role of other cell types, however, remains unclear or completely unknown. For example, lymphocytes, have been proposed to be circulating progenitor cells [22]; vibratile cells which are thought to be involved in coelomic fluid movement and clotting reactions [23]; and finally, crystal cells whose role is not yet totally understood [22].

The echinoderm immune system can recognize a diverse set of non-self molecules as determined by allograft rejection studies [5,6], and their ability to initiate a response when challenged with pathogen-associated molecular patterns (PAMPs). PAMPs represent common molecular features of potential pathogens, like bacteria (lipopolysaccharides for gram-negative bacteria, and peptidoglycan for gram-positive), viruses (dsRNA) or fungi (mannan, chitin), which are recognized by molecules known as pathogen recognition receptors (PRRs) [24,25]. In the sea urchin, several PAMPs have been shown to induce immune responses, e.g. lipopolysaccharides (LPS) [26], β1-3-glucan and dsRNA can induce the expression of the 185/333 transcripts, a well-known family of sea urchin immune response genes [27].

To understand the proper function of the immune response in other non-echinoid echinoderms, it is important to determine their coelomocyte composition. The present study shows a first approach towards the characterization of the immune responses of the sea cucumber *Holothuria glaberrima*. Using classical histological methods and immunological markers we have identified several populations of coelomocytes and shown how they change upon stimulation with three PAMPs, i.e. *Escherichia coli* LPS, heat-killed *Staphylococcus aureus* (SA) and dsRNA. We have found distinct coelomocyte populations that can be recognized using specific markers and have defined the response of these populations to the different PAMPs. Our data increases the available knowledge of echinoderm immunity and shows a hitherto unexpected level of complexity.

## 2. Materials and methods

### 2.1. Animals

Adult sea cucumbers 10–12 cm long (*H. glaberrima*) were collected from the rocky shores of northeastern Puerto Rico (18°28′12.23“N, 66°7′8.99”W). Animals were kept in seawater aquaria at 20–24 °C for a week for acclimation before initiating the experiments.

### 2.2. Coelomocyte preparation

Coelomocytes were collected into ice-cold calcium- and magnesium-free artificial seawater with 30 mM EDTA and 50 mM Imidazole pH 7.4 (CMFSW-EI) as described previously [28–30]. Cells were centrifuged at 450 g for 10 min at 4 °C then washed twice with 5 ml CMFSW-EI and fixed in 4% paraformaldehyde diluted in CMFSW-EI for 15 min. Fixative was removed by pelleting cells and washing them twice with CMFSW-EI. Fresh coelomocyte preparations were also used to observe cell morphology of live cells. Briefly, 10 ul of freshly collected coelomic fluid was drawn onto a hemocytometer and allowed to settle for 10 min, after which microscopic observations were made.

### 2.3. Immunological challenge

Sea cucumbers were immunologically activated by injections of: 0.5 mg of LPS from *E. coli* (L2630, Sigma, St Louis, MO), $1 \times 10^9$ cells of heat-killed *S. aureus* or 6.5 ug of dsRNA. Each PAMP was diluted in filtered seawater to a final volume of 100 uL. Double stranded RNA was prepared with the MEGAscript® kit (Ambion/Applied biosystems, Austin, TX) using an empty pBluescript plasmid as a template to generate a 400 bp dsRNA, plasmid sequence and the primers used to amplify this region are presented in Supplementary material S1. Control animals were injected with equivalent volumes of filtered seawater. Three animals were used per treatment.

### 2.4. Phagocytosis assays

Phagocytic activity (PA) was determined as described in a previous publication [28]. Briefly, 48 h after the injection of the PAMPs, the animals were injected with 500 uL of a 1/1000th dilution of fluorescent beads (Polysciences, Warrington, PA) in filtered seawater. Animals were then left undisturbed for 2 h, after which coelomocytes were extracted as mentioned in Section 2.3. The phagocytic activity (PA) was determined by dividing the number of cells with incorporated fluorescent beads by the total number of cells and multiplying by 100. *T*-tests were conducted to determine the statistical significance of the difference between control and experimental animals.

### 2.5. Morphological studies

Cells were observed under DIC microscopy and classified according to their size and morphology. Cell numbers were determined using a hemocytometer. Morphological classification was done by staining coelomocyte cell spreads with toluidine blue. Additional morphological characterization was also done by staining the cell spreads with fluorescent phalloidin-tetramethylrhodamine B isothiocyanate (TRITC) conjugate from *Amanita phalloides* (cat No. 77418, Sigma, St Louis, MO). Phalloidin binds to filamentous actin, allowing the visualization of the actin cytoskeleton and thus of the overall cellular morphology. Nuclei were stained using DAPI mounting media (buffered glycerol with 2 ug/mL DAPI). Digital micrographs were taken on a Nikon Eclipse E600 fluorescent microscope with FITC, R/DII and DAPI filters and the Metaview software (V 6.0; Universal Imaging) was used for image analysis and processing.

### 2.6. Immunohistochemistry

The immunohistochemical procedures have been described in a previous publication [31]. Briefly, coelomocyte cell spreads were left to air dry for 5 min and then treated with goat serum (1:50) for 1 h, then permeabilized with 0.5% Triton X-100 and incubated overnight in a humid chamber with the corresponding primary antibody. Anti-MYP was kindly donated by Dr. Gary M. Wessel and used accordingly [32]. Monoclonal antibodies were obtained from fusions of spleens from mice immunized with various antigens. The immunogens used were as follows: for Sph1 and Sph4, homogenates of intestinal tissue [33]; Sph2, an acid-extracted and semipurified holothurian collagen fraction [34]; and for Sph3, a Holothurian homeobox peptide [35] coupled to bovine serum albumin. For Sph5, an aqueous extraction of body wall coelomic epithelium of a 7 days regenerating neuromuscular wound [36]. For Coel1, an ethanol-extraction of coelomic epithelium of non-regenerating intestinal tissue, and for Ly1, homogenates of intestinal tissue. Anti-Ly1, anti-Coel1, anti-Sph2, anti-Sph3, anti-Sph4 and anti-Sph5 supernatants were applied undiluted to the cell spreads. The following day, the slides were rinsed in PBS and incubated 1 h with Cy3-conjugated goat-anti rabbit and goat-anti mouse secondary antibodies (1:2000) (Biosource/Invitrogen). Slides were rinsed again in PBS and mounted in buffered glycerol with DAPI (2 ug/ml).

