Comparison of Phagocytosis in Three Caribbean Sea Urchins

John DeFilippo, John Ebersole, and Gregory Beck*

Department of Biology
University of Massachusetts at Boston
Boston, MA 02125-3393 USA

Running Headline: Phagocytosis in Caribbean Sea Urchins

*Correspondence to: Dr. Gregory Beck
Department of Biology
University of Massachusetts at Boston
100 Morrissey Blvd.
Boston, MA 02125-3393 USA
(617) 287-6619, (617) 287-6650 (*FAX*), e-mail: greg.beck@umb.edu

Abstract

In 1983 large numbers of the sea urchin *Diadema antillarum* unexplainably began showing signs of illness and dying in the Caribbean, and over the next year they came close to extinction, making it one of the worst mass mortality events on record. Present evidence suggests a water-borne pathogen as the etiological agent. Decades later *Diadema* densities remain low, and its near extinction has been a major factor in transforming living coral reefs in the Caribbean to barren algae-covered rock. In the ensuing decades, no solid explanation has been found to the questions: what killed *Diadema*; why did *Diadema* succumb while other species of urchins on the same reefs did not; and why has *Diadema* still not recovered? A recent hypothesis posited by our lab as to *Diadema's* vulnerability was directed at possible compromised immunity in *Diadema*, and experimental results found a significantly impaired humoral response to a key component of gram-negative bacteria. Here we use flow cytometry to examine the cellular arm of invertebrate immunity. We performed cytotoxicity and phagocytosis assays as a measure of the cellular immune responses of cells from *Diadema* and two other species of sea urchins not affected by the die-off. Despite our previous findings of in impaired humoral response, our study found no apparent difference in the cellular phagocytic response of *Diadema* compared to the other urchin species studied.

Keywords:

Sea urchin, *Diadema antillarum*, marine mass mortality, invertebrate innate immunity, cytotoxicity, phagocytosis, flow cytometry

1. Introduction

In the 1980's, the black-spined sea urchin *Diadema antillarum* suffered one of the most extensive mass mortality events on record for a marine invertebrate. *Diadema* was one of the most abundant and ecologically important herbivores on Caribbean coral reefs, maintaining reef-building coralline algae by their grazing of fleshy algae (Carpenter, 1981; Ogden and Lobel, 1978; Sammarco, 1982; 1980; Sammarco and Levinton, 1974). In 1983, *Diadema* began showing signs of illness, and quickly died (Lessios et al., 1984). Over the course of a year the outbreak spread over 3.5 million square kilometers, with 95% mortality and no populations of *D. antillarum* spared (Hughes et al., 1985). No causative agent has been discovered, but the outbreak followed water currents and mortality did not decrease with distance, suggesting a water-borne pathogen (Lessios, 1988). Two species of bacteria capable of killing *Diadema* were associated with dying urchins in the laboratory, but no bacteria have been isolated from wild individuals (Bauer and Agerter, 1987).

No other species of urchins were affected, nor were Pacific *Diadema* populations of *D. mexicanum* near the initial outbreak, suggesting that the etiological agent was a species-specific pathogen. The loss of this keystone grazer facilitated a phase shift from hard coral to algal dominated reefs throughout the Caribbean (Carpenter, 1990; Hughes, 1994), and *Diadema* densities have failed to recover in the ensuing thirty years, remaining more than 85% below their pre-epizootic levels (Lessios, 2015).

Along with *Diadema*, we examined the immune response of two other major cosmopolitan species of sea urchins in the Caribbean Sea (*Tripneustes ventricosus* and *Echinometra lucunter*). The phylogenetic position of echinoderms makes them an important group for comparative immunological studies (Binyon, 1972; Lin et al., 2001). Sea urchins are in the phylum Echinodermata, class Echinoidea (Harvey, 1956; A. B. Smith and Kroh, 2013). The phylogenetic relationships based on 18S-like small and 28S-like large rRNA show the Diadematoida order of *Diadema* to be one of the more basal, with order Carinacea diverging from Diadematoida and radiating into the large Echinacea clade (A. B. Smith and Kroh, 2013). From within Echinacea were splits that included the Toxopneustidae clade that one of our experimental species, *Tripneustes* ventricosus, belongs to, and another branch that later gave rise

91 to the Echinometridae clade of our experimental *Echinometra lucunter* (and still later the
92 Strongylocentrotidae clade) (A. B. Smith and Kroh, 2013).
93 Sea urchins clear pathogens from coelomic fluid (CF) efficiently and CF cells
94 (coelomocytes) mount immune responses (Lin et al., 2001). Progress has been made on
95 deciphering the immune response of sea urchins (Lin et al., 2001; Materna and Cameron, 2008;
96 Rast and Messier-Solek, 2008; Rast et al., 2006; L. C. Smith et al., 2006), and with the
97 sequencing of the genome of the purple sea urchin *Strongylocentrotus purpuratus*, new
98 information will follow (Hibino et al., 2006; Materna and Cameron, 2008; Sodergren et al.,
99 2006). It has been hypothesized that ~5% of the genes in the sea urchin are related to the immune
100 response (Hibino et al., 2006; Rast et al., 2006; L. C. Smith et al., 2006; Sodergren et al., 2006).
101 Innate immunity involves anatomical, physiological, phagocytic and inflammatory
102 barriers deployed before infection, is capable of rapid response to pathogens, and reacts in
103 mostly the same way to repeated infections (Akira et al., 2006; Medzhitov and Janeway, 2002).
104 These innate responses in invertebrates are activated when microbes breach anatomical barriers.
105 Innate immunity exists in vertebrates, invertebrates, and to a certain extent in plants, however for
106 invertebrates it is the only line of defense. Invertebrate immunity is nonspecific [variable
107 immune-like molecules, and alternative anticipatory and memory-like immune mechanisms are
108 found in some invertebrates (Cerenius and Soderhall, 2013)], based on activation of immune
109 effector cells, and is mediated to a large extent by circulating coelomocytes (Bochud et al., 2007;
110 Lin et al., 2001; Medzhitov and Janeway, 2002). Even though innate immunity lacks the
111 elegance of genetic recombination and shows no memory, the view of innate immunity
112 inconsequentiality is out of date (Akira et al., 2006; Medzhitov and Janeway, 2002). As in
113 vertebrates, the innate response of invertebrates has both a molecular (secreted protein) and cell-
114 mediated component (Beck and Habicht, 1996; Lin et al., 2001).
115 Sea urchins (as most invertebrates) have an open circulatory system, and the blood cells
116 of their coelomic fluid are referred to as coelomocytes (Chia and Xing, 1996). Their importance
117 in immunity has been well described (Chia and Xing, 1996; Coffaro and Hinegardner, 1977; de
118 Faria and da Silva, 2008; Mangiaterra and Silva, 2001; Matranga et al., 2005; Silva, 2000; V. J.
119 Smith, 1981). There has not been consistent nomenclature and classification of coelomocytes
120 among types of echinoderms (Boolootian and Giese, 1958; Chia and Xing, 1996; Coulter, 1956;
121 1953; Edds, 1993; Gross et al., 1999; Matranga et al., 2005; 2006; L. C. Smith et al., 2010; V. J.

122 Smith, 1981), but sea urchins are generally described as having at least four types of
123 coelomocytes, with variable population percentages in different species of sea urchins (L. C.
124 Smith et al., 2010). Historically these have often been called phagocytes (sometimes further
125 divided between large and small phagocytes, and also called petaloid/filopodial phagocytes, and
126 phagocytic or bladder amoebocytes), with populations given as between 40-80%, red spherule
127 (or morula) cells (sometimes called pigment cells) (4-40%), colorless or white spherule (or
128 morula) cells (5-25%), and vibritile cells (8-20%) (Bertheussen and Seijelid, 1978; Chia and
129 Xing, 1996; Edds, 1993; Gross et al., 2000; Johnson, 1969; Mangiaterra and Silva, 2001; L. C.
130 Smith et al., 2010; V. J. Smith, 1981; Standerholen et al., 2014; Terwilliger et al., 2004).
131 While often referred to as phagocytic amoebocytes, phagocytic coelomocytes cells have
132 been reported to not be very mobile, and thus 'amoebocytes' may be a misnomer (Matranga et
133 al., 2006; 2005). Conversely, the lack of motion generally reported for red and white cells is
134 thought by some investigators to be due to EDTA in cell preparations, as fresh preparations show
135 them to be so swiftly mobile that these are the cells that should correctly be called amoebocytes
136 (Matranga et al., 2006; 2005). Spherule cells are also reported as not actually being spherical in
137 fresh preparations, and their description as such is likely due to fixatives or anti-coagulants
138 (Matranga et al., 2006; 2005).
139 Phagocytic cells are reported to exist in three forms, large discoidal and polygonal forms
140 responsible for phagocytosis, and a small filopodial form involved in clotting (Brockton et al.,
141 2008; Gross et al., 2000; Majeske et al., 2014; V. J. Smith, 1981), and that possibly these are one
142 cell type that transitions under environmental influences (Edds, 1993; 1985; Henson et al., 1999).
143 Phagocytes are the only cells found to be capable of phagocytosis (Matranga et al., 2006; L. C.
144 Smith et al., 2010), with red and white cells believed to be responsible for releasing humoral
145 components (Matranga, 1996; Matranga et al., 2006; 2005; L. C. Smith et al., 2010). Some
146 reports state phagocytes are also the only cells capable of clotting (Matranga et al., 2006), while
147 others suggest white cells may also be involved (L. C. Smith et al., 2010).
148 Phagocytosis (the engulfment of foreign material by specialized phagocytic cells) is the
149 predominant cellular defense mechanism in vertebrates and invertebrates (Beck and Habicht,
150 1996; Cooper, 1976; Marchalonis and Schluter, 1990a). In his studies of invertebrates in the late
151 19th century, Metchnikoff established the role of phagocytes in host defense, studies that began
152 the field of cellular immunology (Beck and Habicht, 1996). Some of his earliest studies were on

153 the response of echinoderm phagocytes to injury. He proposed that all animals use phagocytosis
154 as a general defense mechanism (Beck and Habicht, 1996; Binyon, 1972; Flajnik and L. Du
155 Pasquier, 2004).
156 Phagocytes and cytotoxic cells have been reported in most every invertebrate phylum
157 (Beck and Habicht, 1991; Beck et al., 1993; Blanco et al., 1997; Franceschi et al., 1991).
158 Bertheussen showed that coelomocytes of the sea urchins *Echinus esculentus* and *S.*
159 *droebachiensis* were cytotoxic towards allogenic coelomocytes (Bertheussen, 1979). Hemocyte-
160 mediated cytotoxicity may involve ROI and/or lysosomal enzymes (Peddie and V. J. Smith,
161 1994). We have shown that coelomocytes from the purple sea urchin (*Arbacia punctulata*)
162 exhibit cellular cytotoxic activity against vertebrate target cells *in vitro* (Lin et al., 2001). We
163 have also demonstrated that phagocytic amebocytes are the effector cells of the cytotoxic activity
164 (Lin et al., 2001). In addition, we have shown that these cells are phagocytic and can be
165 stimulated to release host defense molecules (Beck et al., 1993; Lin et al., 2001).
166 Previously we asked whether an impaired immune response could be responsible for the
167 susceptibility of *Diadema* to some pathogen. In that study, we employed several assays (reactive
168 oxygen and nitrogen intermediate generation, phenoloxidase responses, release of antimicrobial
169 peptides, and iron sequestration) to investigate the humoral immune response of the 3
170 cosmopolitan sea urchin species (*Diadema, T. ventricosus* and *E. lucunter*). We found that
171 *Diadema's* humoral response to LPS, a component of gram-negative bacteria, was absent (Beck
172 et al., 2014; 2008). In the present study, we examine *Diadema*'s cellular immune response by
173 assessing cytotoxicity and phagocytosis using flow cytometry.
174
175
176 **2. Materials and Methods**