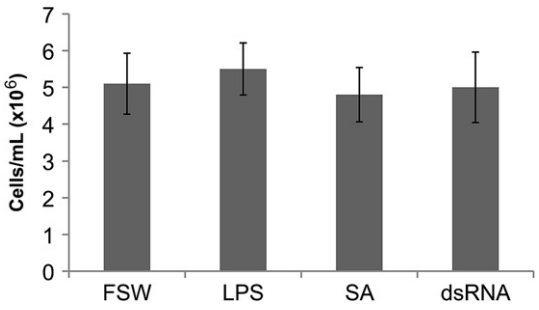


**Fig. 1.** Coelomocyte concentration of sea cucumbers injected either with filtered seawater (FSW) or three different pathogen-associated molecular patterns (PAMPs): lipopolysaccharides (LPS), heat-killed *S. aureus* (SA) and dsRNA. Columns represent averages from three animals. Bars represent the Standard Error (SE).

## 3. Results

### 3.1. Coelomocytes from H. glaberrima

Initial results focused on coelomocyte cell types in *H. glaberrima* and how these might change upon stimulation with different PAMPs. Similar sized animals (10–12 cm approx) were chosen to avoid big fluctuations in coelomic fluid content. On average, animals had a coelomic fluid volume that ranged between 5 and 7 ml with no significant differences among control groups (5.9 ± 0.8 mL mean ± S.E., $n = 3$ animals), LPS-treated animals (6.0 ± 1.0 mL), SA-treated animals (5.2 ± 0.3 mL) and dsRNA-treated animals (6.0 ± 0.5 mL). Animals' coelomocyte content averaged around 5 million coelomocytes per ml and injection of PAMPs did not cause significant changes in their number (Fig. 1).

Three of the five cell types reported in the literature were readily distinguished in our coelomocyte preparations and named according to the terminology used by Endean [22] and Hetzel [16], These cell types were: lymphocytes, phagocytes and spherule cells; a new type of cell not reported previously was also found and named "giant" cells.

#### 3.1.1. Lymphocytes

Lymphocytes were small spherical cells, with an average size of 6.2 ± 0.8 um in diameter (mean ± SD, $n = 10$ cells). They had a large nucleus surrounded by a very thin layer of cytoplasm with no visible organelles or inclusions (Fig. 2A). In control animals they accounted for over 60% of the total cell population. After toluidine blue staining two kinds of lymphocytes were identified: basophilic lymphocytes that had purple-colored nuclei and neutrophylic lymphocytes that had blue-colored nuclei (Fig. 2B).

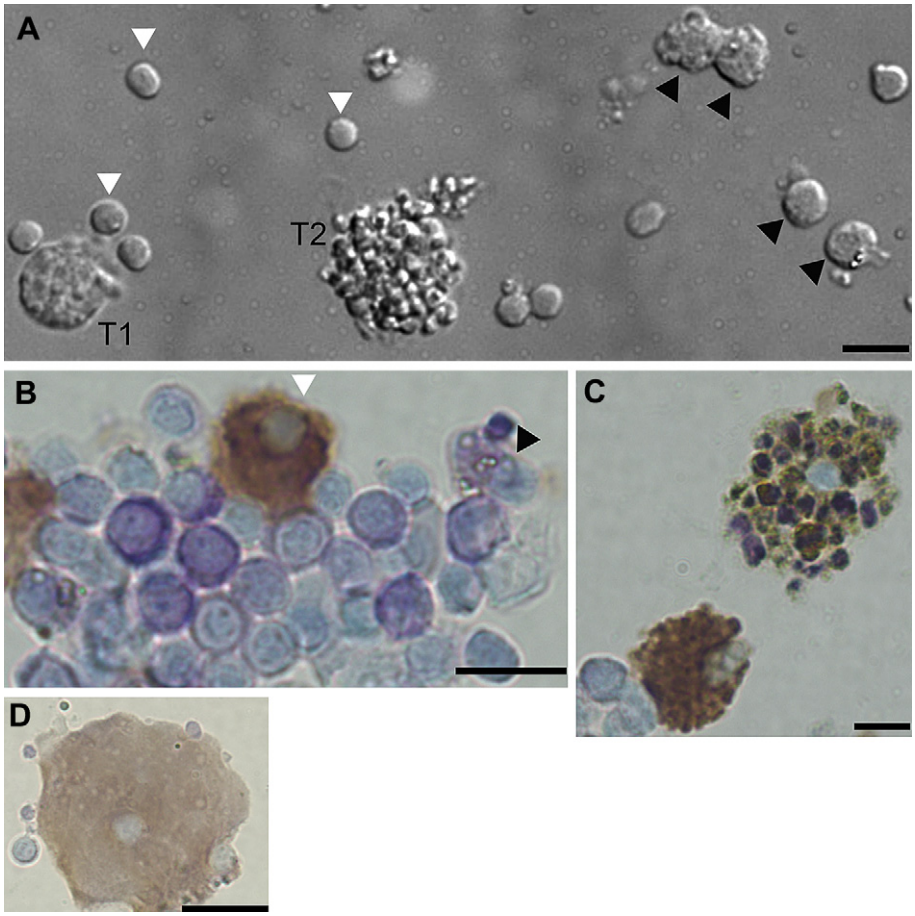


**Fig. 2.** Micrographs of the different cell types found in the coelomic fluid of *Holothuria glaberrima*. A. DIC micrograph of several coelomocyte types. T1 and T2 represent the two types of spherulocytes. White arrowheads show lymphocytes and black arrowheads show the phagocytes. B-D Toluidene blue staining showing (B). a Type 1 spherulocyte (white arrowhead), and lymphocytes both basophilic (purple) and neutrophilic (light blue) and a basophilic phagocyte (black arrohhead), (C) two spherulocytes, (upper right a type 2 and lower left a type 1 spherulocyte) and (D) a giant cell. Scale bars = 10 um.

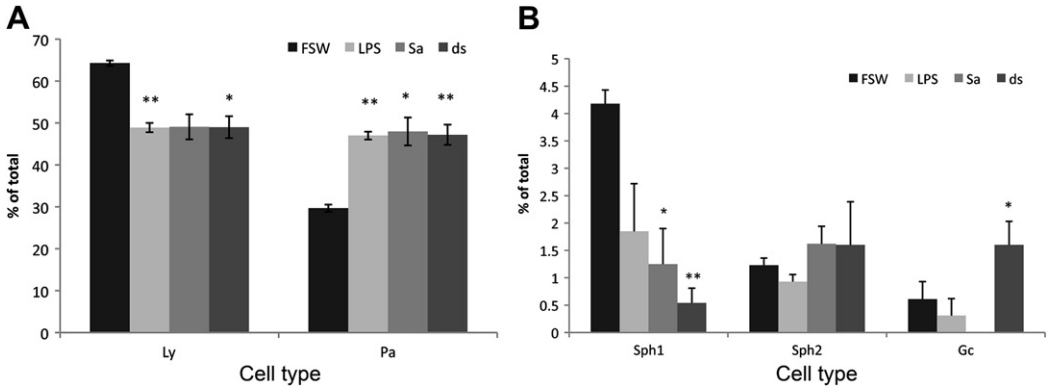


**Fig. 3.** Distribution of coelomocyte cell types in *H. glaberrima* after injection with pathogen-associated molecular patterns (PAMPs). A. lymphocytes and phagocytes. B. Spherulocytes and giant cell types. Numbers represent percentages averaged from three animals for each treatment. Bars indicate the standard error. Asterisks represent *t*-test values for statistical significance of difference between each treatment and controls (*$P < 0.05$; **$P < 0.001$). Sph1: Type 1 spherulocyte; Sph2: type 2 spherulocyte; Gc: giant cell; Ly: lymphocyte; Pa: phagocyte. FSW: filtered seawater; L: LPS; Sa: Heat-killed *S. aureus*; ds: dsRNA.

### 3.1.2. Phagocytes

Phagocytes were 10.8 ± 2.3 um in diameter ($n = 10$ cells), with round or irregular shapes (Fig. 2A). These represented almost 30% of the total cells in control animals. When stained with toluidine blue, two types of cells were also found: basophilic phagocytes with purple-stained cytoplasm and neutrophylic phagocytes with clear cytoplasm where only the nuclei were colored blue. Phagocytes, as their name imply, and as will be shown in Section 3.3, engulf foreign particles.

### 3.1.3. Spherule cells

The spherule cells (spherulocytes) were spherical cells whose size averaged 13.8 ± 2.7 in diameter ($n = 10$ cells) (Fig. 2A). Each contained a variable number of tightly packed spherules in their cytoplasm forcing the small nucleus to an eccentric position. Under the light microscope, these spherules appear translucent or with a dark tint. Two type of spherulocytes were identified: Type 1 had spherules that were clearly round in shape and their spherules were enclosed within the cytoplasm; and Type 2, which had spherules that "protruded" through the plasma membrane giving the cell a "morula" shape. These two types of spherule-containing cells comprise approximately 5% of the cells observed in control animals, being the type 1 the most abundant (4% of the cell population) and the type 2 the most rare (1% of cell population). When stained with toluidine blue, these two cell types stained differentially: type 1 spherulocytes stained uniformly from pink to deep red and type 2 stained heterogeneously with their internal spherules colored in various colors that could include yellow, green and violet (Fig. 2C).

### 3.1.4. Other cell types

The fourth type of coelomocyte was a large type of cell, 21.7 ± 0.7 um in diameter ($n = 7$ cells) referred here as "giant" cell. This cell type showed a flattened circular shape with a centrally located nucleus and large amount of cytoplasm, giving the cell the aspect of a "fried egg" (Fig. 2D). This cell type was the least common, making up roughly 1% of the total cells. Under light microscopy the cytoplasm of these cells appeared clear with no visible inclusions or vesicles, and when stained with toluidine blue the cytoplasm appeared uniformly pink with the nucleus stained blue.

Two other types of cells were also present in control animals but were only observed on freshly drawn live coelomocyte preparations. These were: vibratile cells, which had an average size of 7.6 ± 0.4 um in diameter ($n = 5$ cells), had a spherical shape with a moving flagellum, that made them move in circles in the preparations. The other cells were crystal cells; these were small cells with a rhomboidal crystal inclusion in their cytoplasm, with an average size of 5.4 ± 0.8 um in diameter ($n = 5$). Together, these two cell types roughly represented less than 0.5% of observed cells. No further data for these cells was included here, because they were not common and were not evaluated in PAMP-activated animals.

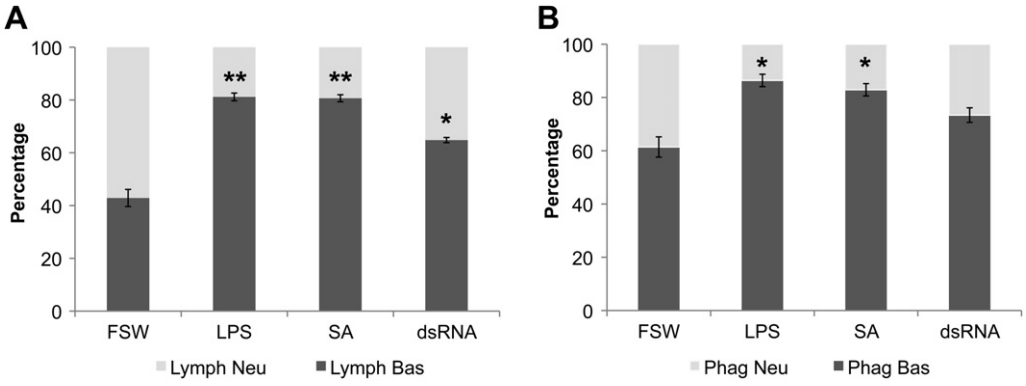


**Fig. 4.** Percentage of toluidine-stained basophilic and neutrophilic lymphocytes (A) and phagocytes (B) in animals treated with different PAMPs. Numbers represent percentages averaged from three animals for each treatment. Bars indicate the standard error. Asterisks represent *t*-test values for statistical significance of difference between each treatment and controls (*$P < 0.05$, **$P < 0.01$). FSW: filtered seawater; L: LPS; Sa: Heat-killed *S. aureus*; ds: dsRNA.

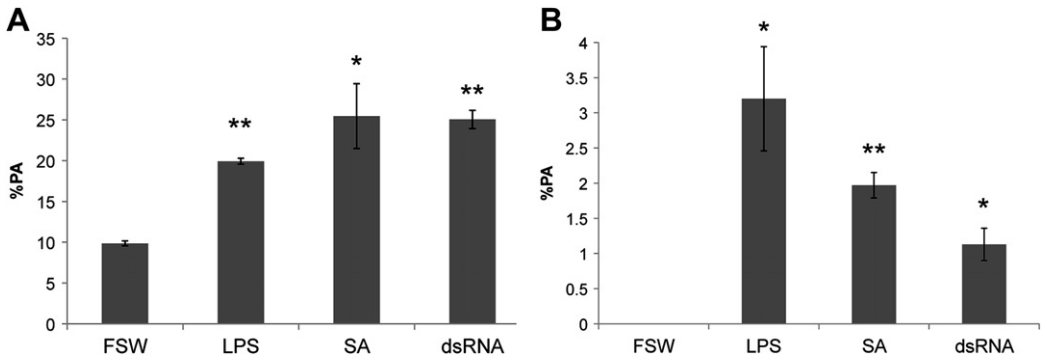


**Fig. 5.** A. Total Phagocytic Activity (PA) of *H. glaberrima* coelomocytes after treatment with three PAMPs. B. Phagocytic activity of lymphocytes. Values shown are averages of PA of three animals per treatment. Bars indicate mean ± standard error (SE). Asterisks indicate *t*-test values when compared against control animals ($^{*}P < 0.05$, $^{**}P < 0.01$). No significant differences were found between treatments. FSW: Filtered seawater; LPS: lypopolysaccharides; SA: heat-killed *S. aureus*.