177 2.1. *Harvesting of sea urchin coelomocytes*

178 For testing cellular immune responses (phagocytosis and cytotoxicity), coelomic fluid
179 (CF) was drawn from sea urchins (thirty *D. antillarum,* five *E. lucunter,* and four *T. ventricosus*)
180 collected from seven study sites on St. Croix (Beauregard Bay, Butler Bay, Cramer's Point,
181 DIVI, Gentle Winds, Grassy Point, Sprat Hole) (Beck et al., 2014; 2008). The soft tissue surrounding
182 Aristotle's lantern was pierced with a 20-gauge needle and 5-8 ml CF was drawn into a 10-mL

183 syringe containing 4 mL of ice-cold anticoagulant [Marine Alsever's solution (0.12M glucose,
184 0.03M sodium citrate, 9mM EDTA, 0.38M NaCl), (pH 7.4)] (Bachere et al., 1988; Bowdish and
185 Gordon, 2009; Sarrias et al., 2004; Whelan et al., 2012). All sea urchins recovered successfully
186 from CF extraction and were returned to the sites they were taken from. Collected CF was used
187 within 2 h of collection.

188

189 2.3. *Phagocytosis assay*

190 The coelomocytes were diluted to yield a concentration of ~1x10^6/ml with DMEM. We added 20
191 µl fluorescently labeled latex beads (1:100 in seawater) [Fluospheres (Carboxylate 1.0 µm,
192 yellow-green) Molecular Probes)] with excitation/emission maxima of 505/515 nm, to 1.0 ml
193 cells (these beads have not been associated with any PAMP-like molecules.). Either 10 µl
194 (1mg/ml) of a known immune stimulant: laminarin [from *Laminaria digitata* (Sigma L9634)],
195 lipopolysaccharide (LPS) [from *Escherichia coli* 0111:B4 (Sigma L2630)], or peptidoglycan
196 (PGN) [from *Bacillus subtilis* (Sigma 69554)], or sea water (as control) were then added (Beck et
197 al., 2014; 2008). The samples were incubated for 3 h at 25°C, and then centrifuged at 5,000 x g
198 for 5 min to pellet the cells. The cells were re-suspended in 1% formalin in 1X PBS and kept at
199 4° C until analyzed. Representative experimental treatment (or control) was checked using
200 Trypan blue to assess coelomocyte viability. Only cells with viabilities of >95% were used.
201 Phagocytic activity was calculated as the number of coelomocytes that had ingested
202 particles relative to the total cell population viewed under 1,000× phase contrast microscopy as
203 we have described previously (Beck et al., 1993; Raftos et al., 1992a). A minimum of 100 cells
204 were counted. Experiments were carried out double-blind. Data are presented as the
205 phagocytic stimulation index (P.S.I.) such that:

206

207 $$\text{P.S.I.} = \frac{\%\ \text{phagocytic cells in experimental trials}}{\%\ \text{phagocytic cells in controls incubated with seawater alone}}$$

208

209

210 Mean values for each trial were calculated from P.S.I. for individual replicates.

211

212

213

214

215

2.4. *Fluorescent Microscopy*

Cells exposed to fluorescently labeled beads were viewed under a Nikon Eclipse E200 microscope with 100-watt mercury lamp and a GFP filter. Photographs were taken with a clamp-mounted Canon PowerShot G10 connected to Breeze Systems PSRemote software.

2.5. *Phagocytosis Analysis by Flow Cytometry*

Samples (n = 4) were analyzed using flow cytometry on a Beckman Coulter Cell Lab Quanta SC-MPL with a 488 nm laser, software version 1.0. The Quanta SC flow cytometer employs the Coulter Principle (Coulter, 1956; 1953) to measure cell size by electronic volume (EV) rather than FSC (Graham, 2003; Hynes, 1992). EV has been found to be a more accurate measure of cell size (Krishan and Cabana, 2004; Standerholen et al., 2014). QC was first performed using Beckman Coulter Flow-Check Fluorospheres. Sample collection was set for 100,000 cells. To quantify phagocytosis of fluorescent beads, fluorescence was measured using the 525BP FL1 filter. Representative experimental treatment (or control) was checked using Trypan blue to assess coelomocyte viability. Only cells with viabilities of >95% were used. A cell sorter was not employed to separate individual cell populations. Variation in phagocytic uptake between individual cells was not analyzed.

2.6. *Analysis of Flow Cytometry Data*

Data analysis was done in R version 3.2.1 (R Core, 2015) on an x86_64-apple-darwin14.3.0 (64-bit) platform. Bioconductor version 3.1 (Gentleman et al., 2004) flow cytometry packages used in the analysis and plotting were flowCore version 1.34.3 (Ellis et al., 2015), flowViz version 1.32.0 (Ellis et al., 2008), and flowDensity version 1.2.0 (Taghiyar and Malek, 2012).

A one-factor analysis of variance (ANOVA) was performed for each of the four treatments in the phagocytosis assays, with sea urchin species as the independent factor, and the mean FL1+ as the dependent response variable.

2.7 *Cytotoxicity Assay*

Cytotoxic activity was assayed by incubating sRBC target cells with coelomocytes (Fuson et al., 1979; Parrinello and Arizza, 1992; Parrinello and Rindone, 1981; Porchet-Henneré

et al., 1992; Wittke and Renwrantz, 1984). Sheep red blood cells (sRBC) were prepared by washing stock sRBC (Colorado Serum Co., Denver, CO) in 40 ml of PBS three times. The last supernatant wash fraction was discarded and the cells were resuspended to 1% with PBS and kept on ice until use. The coelomocytes were diluted to yield a concentration of ~$1x10^6$/ml with Dulbecco's Modified Eagle Medium (DMEM). We cultured the cells with 10 µl of stimulant [lipopolysaccharide (LPS) and zymosan] or marine Alsever's solution (negative control). The assay was performed with an effector to target cell ratio of 2 to 1. The effector cells and target cells, or target cells only for control, or target cells only for target spontaneous release, or effector cells only for effector spontaneous release, or medium only for background control were also used (Lin et al., 2001; 2007).

The cultures were incubated for 4 hrs at 22°C. The cells were then centrifuged for 5 min at 6,000 x g to pellet intact sRBC and the supernatant was transferred from each tube into a clean glass tube and the absorbance was measured with a spectrophotometer at a wavelength of 405 nm.

The percentage of specific cytotoxicity (S.P.) is calculated using the following formula:

$$\% \text{ S.P.} = \frac{(\text{experimental} - \text{effector spontaneous release} - \text{target spontaneous release}) * 100\%}{(\text{target maximum release} - \text{target spontaneous release})}$$

Mean values for each trial were calculated for individual replicates ($n \geq 6$). Representative experimental treatment (or control) was checked using Trypan blue to assess coelomocyte viability. Only cells with viabilities of >95% were used. Significance of differences among samples was assessed by Student's *t*-test.

3. Results

3.1. *Phagocytosis Assay and Fluorescent Microscopy*

Phagocytic activity of the coelomocytes was estimated using fluorescently labeled latex beads (Figure 1). We used identical view observations between bright field and fluorescence microscopy. The phagocytosis index that we employed was determined as the percentage of cells that engulfed at least 2 labeled beads. We routinely recorded phagocytosis indexes of > 20 (data not shown). To characterize further the phagocytic response of the sea urchins we employed flow cytometry to analyze phagocytosis.

3.2. *Phagocytosis Assay and Flow Cytometry*

Based on initial plotting, cells with a side-scatter (SC) value of 1 were considered margin events and removed. Remaining cells were transformed using an estimateLogicle biexponential function. Cells were plotted for FL1 fluorescent emission (FL1), both with one parameter histograms of channel number vs. number of events using flowViz densityplot, as well as two parameter FL1 vs. SS scatter plots using flowDensity. Density plot gates and cell proportions were partly calculated using deGate. Based on the FL1 density plots, the separation between FL1– and FL1+ cells was determined to be 2.7.

FL1+ cells were those that were positive for phagocytosis of the fluorescently labeled latex beads. Proportions of coelomocytes that were FL1+, as well as cell populations based on granularity and mean FL1 response, were very similar for all urchin species to all treatments (Table 1 and Figure 2; Table 2 and Figure 3).

With no immune stimulant added (seawater only) *Diadema* had 0.05% FL1+ cells, *Echinometra* 0.02%, and *Tripneustes* 0.03%. With LPS stimulation *Diadema* had 15% FL1+ cells, *Echinometra* 14%, and *Tripneustes* 19%. With laminarin stimulation *Diadema* had 13% FL1+ cells, *Echinometra* 16%, and *Tripneustes* 22%. And with peptidoglycan stimulation *Diadema* had 10% FL1+ cells, *Echinometra* 13%, and *Tripneustes* 14% (Table 1, Figure 2). LPS, laminarin, and PGN are known immune stimulants, and we have previously demonstrated that these PAMPs elicited a humoral immune response in all three urchin species, although *Diadema's* response to LPS was significantly impaired (Beck et al., 2014; 2008).

To reflect the morphology of phagocytic vs. non-phagocytic cell populations, 2D scatter plots of side-scatter (SS) and FL1 fluorescence were performed, and the percent of cells in each quadrant were determined. The quadrants represent relatively granular non-phagocytic cells, relatively granular phagocytic cells, relatively non-granular non-phagocytic cells, and relatively non-granular non-phagocytic cells (Table 2, Figure 4). In *Diadema*, 80-85% of phagocytic cells were non-granular; in *Echinometra*, 85-88% were non-granular, and in *Tripneustes* 84-91% were non-granular. We have previously shown in the purple sea urchin *Arbacia punctulata* that phagocytic amoebocytes are phagocytic, while red and white spherule cells are granular (Lin et al., 2007; 2001). Our findings here also seem to show that phagocytic coelomocytes in these three urchin species tend to be non-granular.

One-factor ANOVA showed that the effect of laminarin on mean FL1 was significant, $F(2, 31) = 3.582$, $p = 0.0399$, as was the effect of LPS, $F(2, 33) = 4.133$, $p = 0.025$, and peptidoglycan, $F(2, 34) = 3.565$, $p = 0.0393$. Treatments with Pr(>F) less than or equal to 0.05 had their adjusted p-values determined by Tukey multiple comparisons of means with a 95% family-wise confidence level. Tukey's HSD showed that the only significant difference in the mean FL1 response was to LPS between *D. antillarum* and *E. lucunter*, p(adj) = 0.0469.

3.3 *Cytotoxicity Assay*

A cytotoxicity assay was used as another measure of the activity of the cellular immune response as we have described in characterization of cytotoxic cells in another sea urchin (the purple sea urchin, *Arbacia punctulata*) (Lin et al., 2007; 2001). As seen in Figure 5 the coelomocytes from all three urchin species were capable of recognizing and lysing the xenogenic sRBC after incubation for 4 hrs. Background sea water controls for each urchin species in the experiments were approximately 15%. When exposed to the stimulatory agents (LPS and zymosan) the coelomocytes from each of the 3 different urchin species responded with an increase of cytotoxic activity (ranging from 30 to 50%).

4. Discussion

Coelomocytes of all sea urchins in these experiments (*Diadema antillarum, Tripneustes ventricosus* and *Echinometra lucunter*) showed a consistent phagocytic response to all stimulants. In addition, the coelomocytes exhibited cytotoxic activities towards xenogenic cells. Based on these results, it appears that *Diadema* is no less capable of microbial recognition, phagocytosis, and cytotoxic responses - all hallmarks of the cellular immune response – than any of the other urchins studied that did not suffer mass mortality in 1983. While we previously observed a diminished humoral response by *Diadema* to LPS (Beck et al., 2014), we found no difference in its cellular host defense responses of phagocytosis and cytotoxicity stimulated by LPS. Both phagocytosis and cytotoxicity in *Diadema,* and the other urchins studied, are stimulated by other experimental immune stimulants we employed as well.

Given our previous results with the humoral response, the apparent normal cellular response in *Diadema* was surprising. Is the cellular immune response in *Diadema* normal, despite its die-off, poor recovery, and reduced humoral response? Or could the phagocytotic engulfment that our experiments tested occur normally, but without associated microbicidal action and thus diminished host defense? And if so, what signaling pathways might be responsible for a failure of microbicidal action following normal phagocytic engulfment? As more is known about phagocytosis and its associated signaling pathways in insects and higher organisms, more recently proposed models in these organisms may offer some possible explanations.

The most basic function of host defense is to distinguish self from non-self (*i.e.,* viruses, bacteria, protozoa, fungi, and other non-host proteins) and altered-self (i.e., damaged host cells, transformed cells) (Medzhitov and Janeway, 2002). To accomplish this, all gnathostomes possess both an innate and adaptive immune system (L. Du Pasquier, 2001). A distinguishing feature of adaptive immunity is the somatic rearrangement of genes for specific recognition molecules, which provides almost infinite receptor variability to distinguish and bind foreign antigens (L. D. Pasquier, 2006). Another key characteristic of adaptive immunity is the production of memory cells with receptors specific to a previously encountered pathogen, expediting host defense (Beck and Habicht, 1996).

Although true adaptive immunity is confined to higher vertebratesj, all living organisms possess some type of innate immune system as a first line of defense (Beck and Habicht, 1996; Cooper, 2003; Marchalonis and Schluter, 1990b). For invertebrates to mount a successful immune response, their innate immune system must first be able to recognize a pathogen, and then take appropriate action to eliminate it by way of humoral and cellular effector mechanisms (Beutler, 2004). Non-self recognition in invertebrates typically occurs via germ-line encoded pattern recognition receptors (PRRs) (Janeway, 1992), and selective pressure would likely drive an expansion of that repertoire (Bayne, 2003; Buchmann, 2014; L. D. Pasquier, 2006; Zhang et al., 2015). Indeed, the first sea urchin to be sequenced revealed a wide diversity of immune-related genes (Buckley and Rast, 2012; Hibino et al., 2006; Rast et al., 2006; Sodergren et al., 2006), making up perhaps 4-5% of the genome (Silva, 2013). Other than Fcγ receptors used by vertebrates to bind antibody-opsonized particles, homologs of all PRRs have been found in invertebrates (Buckley and Rast, 2015). While their function in host defense has been shown for some (L. C. Smith et al., 2001), most others are still to be determined.

PRRs act as the afferent (sensing) cellular components of the innate immune system, and in sea urchins as in other organisms they would play an important role in initiating phagocytosis. Any breakdown between these afferent cellular components of the innate immune system could result in an impaired efferent (effector) cellular response. Additionally, the humoral and cellular arms of the immune system are thought to work cooperatively. Interaction between a receptor and its cognate ligand activates downstream signaling events that lead to various humoral or cellular immune responses (Beutler, 2004; Leclerc, 1996; Matranga, 1996; Ottaviani et al., 2003; Raftos et al., 1992b). Some of the most studied inflammatory signaling pathways arise from TLRs (Kawai and Akira, 2011; Moresco et al., 2011), and their downstream signal transduction requires engagement of adaptor molecules (Akira et al., 2003; Kenny and O'Neill, 2008) to activate kinase cascades to transcription factors (Lee and Kim, 2007; O'Neill et al., 2013; L. C. Smith et al., 2010). Activation of NF-kB results in gene transcription of cytokines or invertebrate cytokine-like signal molecules that elicit an inflammatory response (Beck, 1998; Beck et al., 1993; Beck and Habicht, 1996; Gantner et al., 2003; Malagoli, 2010; Moresco et al., 2011; Raftos et al., 1991; L. C. Smith et al., 2010). A break in any link of this chain, especially involving the TLR pathway, might alter an organism's phagocytic response.

386 The few sea urchin genomes that have been sequenced show great diversity in their TLR
387 genes. Compared to thirteen mammalian TLR genes (O'Neill et al., 2013), three species from the
388 Strongylocentrotidae family have well over two-hundred, while another belonging to the more
389 basal Toxopneustidae clade has one-quarter as many (Buckley and Rast, 2012; Hibino et al.,
390 2006; Rast et al., 2006; Sodergren et al., 2006). Although their cognate ligands and function are
391 still unknown, their enhanced expression in phagocytic coelomocytes suggests a role in immune
392 function (Buckley and Rast, 2012; Leulier and Lemaitre, 2008). Invertebrate TLRs from a
393 protochordate that were transfected into mammalian cells have been found to activate NF-kB
394 pathways in response to known mammalian ligands (Sasaki et al., 2009).
395 TLR signaling has been shown to be intimately involved with phagocytosis in higher
396 organisms. Phagocytosis represents one of the most ancient, highly conserved, and important
397 first line microbial defenses, and is the primary cellular defense for invertebrates (Beck and
398 Habicht, 1996; Marchalonis and Schluter, 1990b). Phagocytosis begins by interaction of
399 microbial ligands with multiple and varied PRRs on phagocytic cells (Aderem and Underhill,
400 1999), either directly, or indirectly through opsonin and complement receptors (Underhill and
401 Ozinsky, 2002).
402 Signaling following phagocyte binding is facilitated by receptor clustering at the binding
403 site (Greenberg, 1995; Kwiatkowska and Sobota, 1999). The formed phagosome fuses with a
404 lysosome, where it acquires hydrolase enzymes necessary for particle breakdown, thus creating
405 the mature phagolysosome (Flannagan et al., 2012; Steinberg, 2009). Thus, downstream
406 signaling following phagocytic engulfment is necessary for the microbicidal effect of
407 phagocytosis. As a result, any interference in the signaling pathway could impair normal immune
408 function even if phagocytic engulfment successfully occurred.
409 Due to the great expansion of the TLR repertoire in sea urchins their function in
410 echinoderms is poorly understood at present, while TLRs in higher organisms have been greatly
411 studied. In vertebrate phagocytes, microbial engulfment is accompanied by trafficking of
412 membrane TLRs and adapter proteins to the phagosome, where TLR signaling facilitates fusion
413 with lysosomes and phagosome maturation (Jaumouillé and Grinstein, 2011). This may be
414 unique to higher organisms, where phagocytosis also represents a means of antigen presentation
415 and activation of cells of the adaptive immune system (Blander, 2008; 2007a; Blander and
416 Medzhitov, 2006a; 2006b; 2004). Phagolysosome formation has been shown to occur without

TLR signaling from the phagosome, although slower and with impaired bacterial killing (Blander and Medzhitov, 2004). There is still debate as to whether TLR signaling from the phagosome is critical to its maturation (Blander, 2007a; 2007b), or if it proceeds independently (Russell and Yates, 2007; Yates et al., 2005). But if phagosomal maturation is dependent on TLR signaling in sea urchins, a possible defect in the pathway in *Diadema* might result in the normal phagocytic engulfment observed in our experiments, yet a compromised ability to successfully clear certain pathogens.

Indeed, in vertebrates it has been shown that phagocytosis can still occur in the absence of TLR signaling, but without the subsequent inflammatory response. While TLRs are required for inflammatory signaling, they may not function directly as phagocytic receptors. Vertebrate phagocytosis of zymosan and LPS can occur even without the appropriate TLR and adapter proteins, but in their absence gene transcription of inflammatory cytokines does not (Gantner et al., 2003; Peiser et al., 2002; Underhill et al., 1999). TLRs may only be capable of recognizing and binding soluble ligands, with the direct recognition of pathogen surfaces necessary for phagocytosis handled by other phagocyte receptors (Underhill and Gantner, 2004). Whether a similar model is present in sea urchins is unknown, but if so, a TLR defect in *Diadema* could result in normal phagocytotic engulfment without an effective inflammatory response.

Additionally, phagocytotic engulfment might still occur through non-TLR pathways, but without the maturation and signaling necessary for microbicidal activity. In vertebrates, it is thought that microbial binding to phagocytes and subsequent ingestion may be dependent on PRRs other than TLRs, while both phagosomal maturation and downstream inflammatory signaling are TLR-dependent (Moretti and Blander, 2014). Initiation of phagocytosis and activation of inflammatory signaling may be controlled by separate types of PRRs (Moretti and Blander, 2014). True phagocytic receptors, such as mannose and scavenger receptors, trigger actin-polymerization and phagocytosis but cannot activate inflammatory signaling, although they may modulate signaling activated by other PRRs (Moretti and Blander, 2014). Other kinds of receptors such as TLRs cannot initiate phagocytosis but can regulate its rate, and their primary function is downstream inflammatory signaling transduction to transcription factors like NF-kB. And other PRRs initiate both phagocytosis and signaling, such as β-glucan recognition Dectin-1. (Moretti and Blander, 2014). Thus, phagocytosis might occur through PRRs, while some defect affecting TLR signaling could result in either inefficient microbicidal action within phagocytes,

or failure to produce inflammatory cytokines to stimulate humoral mechanisms, leaving the organism susceptible to infection. This signaling defect could be in one or more TLRs or their adapter molecules. While it is unknown if similar mechanisms are involved in sea urchins, the possibility offers an intriguing potential explanation of *Diadema's* immune response.

Another intriguing possibility is the possibility of a defect in an immune gene more unique to sea urchins. In *S. purpuratus*, polygonal and small filopodial phagocytes have been shown to upregulate the immune-related 185/333 [recently renamed Transformer, (Lun et al., 2017; L. C. Smith and Lun, 2017)] genes after immune stimulation with PAMPs such as LPS, PGN, and laminarin (Majeske et al., 2014; 2013; Nair, 2005; Rast et al., 2000; Terwilliger et al., 2007). It is unknown whether 185/333 phagocytes are present in the sea urchin species studied here. And while LPS stimulation has been shown to increase the population of large polygonal and small filopodial 185/333 phagocytes in *S. purpuratus* (Brockton et al., 2008; Majeske et al., 2014; 2013) (0, 23, 29, 30), it is not known whether stimulation with the PAMPs used in our experiments affect either the number of cells or the relative proportion of coelomocytes subtypes in the urchin species we studied. Whether *Diadema* also has 185/333 genes in its immune repertoire is in area worth future investigation.