### 3.2. Changes in cell populations after challenge with PAMPs

Cell types were counted to determine if their numbers changed depending on the PAMP challenge. The three treatments showed significant statistical differences when compared to control animals ($P < 0.05$). In general, PAMP injection affected all cell types composition; the most evident change was in the lymphocyte and phagocyte numbers. Interestingly, while lymphocyte numbers decreased after all three PAMP treatments, phagocytes increased (Fig. 3A). Lymphocytes went from 65% in control animals to around 50% after the three treatments. On the other hand, phagocytes increased from 30% in control animals to 50% approximately in the PAMP-treated animals. The type 1 spherulocyte population also changed, decreasing to almost half (from 4% in control animals, to less than 2% after PAMP treatment). The giant cell population increased significantly ($P < 0.05$) in dsRNA where it reached 1.6%, compared with the 0.6% in control animals. This cell type disappeared in SA-treated animals but was not significantly different from control since giant cells were not found in some control animals (Fig. 3B).

PAMP challenge also caused changes in the number of basophilic lymphocytes and phagocytes (stained with toluidine blue). These numbers increased significantly after challenges. Basophilic lymphocytes increased from around 40% of the lymphocyte population in control animals to up to 80% in challenged animals (Fig. 4A) and basophilic phagocytes increased from 60% of the phagocyte population in control animals to around 80% in PAMP challenged animals (Fig. 4B).

### 3.3. Phagocytic activity

The basal phagocytic activity (PA) of holothurian coelomocytes was measured in control animals (seawater injected) and averaged a mean of 9.9% (S.E. ± 0.54). PAMP injection increased significantly the PA ($P < 0.05$) of phagocytic cells; LPS increased PA to 20% (±0.5), heat-killed *S. aureus* to 24.8% (±4.9) and dsRNA to 25.1% (±1.9) (Fig. 5A). An interesting finding was that while in control animals only phagocytes undergo phagocytosis, in PAMP-treated animals a small percentage of lymphocytes were also observed to phagocytose. These numbers went from 0% in control animals to 3.2%

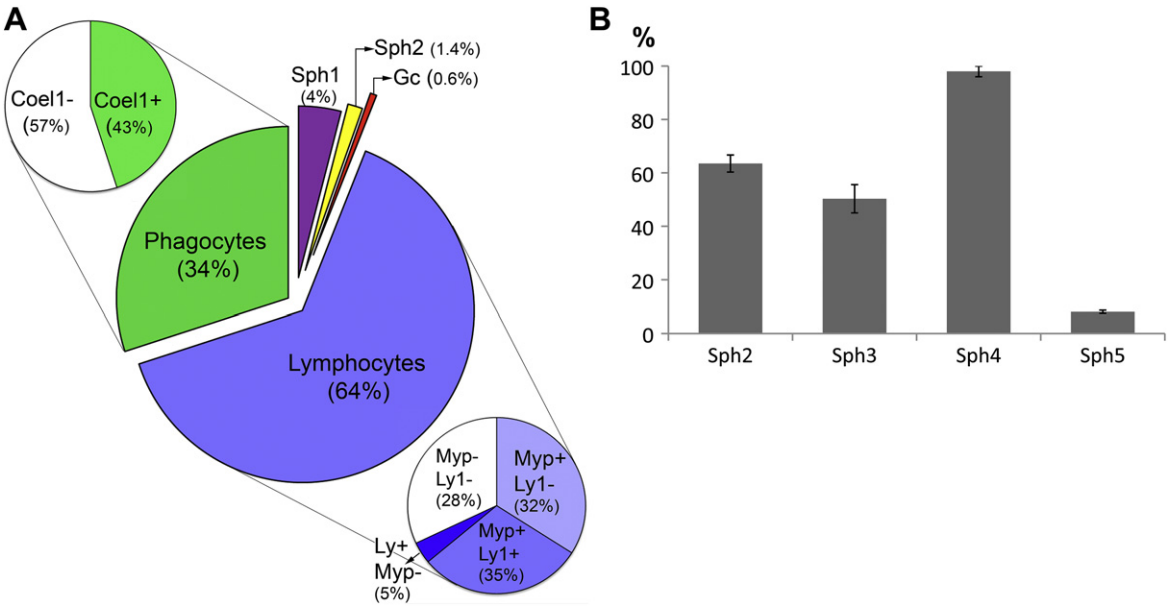


**Fig. 6.** A. Cell population distribution in sea cucumbers injected with filtered seawater. Antibodies labeling the lymphocyte and phagocyte population are also included. Sph1: Spherulocyte type 1; Sph2: Spherulocyte type 2; Gc: Giant cell. Coel1+: Phagocytes positively labeled with the Coel1 antibody. MYP+: Lymphocytes labeled only with the MYP antibody; Ly1+: Lymphocytes labeled only with the Ly1 antibody; MYP + Ly1+: Lymphocytes labeled with both Ly1 and MYP + antibodies. B. Percentages of Type 1 Spherulocytes labeled with monoclonal antibodies. Bars indicate mean ± standard error (SE).

(±0.74) in LPS injected, 2.0% (±0.18) in SA injected and 1.1% (±0.23) in dsRNA injected (Fig. 5B).

### 3.4. Coelomocyte sub-populations as defined by antibody markers

Immune cell types are commonly characterized by the expression of antigenic markers. To determine if different cell populations or sub-populations could be found in *H. glaberrima*, we used six monoclonal antibodies produced in our laboratory against holothurian tissues. Four of these antibodies labeled the spherulocyte population (Sph2 to 5) labeling only Type 1 spherulocytes, the other two labeled phagocytes and/or lymphocytes. A distribution of the cell types found in the coelomic fluid of the holothurian is shown in Fig. 6A together with the proportion of lymphocytes, phagocytes and spherulocytes labeled with these antibodies.

The antibody Sph2 labeled 63.2% (±3.2) of type 1 spherulocytes (mean ± SE $n = 3$ animals), Sph3 50.3% (±5.3), Sph4 98% (±2) and Sph5 8% (±0.6) (Fig. 6B). These antibodies clearly labeled the internal vesicles/spherules inside the cytoplasm, each one in slightly different ways. For example, Sph2 antibody showed a punctuated labeling of the internal vesicles (Fig. 7A) while Sph3 labeled them homogeneously (Fig. 7B). Sph4 and Sph5 labeled the membranes of the vesicles, the first one in a diffuse pattern and the second one being stronger and more defined (Fig. 7C and D).

The remaining two antibodies labeled other coelomocyte populations, i.e. Coel1 mainly labeled phagocytes and Ly1 only labeled lymphocytes. Cells labeled with Coel1 showed a cytoplasmic labeling seen as multiple dots dispersed in the cytoplasm (Fig. 8A). This antibody labeled 13% (±0.1) of total coelomocytes, which corresponds to 43% of the phagocyte population. In phagocytosis

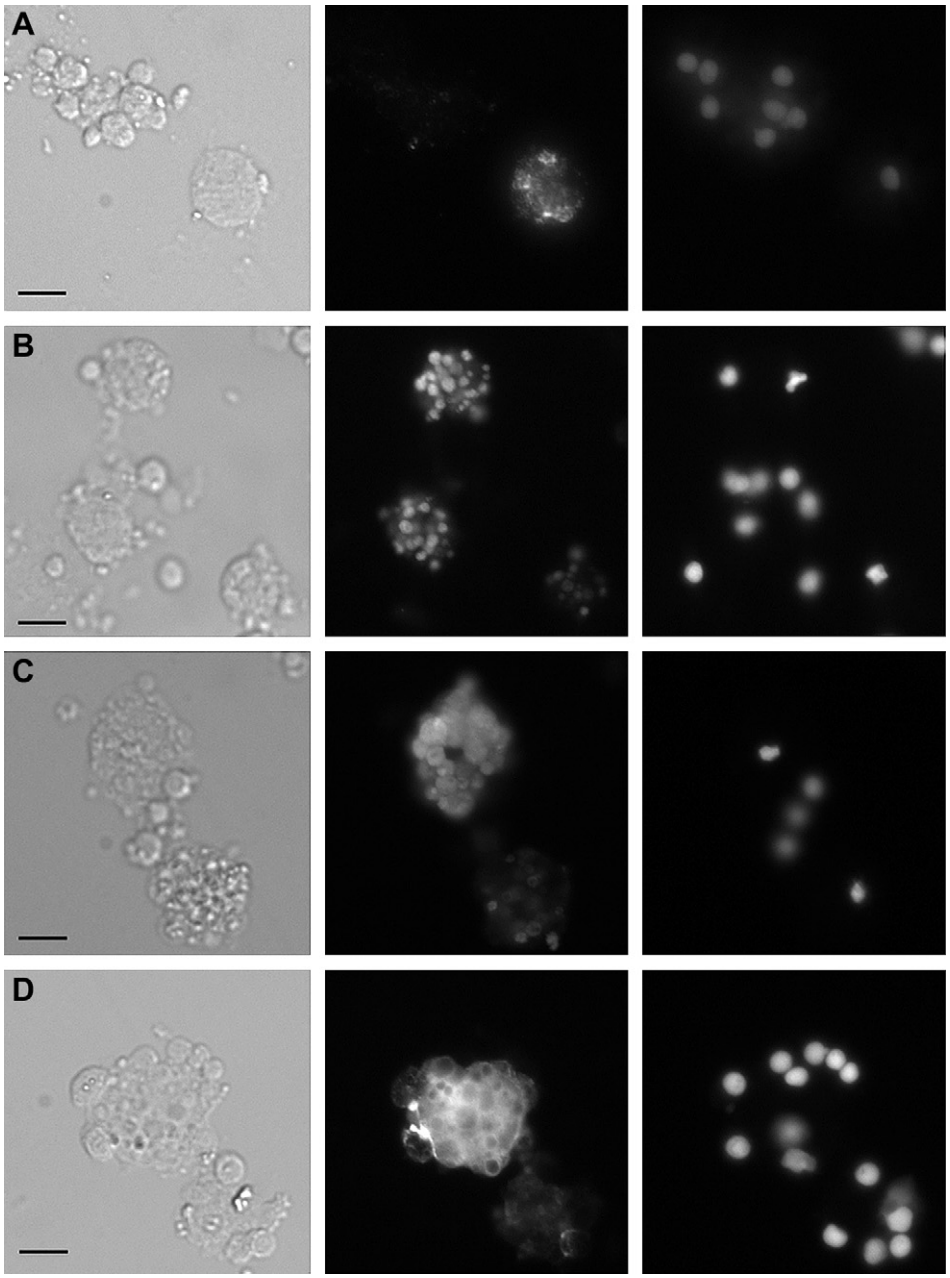


**Fig. 7.** Spherulocytes labeled with monoclonal antibodies. A. Sph2. B. Sph3. C. Sph4. D. Sph5. Left panel are DIC micrographs of spherulocytes, middle panel shows the immunofluorescence and right panel shows the nuclei as stained with DAPI. Scale bars:10 um.

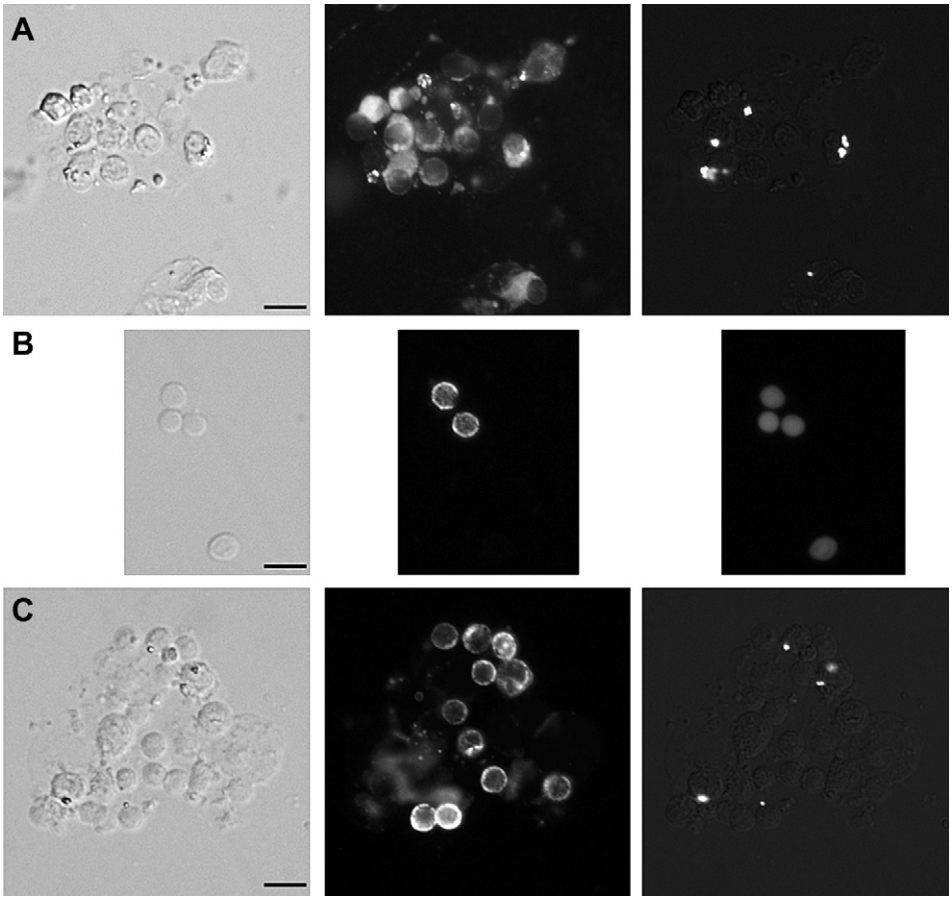


**Fig. 8.** Labeling of phagocytes and lymphocytes with monoclonal antibodies Coel1 (A) and Ly1 (B), and polyclonal antibody against sea urchin MYP (C). Left panel show DIC micrographs of coelomocytes, middle panel show the corresponding antibody channel (Cy3) and right panel the green channel (FITC) to show phagocytosed beads. In B the right panel shows the DAPI channel. Scale bars:10 um.