It is also possible that *Diadema's* cellular immune response is in fact normal, and that only its humoral arm is impaired. Research on insects has challenged the traditional cooperative model of the humoral and cellular arms of the immune response. In this new paradigm, humoral immune activation is seen as a later and secondary response to phagocytosis (Makarova et al., 2016; Matranga et al., 2005; Mills et al., 2015; Tsuzuki et al., 2014). As such, phagocytosis might occur independently of any defect in the humoral response.

It is also possible that phagocytosis was impaired in *Diadema* during the die-off, but is normal in the recovering population. Given that no immune studies were done on affected *Diadema* during the die-off, this will remain an unanswered question.

Here, we have observed normal phagocytic engulfment in *Diadema*. However, it remains unclear whether phagosomal maturation is necessary for microbicidal activity to occur. Is TLR signaling involved in phagocytosis in sea urchins? And if so, does downstream signaling and gene transcription of inflammatory molecules occur? These are important questions, not only for understanding the *Diadema* mass mortality, but also to further expand our understanding of the immune mechanisms of this important marine invertebrate. A possible future area of

investigation on the cellular immune response of *Diadema* might involve further phagocytosis assays using pH sensitive beads to determine whether phagocytosis is followed by phagosomal acidification. Additionally, quantification of mRNA expression patterns of PRRs and their adapter molecules, in response to challenge with microbial ligands at multiple time intervals, would provide insight into signaling cascades involved in the cellular immune response (manuscript in preparation).

The vast diversity of pathogen recognition receptors discovered in sea urchins makes understanding their immune function critical to unravel the potential relationship between PRR defects and immune related pathology. Given some of the recently discovered roles of TLR signaling in higher organisms, and considering the greatly expanded TLR repertoire in sea urchins, it is essential to gain a better understanding of their immune function.

Diadema suffered a devastating die-off, likely due to an unknown pathogen, while other cosmopolitan species of sea urchins on the same reefs were unaffected. While we previously observed impaired humoral responses in *Diadema*, our results here show normal phagocytic engulfment. The evolutionary events that produced this duality in cellular versus humoral response in *Diadema,* and the relationship of this duality is to the epidemic of 1983-84, are difficult to ascertain but invite speculation. The poor humoral response in *Diadema*, which is not shared by other Caribbean sea urchins, could predate the epidemic and explain why *Diadema* was the only Caribbean urchin to experience mass mortality. In this scenario, the inactive humoral response was preserved in *Diadema* through the epidemic because survivors were in a non-susceptible part of the bi-phasic life cycle, had exceptionally strong cellular responses (perhaps because some other challenge had activated the cellular arm at just the right time), or were simply lucky (did not encounter the pathogen). Alternatively, if mortality during the epidemic resulted from over-activity of the humoral defenses, intense selection for weak humoral response may have produced the "defect" we see today.

Borrowing on research in higher organisms, our current speculation is that during the epidemic there was likely sufficient PRR activation to initiate phagocytosis, but that some defect in downstream signaling, such as in the TLR pathway, prevented a full inflammatory response with microbicidal killing. This could be responsible for *Diadema* succumbing to a pathogen, leading to both the mass die-off and the subsequent lack of recovery. By demonstrating apparently normal phagocytosis in a sea urchin with a proven humoral defect, when the humoral

and cellular arms of the innate immune system are thought to work cooperatively, we have opened areas of further inquiry, especially given recent research in vertebrates and insects in this area.

Acknowledgements

This research was supported by a grant from the NOAA: University of Puerto Rico College SeaGrant Program (UPRSGCP; 2013-2014-012)

520 **References**

521

522

523 Aderem, A., Underhill, D.M., 1999. Mechanisms of phagocytosis in macrophages. Annu. Rev.
524 Immunol. 17, 593–623. doi:10.1146/annurev.immunol.17.1.593
525 Akira, S., Uematsu, S., Takeuchi, O., 2006. Pathogen Recognition and Innate Immunity. Cell
526 124, 783–801. doi:10.1016/j.cell.2006.02.015
527 Akira, S., Yamamoto, M., Takeda, K., 2003. Role of adapters in Toll-like receptor signaling.
528 Biochem. Soc. T. 637–642.
529 Bachere, E., Chagot, D., Grizel, H., 1988. Separation of *Crassostrea gigas* hemocytes by density
530 gradient centrifugation and counterflow centrifugal elutriation. Dev. Comp. Immunol. 12,
531 549–559.
532 Bauer, J.C., Agerter, C.J., 1987. Isolation of Bacteria Pathogenic For The Sea Urchin *Diadema*
533 *Antillarum* (Echinodermata: Echinoidea). B. Mar. Sci. 401, 161–165.
534 Bayne, C.J., 2003. Origins and evolutionary relationships between the innate and adaptive arms
535 of immune systems. Integr. Comp. Biol. 43, 293–299. doi:10.1093/icb/43.2.293
536 Beck, G., 1998. Macrokines: Invertebrate cytokine-like molecules? Front. Biosci. 3, d559–69.
537 Beck, G., Habicht, G.S., 1996. Immunity and the Invertebrates. Sci. Am. 60–71.
538 Beck, G., Habicht, G.S., 1991. Purification and biochemical characterization of an invertebrate
539 interleukin 1. Mol. Immunol. 28, 577–584.
540 Beck, G., Miller, R., Ebersole, J., 2014. Mass mortality and slow recovery of *Diadema*
541 *antillarum*: Could compromised immunity be a factor? Mar. Biol. 161, 1001–1013.
542 doi:10.1007/s00227-013-2382-6
543 Beck, G., Miller, R., Ebersole, J., 2008. Significance of immune responses in *Diadema*
544 *antillarum* to Caribbean-wide mass mortality. Proc. 11th Int. Coral Reef Symp. 221–225.
545 Beck, G., O'Brien, R.F., Habicht, G.S., Stillman, D.L., Cooper, E.L., Raftost, D.A., 1993.
546 Invertebrate Cytokines III: Invertebrate Interleukin-1-like Molecules Stimulate Phagocytosis
547 by Tunicate and Echinoderm Cells. Cell. Immunol. 146, 284–299.
548 Bertheussen, K., 1979. The cytotoxic reaction in allogeneic mixtures of echinoid phagocytes.
549 Exp. Cell Res. 120, 373–381.
550 Bertheussen, K., Seijelid, R., 1978. Echinoid phagocytes in vitro. Exp. Cell Res. 111, 401–412.
551 Beutler, B., 2004. Innate immunity: an overview. Mol. Immunol. 40, 845–859.
552 doi:10.1016/j.molimm.2003.10.005
553 Binyon, J., 1972. Physiology of Echinoderms. Pergamon Press, Oxford.
554 Blanco, G., Escalada, A.M., Alvarez, E., 1997. LPS-induced stimulation of phagocytosis in the
555 sipunculan worm *Themiste petricola*: possible involvement of human CD14, CD11B and
556 CD11C cross-reactive molecules. Dev. Comp. Immunol. 21, 349–362.
557 Blander, J.M., 2008. Phagocytosis and antigen presentation: a partnership initiated by Toll-like
558 receptors. Ann. Rheum. Dis. 67, iii44–iii49. doi:10.1136/ard.2008.097964
559 Blander, J.M., 2007a. Coupling Toll-like receptor signaling with phagocytosis: potentiation of
560 antigen presentation. Trends Immunol. 28, 19–25. doi:10.1016/j.it.2006.11.001
561 Blander, J.M., 2007b. Signalling and phagocytosis in the orchestration of host defence. Cell.
562 Microbiol. 9, 290–299. doi:10.1111/j.1462-5822.2006.00864.x
563 Blander, J.M., Medzhitov, R., 2006a. Toll-dependent selection of microbial antigens for
564 presentation by dendritic cells. Nature 440, 808–812. doi:10.1038/nature04596

565 Blander, J.M., Medzhitov, R., 2006b. On regulation of phagosome maturation and antigen
566 presentation. Nat. Immunol. 7, 1029–1035. doi:10.1038/ni1006-1029
567 Blander, J.M., Medzhitov, R., 2004. Regulation of phagosome maturation by signals from toll-
568 like receptors. Science 304, 1014–1018. doi:10.1126/science.1096158
569 Bochud, P.-Y., Bochud, M., Telenti, A., Calandra, T., 2007. Innate immunogenetics: a tool for
570 exploring new frontiers of host defence. Lancet Infect. Dis. 7, 531–542. doi:10.1016/S1473-
571 3099(07)70185-8
572 Boolootian, R.A., Giese, A.C., 1958. Coelomic corpuscles of echinoderms. Biol. Bull. 115, 53–
573 63.
574 Bowdish, D.M.E., Gordon, S., 2009. Conserved domains of the class A scavenger receptors:
575 evolution and function. Immunol. Rev. 227, 19–31. doi:10.1111/j.1600-065X.2008.00728.x
576 Brockton, V., Henson, J.H., Raftos, D.A., Majeske, A.J., Kim, Y.O., Smith, L.C., 2008.
577 Localization and diversity of 185/333 proteins from the purple sea urchin - unexpected
578 protein-size range and protein expression in a new coelomocyte type. J. Cell Sci. 121, 339–
579 348. doi:10.1242/jcs.012096
580 Buchmann, K., 2014. Evolution of innate immunity: clues from invertebrates via fish to
581 mammals. Front. Immunol. 1–8. doi:10.3389/fimmu.2014.00459/abstract
582 Buckley, K.M., Rast, J.P., 2015. Diversity of animal immune receptors and the origins of
583 recognition complexity in the deuterostomes. Dev. Comp. Immunol. 49, 179–189.
584 doi:10.1016/j.dci.2014.10.013
585 Buckley, K.M., Rast, J.P., 2012. Dynamic evolution of toll-like receptor multigene families in
586 echinoderms. Front. Immunol. 3, 1–16. doi:10.3389/fimmu.2012.00136/abstract
587 Carpenter, R.C., 1990. Mass mortality of *Diadema antillarum* I. Long-term effects on sea urchin
588 population dynamics and coral reef algal communities. Mar. Biol. 104, 67–77.
589 Carpenter, R.C., 1981. Grazing by *Diadema antillarum* (Philippi) and its effects on the benthic
590 algal community [Sea urchin damage]. Journal of Marine Research.
591 Cerenius, L., Soderhall, K., 2013. Variable immune molecules in invertebrates. J. Exp. Biol. 216,
592 4313–4319. doi:10.1242/jeb.085191
593 Chia, F.S., Xing, J., 1996. Echinoderm coelomocytes. Zool. Stud. 35, 231–254.
594 Coffaro, K.A., Hinegardner, R.T., 1977. Immune response in the sea urchin *Lytechinus pictus*.
595 Science 197, 1389–1390.
596 Cooper, E.L., 2003. Comparative immunology. Curr. Pharm. Design 9, 119–131.
597 Cooper, E.L., 1976. Comparative immunology. Prentice Hall.
598 Coulter, W.H., 1956. High speed automatic blood cell counter and cell size analyzer. Proc Natl
599 Electron Conf.
600 Coulter, W.H., 1953. Means for counting particles suspended in a fluid. United States Patent
601 Office. 2656508.
602 de Faria, M.T., da Silva, J.R.M.C., 2008. Innate immune response in the sea urchin E*chinometra*
603 *lucunter* (Echinodermata). J. Invertebr. Pathol. 98, 58–62. doi:10.1016/j.jip.2007.10.004
604 Edds, K.T., 1993. Cell biology of echinoid coelomocytes: I. Diversity and characterization of
605 cell types. J. Invertebr. Pathol. 61, 173–178.
606 Edds, K.T., 1985. Morphological and cytoskeletal transformation in sea urchin coelomocytes.
607 Blood Cells of Marine Invertebrates: Experimental Systems in Cell Biology and
608 Comparative Physiology.
609 Ellis, B., Gentleman, R., Hahne, F., Meur, N.L., Sarkar, D., 2008. flowViz: Visualization for
610 flow cytometry. Bioinformatics 24, 878–879. doi:10.1093/bioinformatics/btn021