studies, only 6% percent of the Coel1-labeled cells showed phagocytic activity, suggesting that they correspond to immature or non-activated phagocytes. Ly1 positive cells showed a strong cytoplasmic labeling that appeared as a ring surrounding the large nucleus (Fig. 8B). This antibody labeled 25% (±1.6) of total cells, representing 40% of the lymphocyte population.

Additionally, a polyclonal antibody against sea urchin MYP was also tested on holothurian coelomocytes. This antibody labeled

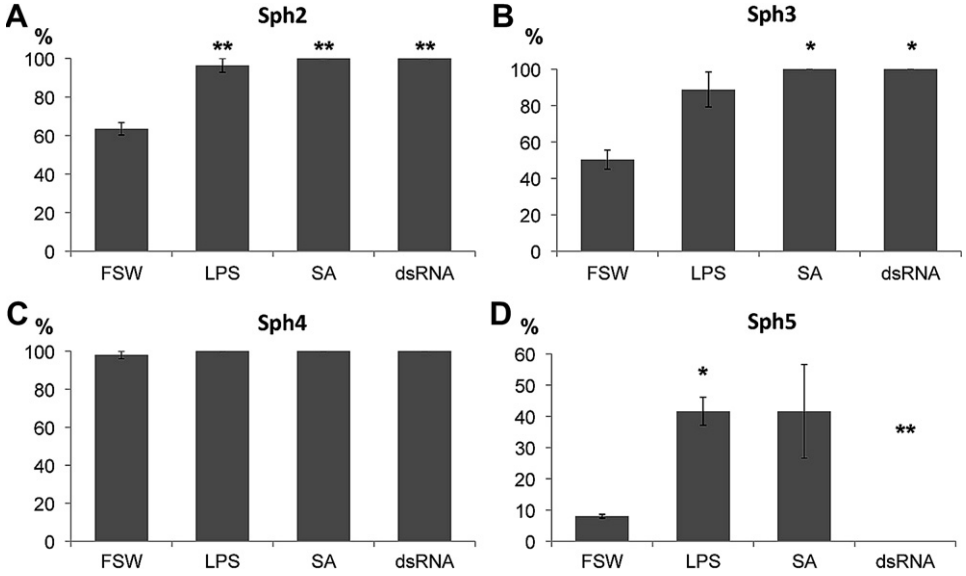


**Fig. 9.** Percentages of type 1 spherulocytes labeled with monoclonal antibodies Sph2 (A), Sph3 (B), Sph4 (C) and Sph5 (D) from animals injected with filtered seawater (FSW), LPS, heat-killed *S. aureus* (SA) and dsRNA. Asterisks indicate significant statistical differences when compared to control animals (seawater) after the *t*-test (*$P < 0.05$, **$P < 0.01$). Bars indicate standard error (SE).

a lymphocyte sub-population, showing a strong cytoplasmic "ring-like" labeling similar to Ly1 (Fig. 8C). This antibody labeled 43% (±1.8) of total cells, accounting for 67% of the lymphocyte population. When double labeling was performed with the Ly1 antibody, 35% of lymphocytes showed double labeling, 5% labeled only with Ly1 and 32% labeled only with MYP (Fig. 6A).

### 3.5. Changes in labeled cell populations after challenge with PAMPs

#### 3.5.1. Spherulocyte markers

Three of the four spherulocyte markers showed changes in the number of cells labeled after challenge with the three different PAMPs. Sph2-positive cells increased significantly ($P < 0.01$) in all three challenges, going from labeling 63.5% in control animals to almost 100% of the spherulocytes in challenged animals (Fig. 9A). Sph3 spherulocytes also increased from 50% in control animals to almost 100% in challenged animals (Fig. 9B). Sph4 continued labeling all S1 spherulocytes after all PAMP treatments (Fig. 9C). For Sph5, the numbers increased from 8% in control animals to 42% in LPS and SA-treated animals but diminished to 0% in dsRNA-treated animals (Fig. 9D).

#### 3.5.2. Lymphocytes

Lymphocytes labeled with MYP decreased significantly ($P < 0.05$) after challenge with PAMPs, going from 43% of total coelomocytes in controls to 30% in LPS, 24% in SA and 21% in dsRNA-treated animals. Additionally a small percentage of these cells (1% approx.) appear to phagocytose in all challenged animals (Fig. 10A). Similarly, Cells labeled with Ly1 also decreased significantly ($P < 0.05$) upon challenge with heat-killed *S. aureus*, with 5% of the cells labeled compared to 25% in controls (Fig. 10B). Other PAMP challenges did not affect the number of Ly1 positive cells.

#### 3.5.3. Phagocytes

Phagocytes labeled with Coel1 increased significantly ($P < 0.05$) after challenges with LPS and heat-killed *S. aureus*, reaching 17% and 20% of the total cells respectively (Fig. 10C). On the other hand, there was a trend (although it was not statistically significantly different from controls) for Coel1-labeled cells to decrease in dsRNA challenged animals (11%). Phagocytic activity of Coel1 positive cells was measured after PAMP challenges, and only showed a significant change ($P < 0.05$) following heat-killed *S. aureus* injection, increasing PA to 9% compared to 6% in the other treatments (Fig. 10C).