611 Ellis, B., Haaland, P., Hahne, F., Meur, N.L., Gopalakrishnan, N., Spidlen, J., 2015. flowCore:
612 data structures package for flow cytometry data 1–36.
613 Flajnik, M.F., Pasquier, Du, L., 2004. Evolution of innate and adaptive immunity: can we draw a
614 line? Trends Immunol. 25, 640–644. doi:10.1016/j.it.2004.10.001
615 Flannagan, R.S., Jaumouillé, V., Grinstein, S., 2012. The Cell Biology of Phagocytosis. Annu.
616 Rev. Pathol.-Mech. 7, 61–98. doi:10.1146/annurev-pathol-011811-132445
617 Franceschi, C., Cossarizza, A., Monti, D., Ottaviani, E., 1991. Cytotoxicity and immunocyte
618 markers in cells from the freshwater snail *Planorbarius corneus* (L.) (*Gastropoda*
619 *pulmonata*): implications for the evolution of natural killer cells. Eur. J. Immunol. 21, 489–
620 493. doi:10.1002/eji.1830210235
621 Fuson, E.W., Shaw, M.W., Hubbard, R.A., Lamon, E.W., 1979. Antibody-antigen complex
622 stimulated lysis of non-sensitized sheep red cells by human lymphocytes. I. Requirements
623 for IgG complexes. Clin. Exp. Immunol. 38, 158.
624 Gantner, B.N., Simmons, R.M., Canavera, S.J., Akira, S., Underhill, D.M., 2003. Collaborative
625 Induction of Inflammatory Responses by Dectin-1 and Toll-like Receptor 2. J. Exp. Med.
626 197, 1107–1117. doi:10.1084/jem.20021787
627 Gentleman, R.C., Carey, V.J., Bates, D.M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B.,
628 Gautier, L., Ge, Y., Gentry, J., Hornik, K., Hothorn, T., Huber, W., Iacus, S., Irizarry, R.,
629 Leisch, F., Li, C., Maechler, M., Rossini, A.J., Sawitzki, G., Smith, C., Smyth, G., Tierney,
630 L., Yang, J.Y., Zhang, J., 2004. Bioconductor: open software development for computational
631 biology and bioinformatic. Genome Biol. 5, R80. doi:10.1186/gb-2004-5-10-r80
632 Graham, M., 2003. The Coulter principle: foundation of an industry. J. Assoc. Lab. Autom. 8,
633 72–81. doi:10.1016/S1535-5535(03)00023-6
634 Greenberg, S., 1995. Signal transduction of phagocytosis. Trends Cell. Biol. 5, 93–99.
635 Gross, P.S., Al-Sharif, W.Z., Clow, L.A., Smith, L.C., 1999. Echinoderm immunity and the
636 evolution of the complement system. Dev. Comp. Immunol. 23, 429–442.
637 Gross, P.S., Clow, L.A., Smith, L.C., 2000. SpC3, the complement homologue from the purple
638 sea urchin, *Strongylocentrotus purpuratus*, is expressed in two subpopulations of the
639 phagocytic coelomocytes. Immunogenetics 51, 1034–1044. doi:10.1007/s002510000234
640 Harvey, E.B., 1956. The American Arbacia and Other Sea Urchins. Princeton University Press,
641 Princeton NJ.
642 Henson, J.H., Svitkina, T.M., Burns, A.R., Hughes, H.E., MacPartland, K.J., Nazarian, R.,
643 Borisy, G.G., 1999. Two components of actin-based retrograde flow in sea urchin
644 coelomocytes. Mol. Biol. Cell 10, 4075–4090.
645 Hibino, T., Loza-Coll, M., Messier, C., Majeske, A.J., Cohen, A.H., Terwilliger, D.P., Buckley,
646 K.M., Brockton, V., Nair, S.V., Berney, K., Fugmann, S.D., Anderson, M.K., Pancer, Z.,
647 Cameron, R.A., Smith, L.C., Rast, J.P., 2006. The immune gene repertoire encoded in the
648 purple sea urchin genome. Dev. Biol. 300, 349–365. doi:10.1016/j.ydbio.2006.08.065
649 Hughes, T.P., 1994. Catastrophes, phase shifts, and large-scale degradation of a Caribbean coral
650 reef. Science 265, 1547–1551. doi:10.1126/science.265.5178.1547
651 Hughes, T.P., Keller, B.D., Jackson, J., Boyle, M.J., 1985. Mass mortality of the echinoid
652 *Diadema antillarum* Philippi in Jamaica. B. Mar. Sci. 36, 377–384.
653 Hynes, R.O., 1992. Integrins: versatility, modulation, and signaling in cell adhesion. Cell 69, 11–
654 25.
655 Janeway, C.A., 1992. The immune system evolved to discriminate infectious nonself from
656 noninfectious self. Immunol. Today 13, 11–16. doi:10.1016/0167-5699(92)90198-G

Jaumouillé, V., Grinstein, S., 2011. Receptor mobility, the cytoskeleton, and particle binding during phagocytosis. Curr. Opin. Cell Biol. 23, 22–29. doi:10.1016/j.ceb.2010.10.006
Johnson, P.T., 1969. The coelomic elements of sea urchins (*Strongylocentrotus*). I. The normal coelomocytes; their morphology and dynamics in hanging drops. J. Invertebr. Pathol. 13, 25–41.
Kawai, T., Akira, S., 2011. Toll-like Receptors and Their Crosstalk with Other Innate Receptors in Infection and Immunity. Immunity 34, 637–650. doi:10.1016/j.immuni.2011.05.006
Kenny, E.F., O'Neill, L.A.J., 2008. Signalling adaptors used by Toll-like receptors: An update. Cytokine 43, 342–349. doi:10.1016/j.cyto.2008.07.010
Krishan, A., Cabana, R., 2004. Flow cytometric analysis of electronic nuclear volume and DNA content in normal mouse tissues. Cell Cycle 3, 380–383.
Kwiatkowska, K., Sobota, A., 1999. Signaling pathways in phagocytosis. BioEssays 21, 422–431. doi:10.1002/(SICI)1521-1878(199905)21:5<422::AID-BIES9>3.0.CO;2-#
Leclerc, M., 1996. Humoral factors in marine invertebrates. Prog. Mol. Subcell. Bio. 15, 1–9.
Lee, M.S., Kim, Y.-J., 2007. Signaling Pathways Downstream of Pattern-Recognition Receptors and Their Cross Talk. Annu. Rev. Biochem. 76, 447–480. doi:10.1146/annurev.biochem.76.060605.122847
Lessios, H., Cubit, J., Robertson, D., Shulman, M., Parker, M., Garrity, S., Levings, S., 1984. Mass mortality of *Diadema antillarum* on the Caribbean Coast of Panama. Coral Reefs 3, 173–182.
Lessios, H.A., 2015. The Great *Diadema antillarum* Die-Off: 30 Years Later. Annu. Rev. Mar. Sci. 8, 267–283. doi:10.1146/annurev-marine-122414-033857
Lessios, H.A., 1988. Mass mortality of *Diadema antillarum* in the Caribbean: what have we learned? Annu. Rev. Ecol. Syst. 19, 371–393.
Leulier, F., Lemaitre, B., 2008. Toll-like receptors--taking an evolutionary approach. Nat. Rev. Genet. 9, 165–178. doi:10.1038/nrg2303
Lin, W., Grant, S., Beck, G., 2007. Generation of monoclonal antibodies to coelomocytes of the purple sea urchin *Arbacia punctulata*: Characterization and phenotyping. Dev. Comp. Immunol. 31, 465–475. doi:10.1016/j.dci.2006.08.001
Lin, W., Zhang, H., Beck, G., 2001. Phylogeny of Natural Cytotoxicity: Cytotoxic Activity of Coelomocytes of the Purple Sea Urchin, *Arbacia punctulata*. J. Exp. Zool. Part B 290, 741–750.
Lun, C.M., Samuel, R.L., Gillmor, S.D., Boyd, A., Smith, L.C., 2017. The Recombinant Sea Urchin Immune Effector Protein, rSpTransformer-E1, Binds to Phosphatidic Acid and Deforms Membranes. Front. Immunol. 8, 349. doi:10.1016/j.bbalip.2012.08.018
Majeske, A.J., Oleksyk, T.K., Smith, L.C., 2013. The Sp185/333 immune response genes and proteins are expressed in cells dispersed within all major organs of the adult purple sea urchin. Innate Immun. 19, 569–587. doi:10.1177/1753425912473850
Majeske, A.J., Oren, M., Sacchi, S., Smith, L.C., 2014. Single Sea Urchin Phagocytes Express Messages of a Single Sequence from the Diverse Sp185/333 Gene Family in Response to Bacterial Challenge. J. Immunol. 1–11. doi:10.4049/jimmunol.1401681/-/DCSupplemental
Makarova, O., Rodriguez-Rojas, A., Eravci, M., Weise, C., Dobson, A., Johnston, P., Rolff, J., 2016. Antimicrobial defence and persistent infection in insects revisited. Philos. T. Roy. Soc. B 371. doi:10.1098/rstb.2015.0296
Malagoli, D., 2010. Cytokine network in invertebrates: the very next phase of comparative immunology. Invertebr. Surviv. J. 7, 146–148.

703 Mangiaterra, M.B., Silva, J., 2001. Induced inflammatory process in the sea urchin *Lytechinus*
704 *variegatus*. Invertebr. Biol. 120, 178–184.
705 Marchalonis, J.J., Schluter, S.F., 1990a. On the relevance of invertebrate recognition and defence
706 mechanisms to the emergence of the immune response of vertebrates. Scand. J. Immunol.
707 13–20.
708 Marchalonis, J.J., Schluter, S.F., 1990b. On the Relevance of Invertebrate Recognition and
709 Defence Mechanisms to the Emergence of the Immune Response of Vertebrates. Scand. J.
710 Immunol. 32, 13–20. doi:10.1111/j.1365-3083.1990.tb02886.x
711 Materna, S.C., Cameron, A., 2008. The Sea Urchin Genome as a Window on Function. Biol.
712 Bull. 214, 266–273.
713 Matranga, V., 1996. Molecular aspects of immune reactions in Echinodermata. Prog. Mol.
714 Subcell. Bio. 15, 235–247.
715 Matranga, V., Pinsino, A., Celi, M., Bella, G.D., Natoli, A., 2006. Impacts of UV-B radiation on
716 short-term cultures of sea urchin coelomocytes. Mar. Biol. 149, 25–34. doi:10.1007/s00227-
717 005-0212-1
718 Matranga, V., Pinsino, A., Celi, M., Natoli, A., Bonaventura, R., Schröder, H.C., Muller,
719 W.E.G., 2005. Monitoring chemical and physical stress using sea urchin immune cells. Prog.
720 Mol. Subcell. Bio. 39, 85–110.
721 Medzhitov, R., Janeway, C.A., 2002. Decoding the Patterns of Self and Nonself by the Innate
722 Immune System. Science 296, 298–300.
723 Mills, C.D., Ley, K., Buchmann, K., Canton, J., 2015. Sequential Immune Responses: The
724 Weapons of Immunity. J. Innate Immun. 7, 443–449. doi:10.1159/000380910
725 Moresco, E.M.Y., LaVine, D., Beutler, B., 2011. Toll-like receptors. Curr. Biol. 21, R488–R493.
726 doi:10.1016/j.cub.2011.05.039
727 Moretti, J., Blander, J.M., 2014. Insights into phagocytosis-coupled activation of pattern
728 recognition receptors and inflammasomes. Curr. Opin. Immunol. 26, 100–110.
729 doi:10.1016/j.coi.2013.11.003
730 Nair, S.V., 2005. Macroarray analysis of coelomocyte gene expression in response to LPS in the
731 sea urchin. Identification of unexpected immune diversity in an invertebrate. Physiological
732 Genomics 22, 33–47. doi:10.1152/physiolgenomics.00052.2005
733 O'Neill, L.A.J., Golenbock, D., Bowie, A.G., 2013. The history of Toll-like receptors -
734 redefining innate immunity. Nat. Rev. Immunol. 13, 453–460. doi:10.1038/nri3446
735 Ogden, J.C., Lobel, P.S., 1978. The role of herbivorous fishes and urchins in coral reef
736 communities. Environ. Biol. Fish. 3, 49–63.
737 Ottaviani, E., Malagoli, D., Franchini, A., 2003. Invertebrate Humoral Factors - Cytokines as
738 Mediators of Cell Survival, in: Progress in Molecular and Subcellular Biology. pp. 1–25.
739 Parrinello, N., Arizza, V., 1992. Cytotoxic activity of invertebrate hemocytes with preliminary
740 findings on the tunicate *Ciona intstinalis*. Itali. J. Zool. 59, 183–189.
741 Parrinello, N., Rindone, D., 1981. Studies on the natural hemolytic system of the annelid worm
742 *Spirographis spallanzanii viviani* (Polychaeta). Dev. Comp. Immunol. 5, 33–42.
743 doi:10.1016/S0145-305X(81)80005-5
744 Pasquier, Du, L., 2001. The immune system of invertebrates and vertebrates. Comp. Biochem.
745 Phys.B 129, 1–15.
746 Pasquier, L.D., 2006. Germline and somatic diversification of immune recognition elements in
747 Metazoa. Immunol. Lett. 104, 2–17. doi:10.1016/j.imlet.2005.11.022
748 Peddie, C.M., Smith, V.J., 1994. Mechanism of cytotoxic activity by hemocytes of the solitary

749 ascidian, *Ciona intestinalis*. J. Exp. Zool. 270, 335–342.
750 Peiser, L., de Winther, M.P.J., Makepeace, K., Hollinshead, M., Coull, P., Plested, J., Kodama,
751 T., Moxon, E.R., Gordon, S., 2002. The Class A Macrophage Scavenger Receptor Is a Major
752 Pattern Recognition Receptor for *Neisseria meningitidis* Which Is Independent of
753 Lipopolysaccharide and Not Required for Secretory Responses. Infect. Immun. 70, 5346–
754 5354. doi:10.1128/IAI.70.10.5346-5354.2002
755 Porchet-Henneré, E., Dugimont, T., Fischer, A., 1992. Natural killer cells in a lower invertebrate,
756 *Nereis diversicolor*. Eur. J. Cell Biol. 58, 99–107.
757 R Core, T., 2015. R: A Language and Environment for Statistical Computing 1–3460.
758 Raftos, D.A., Cooper, E.L., Habicht, G.S., Beck, G., 1991. Invertebrate cytokines: tunicate cell
759 proliferation stimulated by an interleukin 1-like molecule. Proc. Natl. Acad. Sci. U.S.A. 88,
760 9518–9522.
761 Raftos, D.A., Cooper, E.L., Stillman, D.L., Habicht, G.S., Beck, G., 1992a. Invertebrate
762 cytokines II: release of interleukin-1-like molecules from tunicate hemocytes stimulated with
763 zymosan. Lymphokine Cytok. Res. 11, 235–240.
764 Raftos, D.A., Cooper, E.L., Stillman, D.L., Habicht, G.S., Beck, G., 1992b. Invertebrate
765 cytokines II: release of interleukin-1-like molecules from tunicate hemocytes stimulated with
766 zymosan. Lymphokine Cytok. Res. 11, 235–240.
767 Rast, J.P., Messier-Solek, C., 2008. Marine Invertebrate Genome Sequences and Our Evolving
768 Understanding of Animal Immunity. Biol. Bull. 214, 1–10.
769 Rast, J.P., Pancer, Z., Davidson, E.H., 2000. New approaches towards an understanding of
770 deuterostome immunity. Curr. Top. Microbiol. Immunol. 248, 3–16.
771 Rast, J.P., Smith, L.C., Loza-Coll, M., Hibino, T., Litman, G.W., 2006. Genomic insights into
772 the immune system of the sea urchin. Science 314, 952–956. doi:10.1126/science.1134301
773 Russell, D.G., Yates, R.M., 2007. Toll-like receptors and phagosome maturation. Nat. Immunol.
774 8, 217–author reply 217–8. doi:10.1038/ni0307-217a
775 Sammarco, P.W., 1982. Effects of grazing by *Diadema antillarum* Philippi (Echinodermata:
776 Echinoidea) on algal diversity and community structure. J. Exp. Mar. Biol. Ecol. 65, 83–105.
777 Sammarco, P.W., 1980. *Diadema* and its relationship to coral spat mortality: grazing,
778 competition, and biological disturbance. J. Exp. Mar. Biol. Ecol. 45, 245–272.
779 Sammarco, P.W., Levinton, J.S., 1974. Grazing and control of coral reef community structure by
780 *Diadema antillarum* Philippi (Echinodermata: Echinoidea): A preliminary study. J. Mar.
781 Res. 32, 47–53.
782 Sarrias, M.R., Grønlund, J., Padilla, O., Madsen, J., Holmskov, U., Lozano, F., 2004. The
783 Scavenger Receptor Cysteine-Rich (SRCR) domain: an ancient and highly conserved protein
784 module of the innate immune system. Crit. Rev. Immunol. 24, 1–37.
785 Sasaki, N., Ogasawara, M., Sekiguchi, T., Kusumoto, S., Satake, H., 2009. Toll-like receptors of
786 the ascidian *Ciona intestinalis*: prototypes with hybrid functionalities of vertebrate Toll-like
787 receptors. J. Biol. Chem. 284, 27336–27343. doi:10.1074/jbc.M109.032433
788 Silva, J.R., 2000. The onset of phagocytosis and identity in the embryo of *Lytechinus variegatus*.
789 Dev. Comp. Immunol. 24, 733–739.
790 Silva, J.R.M.C., 2013. Immunology in Sea Urchins, in: Lawrence, J.M. (Ed.), Sea Urchins:
791 Biology and Ecology, Developments in Aquaculture and Fisheries Science. Elsevier, pp.
792 187–194. doi:10.1016/B978-0-12-396491-5.00013-7
793 Smith, A.B., Kroh, A., 2013. Phylogeny of Sea Urchins, in: Lawrence, J.M. (Ed.), Sea Urchins:
794 Biology and Ecology, Developments in Aquaculture and Fisheries Science. Elsevier, pp. 1–

14. doi:10.1016/B978-0-12-396491-5.00001-0
Smith, L.C., Chang, L., Britten, R.J., Davidson, E.H., 2001. Sea Urchin Genes Expressed in Activated Coelomocytes Are Identified by Expressed Sequence Tags. J. Immunol. 156, 593–602.
Smith, L.C., Ghosh, J., Buckley, K.M., Clow, L.A., Dheilly, N.M., Haug, T., Henson, J.H., Li, C., Lun, C.M., Majeske, A.J., Matranga, V., Nair, S.V., Rast, J.P., Raftos, D.A., Roth, M., Sacchi, S., Schrankel, C.S., Stensvag, K., 2010. Echinoderm immunity. Adv. Exp. Med. Biol. 708, 260–301.
Smith, L.C., Lun, C.M., 2017. The SpTransformer Gene Family (Formerly Sp185/333) in the Purple Sea Urchin and the Functional Diversity of the Anti-Pathogen rSpTransformer-E1 Protein. Front. Immunol. 8, 355–20. doi:10.3389/fimmu.2017.00725
Smith, L.C., Rast, J.P., Brockton, V., Terwilliger, D.P., 2006. The sea urchin immune system. Invertebr. Surviv. J. 3, 25–39.
Smith, V.J., 1981. The echinoderms, in: Ratcliffe, N.A., Rowley, A.F. (Eds.), Invertebrate Blood Cells. Academic Press, New York, pp. 513–562.
Sodergren, E., Weinstock, G.M., Davidson, E.H., Cameron, R.A., Gibbs, R.A., Angerer, R.C., Angerer, L.M., Arnone, M.I., Burgess, D.R., Burke, R.D., Coffman, J.A., Dean, M., Elphick, M.R., Ettensohn, C.A., Foltz, K.R., Hamdoun, A., Hynes, R.O., Klein, W.H., Marzluff, W., McClay, D.R., Morris, R.L., Mushegian, A., Rast, J.P., Smith, L.C., Thorndyke, M.C., Vacquier, V.D., Wessel, G.M., Wray, G., Zhang, L., Elsik, C.G., Ermolaeva, O., Hlavina, W., Hofmann, G., Kitts, P., Landrum, M.J., Mackey, A.J., Maglott, D., Panopoulou, G., Poustka, A.J., Pruitt, K., Sapojnikov, V., Song, X., Souvorov, A., Solovyev, V., Wei, Z., Whittaker, C.A., Worley, K., Durbin, K.J., Shen, Y., Fedrigo, O., Garfield, D., Haygood, R., Primus, A., Satija, R., Severson, T., Gonzalez-Garay, M.L., Jackson, A.R., Milosavljevic, A., Tong, M., Killian, C.E., Livingston, B.T., Wilt, F.H., Adams, N., Bellé, R., Carbonneau, S., Cheung, R., Cormier, P., Cosson, B., Croce, J., Fernandez-Guerra, A., Geneviere, A.-M., Goel, M., Kelkar, H., Morales, J., Mulner-Lorillon, O., Robertson, A.J., Goldstone, J.V., Cole, B., Epel, D., Gold, B., Hahn, M.E., Howard-Ashby, M., Scally, M., Stegeman, J.J., Allgood, E.L., Cool, J., Judkins, K.M., McCafferty, S.S., Musante, A.M., Obar, R.A., Rawson, A.P., Rossetti, B.J., Gibbons, I.R., Hoffman, M.P., Leone, A., Istrail, S., Materna, S.C., Samanta, M.P., Stolc, V., Tongprasit, W., Tu, Q., Bergeron, K.-F., Brandhorst, B.P., Whittle, J., Berney, K., Bottjer, D.J., Calestani, C., Peterson, K., Chow, E., Yuan, Q.A., Elhaik, E., Graur, D., Reese, J.T., Bosdet, I., Heesun, S., Marra, M.A., Schein, J., Anderson, M.K., Brockton, V., Buckley, K.M., Cohen, A.H., Fugmann, S.D., Hibino, T., Loza-Coll, M., Majeske, A.J., Messier, C., Nair, S.V., Pancer, Z., Terwilliger, D.P., Agca, C., Arboleda, E., Chen, N., Churcher, A.M., Hallbook, F., Humphrey, G.W., Idris, M.M., Kiyama, T., Liang, S., Mellott, D., Mu, X., Murray, G., Olinski, R.P., Raible, F., Rowe, M., Taylor, J.S., Tessmar-Raible, K., Wang, D., Wilson, K.H., Yaguchi, S., Gaasterland, T., Galindo, B.E., Gunaratne, H.J., Juliano, C., Kinukawa, M., Moy, G.W., Neill, A.T., Nomura, M., Raisch, M., Reade, A., Roux, M.M., Song, J.L., Su, Y.-H., Townley, I.K., Voronina, E., Wong, J.L., Amore, G., Branno, M., Brown, E.R., Cavalieri, V., Duboc, V., Duloquin, L., Flytzanis, C., Gache, C., Lapraz, F., Lepage, T., Locascio, A., Martinez, P., Matassi, G., Matranga, V., Range, R., Rizzo, F., Röttinger, E., Beane, W., Bradham, C., Byrum, C., Glenn, T., Hussain, S., Manning, G., Miranda, E., Thomason, R., Walton, K., Wikramanayke, A., Wu, S.-Y., Xu, R., Brown, C.T., Chen, L., Gray, R.F., Lee, P.Y., Nam, J., Oliveri, P., Smith, J., Muzny, D., Bell, S., Chacko, J., Cree, A., Curry, S., Davis, C., Dinh, H., Dugan-Rocha, S., Fowler, J.,