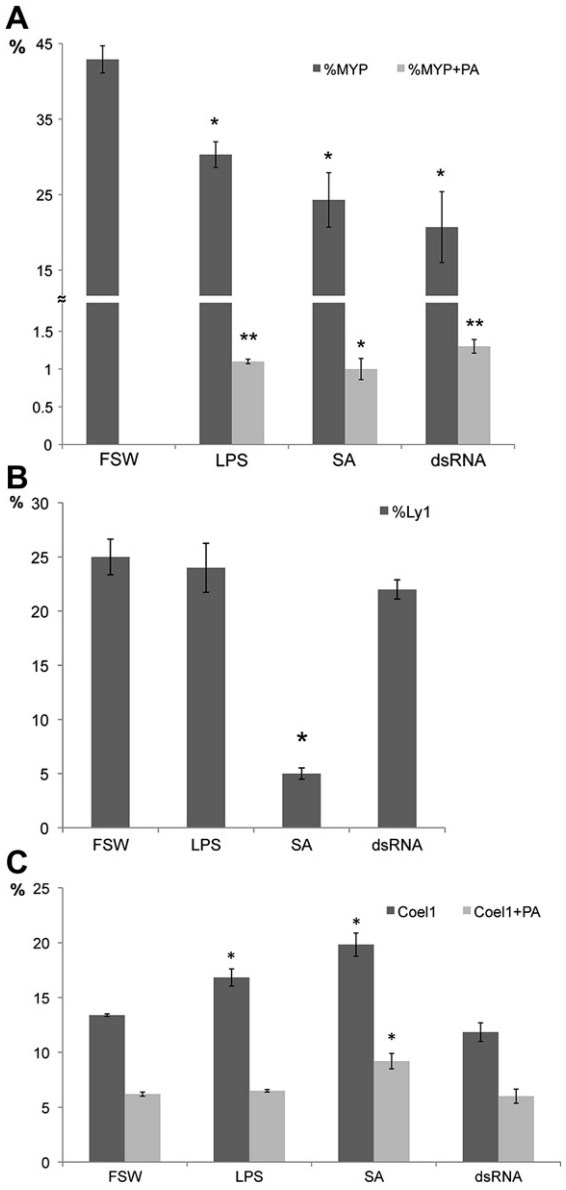


**Fig. 10.** Percentages of cells labeled with three antibodies (anti-MYP, Ly1 and Coe1) from animals challenged with three different PAMPs: filtered seawater (FSW) injection, LPS: lipopolysaccharides (LPS), heat-killed *S. aureus* (SA) and dsRNA. A. Cells labeled with anti-MYP. %MYP + PA column (light grey) represents cells labeled with anti-MYP that showed phagocytic activity. B. Cells labeled with Ly1 antibody. C. Cells labeled with Coel1 antibody. The light grey column represents the cells labeled with Coel1 that showed phagocytic activity (Coel1 + PA). Columns represent averaged percentages from three animals. Bars show the standard error (SE). Asterisks indicate significant statistical differences when compared to control animals (seawater) after the *t*-test ($^{*}P < 0.05$, $^{**}P < 0.01$).

## 4. Discussion

In the present study, we classified the coelomocytes of the sea cucumber *H. glaberrima*, using both morphological and biochemical characteristics (classical histology and immunofluorescence) and by comparisons to descriptions in the scientific literature of other holothurian coelomocytes. The response of these coelomocyte populations to various PAMPs was also determined focusing particularly on changes in their numbers and phagocytic activity.

### 4.1. Coelomocyte populations

The most abundant coelomocyte in the sea cucumber was a cell type similar to the lymphocytes described by Endean [22]. The exact role of these cells is not totally understood and it has even been suggested that these lymphocytes are the progenitor cells of the other coelomocyte types [22]. Three observations from our PAMP challenge experiments lend support to this hypothesis: 1) lymphocyte numbers decrease after challenges; 2) the number of basophilic lymphocytes increase after challenges (we assume that these lymphocytes are more mature and metabolically active) and 3) a small proportion of lymphocytes exhibit phagocytic capacities, an indicative of "maturation" into phagocytes.

The next most abundant cell type in the coelomic fluid of *H. glaberrima* were the phagocytes, these cells correspond to the phagocytes reported by Endean [22] and to the amoebocytes reported by Hetzel [16] in holothurians. They show diverse morphologies and sizes, evidencing their dynamic nature. These cells are actively involved in the immune response, as showed by challenge with PAMPs. First, their numbers increased. Second, their phagocytic responses were enhanced and third there was an

increase in their basophilic staining. Basic stains bind to acid components of the cells, mostly DNA and RNA from the nucleus. But also can bind to other components in the cytoplasm, mostly ribosomes (which are partially made of RNA) and cytoplasmic RNA [37]. Therefore an increase in basophilia suggests an increase in mRNA export and protein synthesis, which can be extrapolated to an increase in cell activity and further increase in metabolism. All these changes are part of an active immune response.

#### 4.1.1. Spherulocytes and giant cells

Less abundant were the spherulocytes, they correspond to the morula cell type described by Endean [22] and Hetzel [38]. These cells have been attributed several roles, including release of bactericidal substances and inflammation [39], formation of brown bodies [40], cell matrix maintenance [41], wound healing [36] and intestinal regeneration [42]. There are no natural color distinctions in *H. glaberrima* spherulocytes, as occurs in sea urchins and some holothurians [22,23], but they differ in cell morphology and histochemical properties [36]. Labeling with different antibodies suggests that these cells may contain different substances in their vesicles as can be deduced by the different staining patterns for each antibody. Additionally, staining with toluidine blue suggests that the two types of spherulocytes differ quite significantly in terms of their chemical properties. A fact that has not escaped our attention is the resemblance of these spherulocytes with vertebrate mast cells. Mast cells play a fundamental role in innate immunity and allergy [43], and when activated by endo- and/or exogenous agents they release their vesicular content, i.e. various pro-inflammatory signal molecules and/or enzymes [44]. In an analogous way, spherulocytes may play a similar role in the sea cucumber defense against pathogens. Evidence of this, are the changes observed in the number of spherulocte type 1 after PAMPs challenges. The reduction of spherulocyte number may be due to the release of the vesicles, therefore the number of "intact" spherulocytes diminish.

A new cell type found in the holothurian was the "giant" cell type, this cell is not described by Endean [22] or Hetzel [16] but a similar-looking cell considered to be a phagocyte enlarged with ingested material was reported in the sea cucumber *Rhabdomolgus ruber*, [45]. This cell type also resembles the sea urchin type 1 discoidal cell reported by Smith et al. [23], with the difference that in *H. glaberrima* this cell type did not show phagocytic activity. In the sea urchin, these cells have been associated with immune function, being able of encapsulation, opsonization, phagocytosis, chemotaxis and graft rejection [23]. Here we showed that this cell type specifically increases after a dsRNA challenge, suggesting a possible role in antiviral responses.