841 Gill, R., Hamilton, C., Hernandez, J., Hines, S., Hume, J., Jackson, L., Jolivet, A., Kovar, C.,
842 Lee, S., Lewis, L., Miner, G., Morgan, M., Nazareth, L.V., Okwuonu, G., Parker, D., Pu, L.-
843 L., Thorn, R., Wright, R., 2006. The genome of the sea urchin Strongylocentrotus
844 purpuratus. Science 314, 941–952. doi:10.1126/science.1133609
845 Standerholen, F.B., Myromslien, F.D., Kommisrud, E., Ropstad, E., Waterhouse, K.E., 2014.
846 Comparison of electronic volume and forward scatter principles of cell selection using flow
847 cytometry for the evaluation of acrosome and plasma membrane integrity of bull
848 spermatozoa. Cytometry 85, 719–728. doi:10.1002/cyto.a.22474
849 Steinberg, B., 2009. A Biophysical Characterization of Phagolysosome Acidification.
850 Taghiyar, M.J., Malek, M., 2012. flowDensity: Sequential Flow Cytometry Data Gating 1–14.
851 Terwilliger, D.P., Buckley, K.M., Brockton, V., Ritter, N.J., Smith, L.C., 2007. Distinctive
852 expression patterns of 185/333 genes in the purple sea urchin, Strongylocentrotus
853 purpuratus: an unexpectedly diverse family of transcripts in response to LPS, β-1,3-glucan,
854 and dsRNA. BMC Mol. Biol. 8, 16. doi:10.1186/1471-2199-8-16
855 Terwilliger, D.P., Clow, L.A., Gross, P.S., Smith, L.C., 2004. Constitutive expression and
856 alternative splicing of the exons encoding SCRs in Sp152, the sea urchin homologue of
857 complement factor B. Implications on the evolution of the Bf/C2 gene family.
858 Immunogenetics 56, 531–543. doi:10.1007/s00251-004-0711-0
859 Tsuzuki, S., Matsumoto, H., Furihata, S., Ryuda, M., Tanaka, H., Sung, E.J., Bird, G.S., Zhou,
860 Y., Shears, S.B., Hayakawa, Y., 2014. Switching between humoral and cellular immune
861 responses in *Drosophila* is guided by the cytokine GBP. Nat. Commun. 5, 1–11.
862 doi:10.1038/ncomms5628
863 Underhill, D.M., Gantner, B., 2004. Integration of Toll-like receptor and phagocytic signaling for
864 tailored immunity. Microbes Infect. 6, 1368–1373. doi:10.1016/j.micinf.2004.08.016
865 Underhill, D.M., Ozinsky, A., 2002. Phagocytosis of microbes: complexity in action. Annu. Rev.
866 Immunol. 20, 825–852. doi:10.1146/annurev.immunol.20.103001.114744
867 Underhill, D.M., Ozinsky, A., Hajjar, A.M., Stevens, A., Wilson, C.B., Bassetti, M., Aderem, A.,
868 1999. The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates
869 between pathogens. Nature 401, 811–815. doi:10.1038/44605
870 Whelan, F.J., Meehan, C.J., Golding, G.B., McConkey, B.J., Bowdish, D.M.E., 2012. The
871 evolution of the class A scavenger receptors. BMC Evol. Biol. 12, 227. doi:10.1186/1471-
872 2148-12-227
873 Wittke, M., Renwrantz, L., 1984. Quantification of cytotoxic hemocytes of *Mytilus edulis* using a
874 cytotoxicity assay in agar. J. Invertebr. Pathol. 43, 248–253.
875 Yates, R.M., Hermetter, A., Russell, D.G., 2005. The Kinetics of Phagosome Maturation as a
876 Function of Phagosome/Lysosome Fusion and Acquisition of Hydrolytic Activity. Traffic 6,
877 413–420. doi:10.1111/j.1600-0854.2005.00284.x
878 Zhang, L., Li, L., Guo, X., Litman, G.W., Dishaw, L.J., Zhang, G., 2015. Massive expansion and
879 functional divergence of innate immune genes in a protostome. Sci. Rep. 5, 8693.
880 doi:10.1038/srep08693
881
882
883
884

885 **TABLES**

886

887

888 **Table 1.** Percentage of FL1+ coelomocytes for three urchin species with four treatments

889

Treatment	Species		
	Diadema	*Echinometra*	*Tripneustes*
Seawater	0.05%	0.02%	0.03%
LPS	15%	14%	19%
Laminarin	13%	16%	22%
Peptidoglycan	10%	13%	14%

890

891

892

893

894 **Table 2.** Percentage of phagocytic coelomocytes based on cell granularity

895

	Species						
	Diadema		***Echinometra***		***Tripneustes***		
Treatment	Granular	Non-granular	Granular	Non-granular	Granular	Non-granular	**Fluorescence**
Seawater	0.03%	0.02%	0.02%	0%	0.02%	0.01%	+
	20%	80%	17%	83%	18%	82%	–
LPS	3%	12%	2%	12%	3%	16%	+
	11%	74%	10%	76%	10%	71%	–
Laminarin	2%	11%	2%	14%	2%	20%	+
	11%	76%	10%	74%	11%	67%	–
Peptidoglycan	2%	8%	2%	11%	2%	12%	+
	10%	80%	11%	76%	7%	79%	–

896

897

898

899

900

901

FIGURES

Figure 1. Representative fluorescent microscopy of phagocytized fluorescently labeled latex beads in sea urchin coelomocytes stimulated with LPS. A: *D. antillarum*; B: *T. ventricosus*; C: *E. lucunter.*

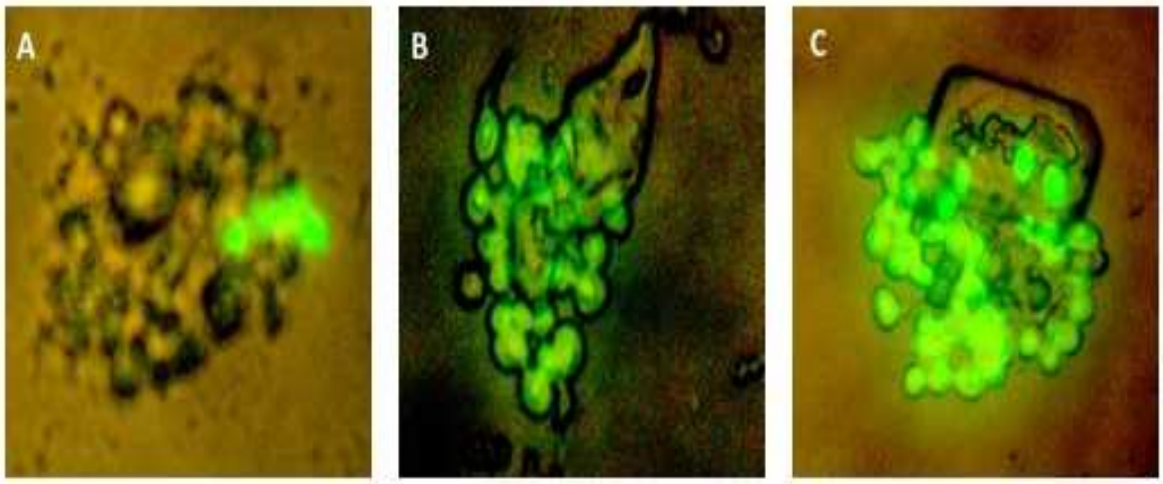


Figure 2. Mean 1D FL1 density plots of *D. antillarum*, *E. lucunter*, and *T. ventricosus* coelomocytes exposed to seawater (A), LPS (B), laminarin (C), and peptidoglycan (D), with the percentage of FL1– and FL1+ cells shown on the cell population peaks.