### 4.2. Phagocytic activity

Previous publications of our group showed that LPS effectively increases phagocytic activity in the sea cucumber [28,29]. Foreign substances have been shown to induce phagocytosis in another sea cucumber species [46], as well as in the sea urchin [18,47,48] and the starfish larva [49] and adult [50]. Our results show that in *H. glaberrima*, other PAMPs (gram-positive bacteria and dsRNA) also activated the immune system. These challenges increased the phagocytic activity of coelomocytes indicating that this organism can recognize and initiate an immune response after these stimuli. An interesting effect of these PAMPs resulted in changes in the distribution of cell sub-populations, where lymphocytes decreased and phagocytes increased. This increment in phagocyte population could not be explained by proliferation of this cell type, since echinoderm coelomocytes do not proliferate in great numbers [22,51]. Therefore, these additional cells could represent a portion of lymphocytes that differentiated into phagocytes as suggested by Endean [52] and Hetzel [38], and coincides with the reduction in the number of lymphocytes. However, in our experiments, lymphocytes decreased by 10% while phagocytes increased by 20%, suggesting that an extra input of coelomocytes might come from other sources. According to a study in the starfish, the coelomic epithelium was identified as one of the sources of new coelomocytes, which proliferates upon LPS injection [51], therefore it is possible that the same phenomenon occurs in the holothurian leading to an extra source of coelomocytes. Additionally, the fact that a small proportion of lymphocytes presented phagocytic activity after PAMP challenge may be indicative of some sort of intermediate state of differentiation, in which the progenitor cells are gaining phagocytic capacity before fully completing morphological differentiation into mature phagocytes. Similar transitional states have also been reported in other holothurians where lymphocytes increase in size and produce more complex system of pseudopodia [38].

### 4.3. Responses to PAMP activation

The three PAMPs used in our study represented three different pathogenic responses: LPS, a gram-negative infection; heat-killed *S. aureus*, a gram-positive infection and dsRNA, a viral infection. The results show that the holothurian immune system is responding differentially to the three PAMPs. However, we have to be cautious when comparing the effects since in one case, LPS is a purified molecule that activate the immune system by means of its pattern recognition receptor (PRR) but there are no bacteria to kill or phagocytose, therefore the cellular response is not the same as when bacteria are injected, e.g. heat-killed *S. aureus*. By injecting heat-killed bacteria, the pathogen is recognized and also a cellular response must be mounted to destroy the bacteria and eliminate the debris.

A differential response to specific PAMPs can be attributed to pathways being activated by different PRRs and not a single (or a few) that possess broad-recognition capability. Analyses from the sea urchin genome had shown that it harbors a highly expanded set of innate receptors [3,4], i.e. 222 TLR gene models, 203 NLRs and 218 SRCRs, which makes this type of reactions highly complex. Assuming that the sea cucumber could harbor a similar set of PRRs, this would make them able to respond to a wide variety of PAMPs in a very specific-manner. Studies showing differential responses to LPS and gram-positive bacteria have been documented in echinoderms. In the starfish, challenge with a gram-positive bacteria (*Micrococcus luteus*) decreased coelomocyte concentrations [50], while in the sea urchin LPS increased coelomocyte concentration [53].

Caution must also be observed in interpreting increases or decreases in cell populations. On one hand a cell population might increase upon stimulation with a particular PAMP. But on the other hand a decrease in cell numbers might also occur if a particular population is being recruited away from the coelomic fluid (e.g. into other organs or tissues). This could be the case if the immune response involves the formation of "brown bodies" in which cells group together around the pathogen to isolate it and eventually kill it. Thus, the reduction in Ly1 labeled lymphocytes or spherulocytes, might be due to the formation of these brown bodies, as has been reported previously [54]. A similar depletion of particular cell types is known to occur in the larvae of the greater wax moth, where after injection of a bacterial suspension the number of plasmatocytes (involved in encapsulation and wound healing responses) were depleted from the haemolymph [55].

Finally, our experiments with dsRNA injection prove that inducers mimicking a viral infection can activate the holothurian

immune response. We found this while trying to knock-down holothurian genes through RNAi using a dsRNA injection. A similar phenomenon occurred in the shrimp *Litopenaeus vannamei* and the moth *Antheraea pernyi*, in those studies while trying to induce RNAi they additionally found an antiviral response with partial protection from viral infection [56,57]. Even though we did not test protection from viral infection in the holothurian we did observe an increase in phagocytosis and an increment in a specific type of coelomocyte, which indicates that the animal has started an immune response.

### 4.4. Antibody characterization of coelomocyte sub-populations

Monoclonal antibodies have been used extensively to characterize the epitopes expressed by immune cells and a clear example of this is the CD (cluster of differentiation) system for human leukocytes [58]. This system allows identification of specific cell populations based on the membrane epitopes they express. Human NK markers, as well as new monoclonal antibodies have been used to characterize sea urchin phagocyte populations [59,60]. Conserved epitopes in these cells allow the identification of a cytotoxic population of phagocytes in the sea urchin [59]. Furthermore, monoclonal antibodies specifically produced against sea urchin phagocytes, also allowed the identification of a population of coelomocytes with cytotoxic activity. These antibodies labeled phagocytes and white spherule cells, identifying these populations as the ones involved in cell-mediated cytotoxicity [60]. The antibodies we used in this study allowed for the identification of coelomocyte sub-populations and provided information on how these sub-populations changed after PAMP challenges.

Similarily, our antibodies appear to label sub-populations of cells and in some cases these sub-populations might be related to the cells maturation stage. For example, the Ly1+ sub-population of lymphocytes may represent lymphocytes in early phases of maturation. A signal of this maturation could be the expression of MYP; therefore Ly1+/MYP + cells may represent early stages of maturity and MYP+/Ly1− further mature cells. In an analogous way, Coel1 antibody, might label immature or non-activated phagocytes since only a small percent of these cells showed phagocytic activity.

Antibodies that recognize type 1 spherulocytes, could also be labeling cells at different maturation stages. The Sph4 antibody seems to recognize a molecule present in all spherulocytes. When some PAMPs are present, spherulocyte numbers decreased to almost half, but this decrease might only be specific to only a particular spherulocyte sub-population (or stage) since cells labeled with other spherulocyte antibodies (Sph2 and 3) show a large increase. In other cases the spherulocyte marker can be directly associated with a particular cell population response. This is the case of the disappearance of Sph5 labeled cells after dsRNA injection, which can be specifically linked to an antiviral response. Nonetheless, further characterization of the antigens recognized by these antibodies is needed to determine the relationship between cell populations and maturation stages.

In summary, we have shown that the immune system of *H. glaberrima* is composed of a variety of morphological cell types with different roles and different histo- and immunochemical characteristics. Challenging the holothurian with three different PAMPs can induce activation of their immune responses and promote changes in coelomocyte populations, and phagocytic activity. We have also shown that antibody markers can be useful tools to discern different sub-populations of coelomocytes. This study presents the cellular mediators of the immune response in the coelomic fluid of *H. glaberrima* as an excellent model to study holothurian immune responses and will serve as a base for future cellular and molecular studies of echinoderm immunology.

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## Appendix. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fsi.2010.03.013.

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