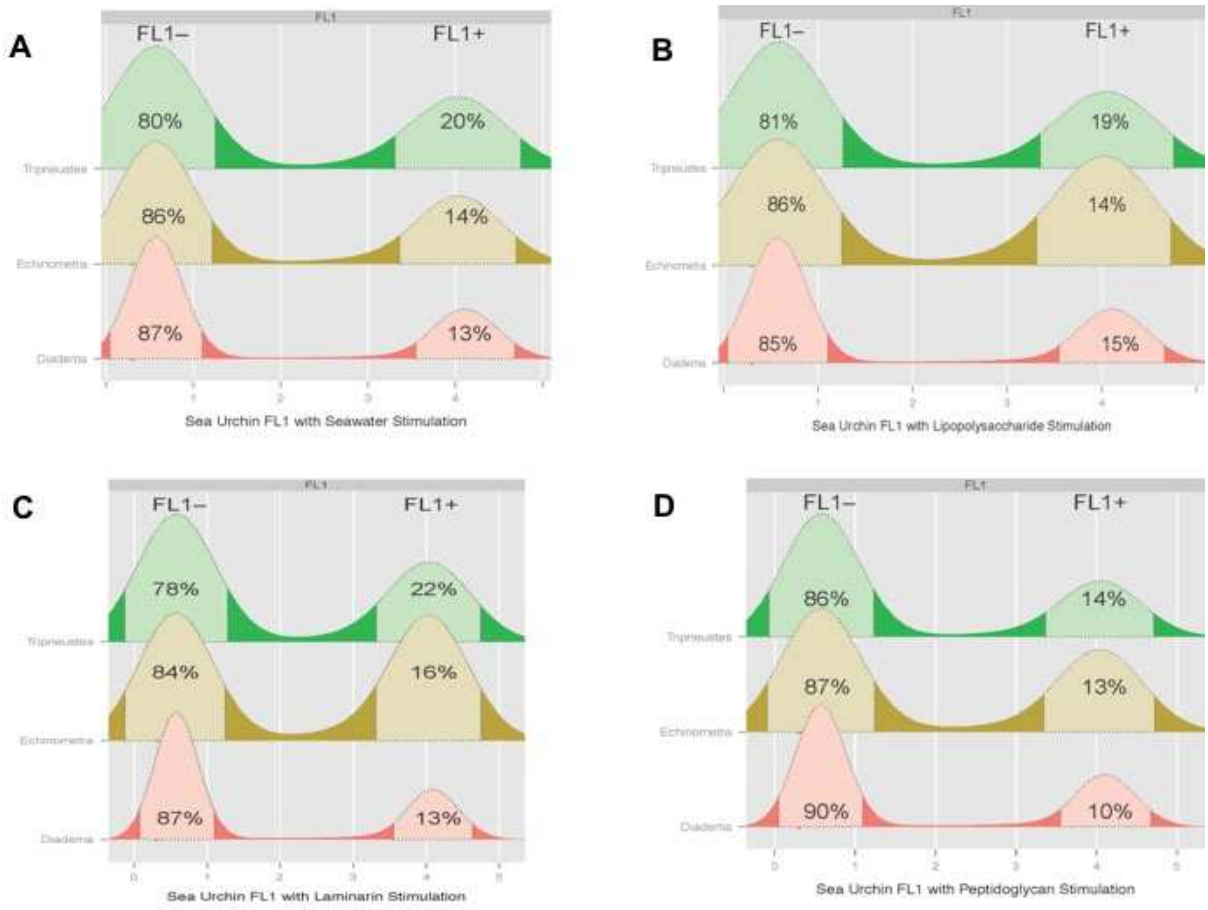
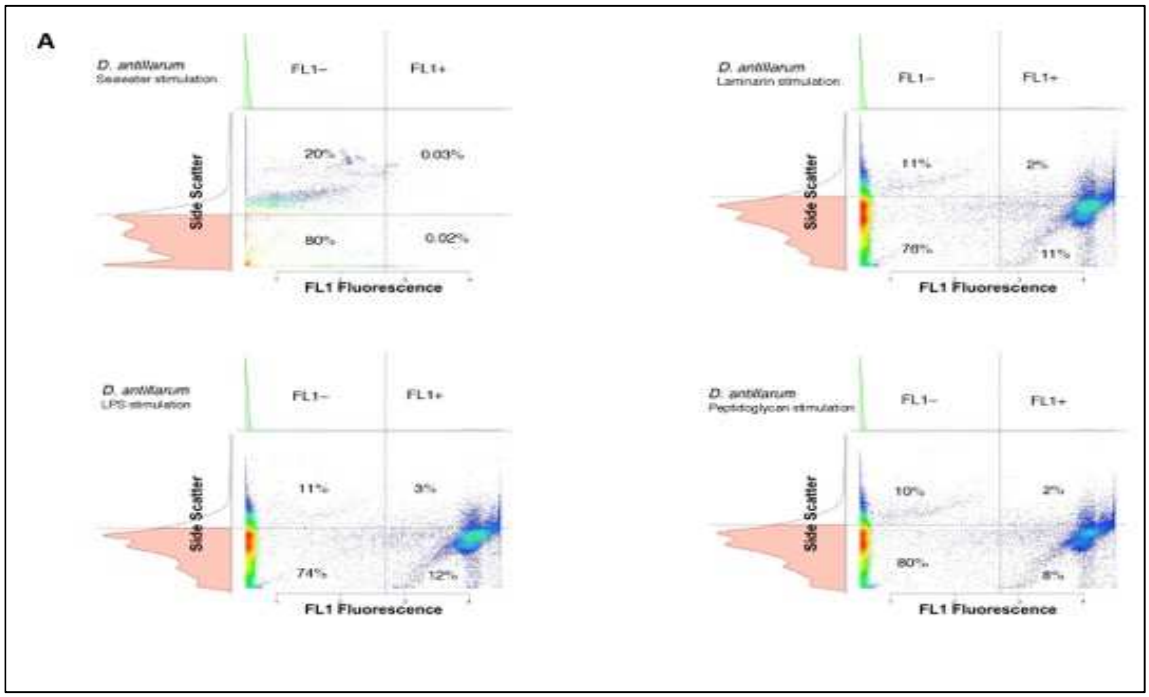
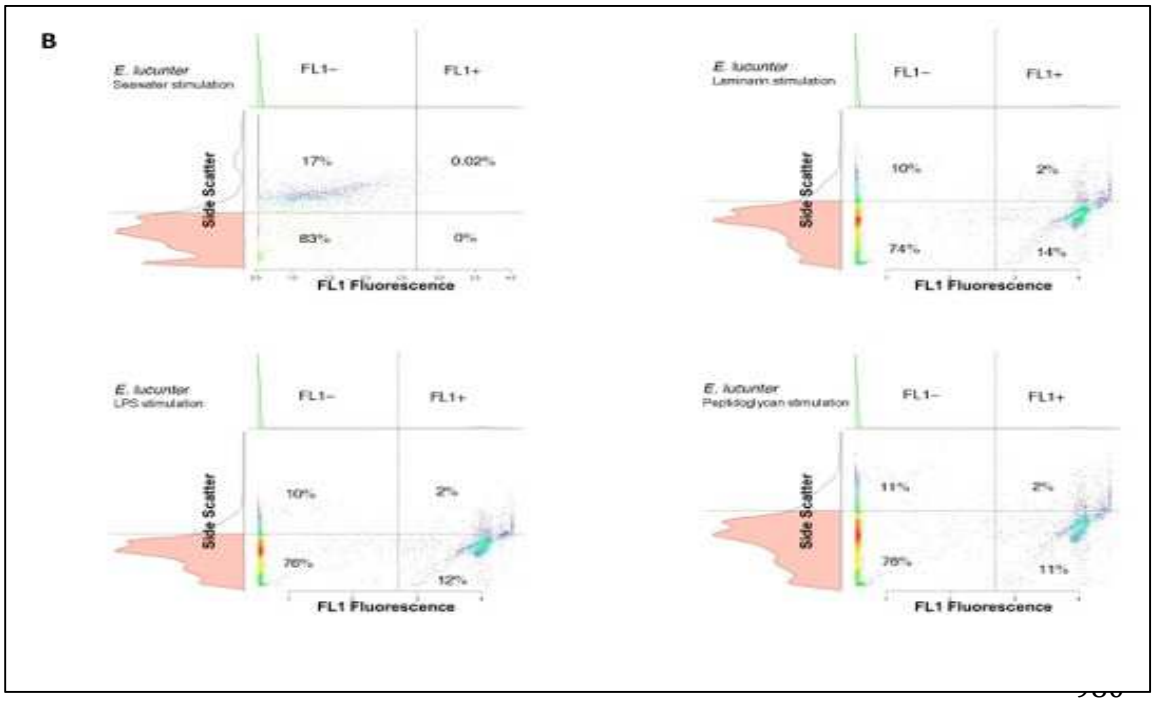


Figure 3. 2D mean SS/FL1 scatter plots of coelomocytes from. (A) *D. antillarum* exposed to seawater, laminarin, LPS, and peptidoclycan; (B) *E. lucunter* exposed to seawater, laminarin, LPS, and peptidoclycan., and (C) *T. ventricosus* exposed to seawater, laminarin, LPS, and peptidoclycan. Each quadrant represents cell populations that were relatively (clockwise from upper left) FL1–/granular, FL1+/granular, FL1+/non-granular, and FL1–/non-granular, and the proportion of the total cells that each cell population represents is shown for each quadrant.

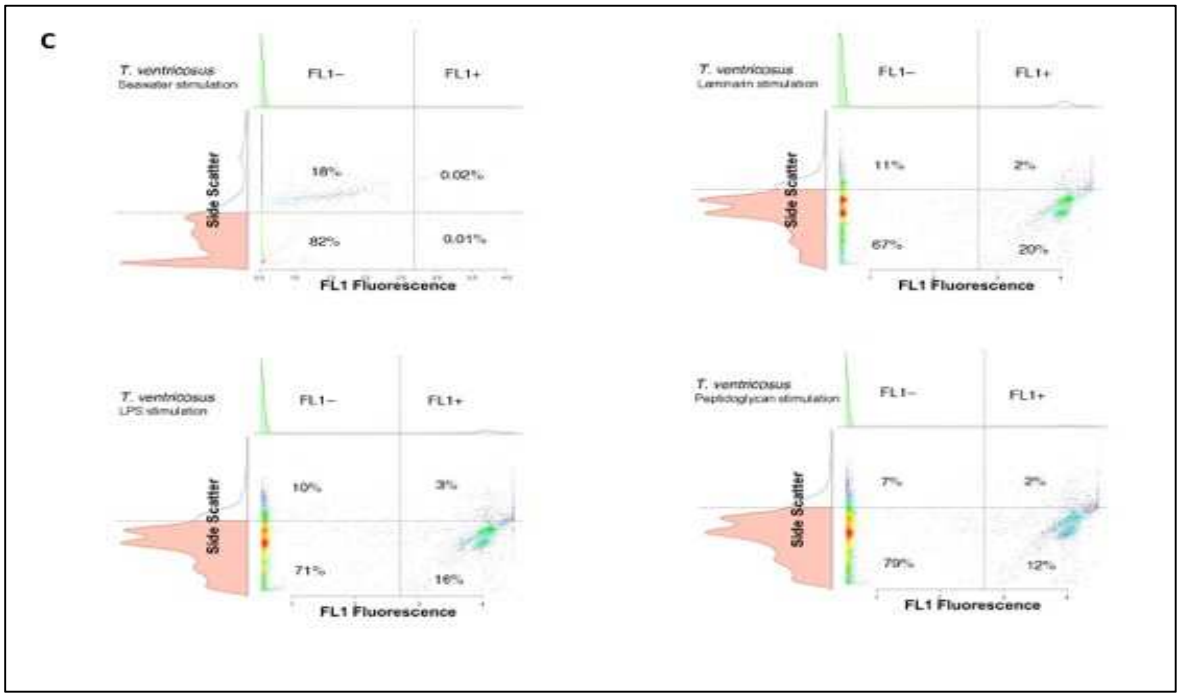


966

967



981



982

983

Figure 4. Mean FL1+ fluorescent response for *D. antillarum* (A), *E. lucunter* (B), and *T. ventricosus* (C), to each treatment (from left to right: seawater, laminarin, LPS, and peptidoglycan).

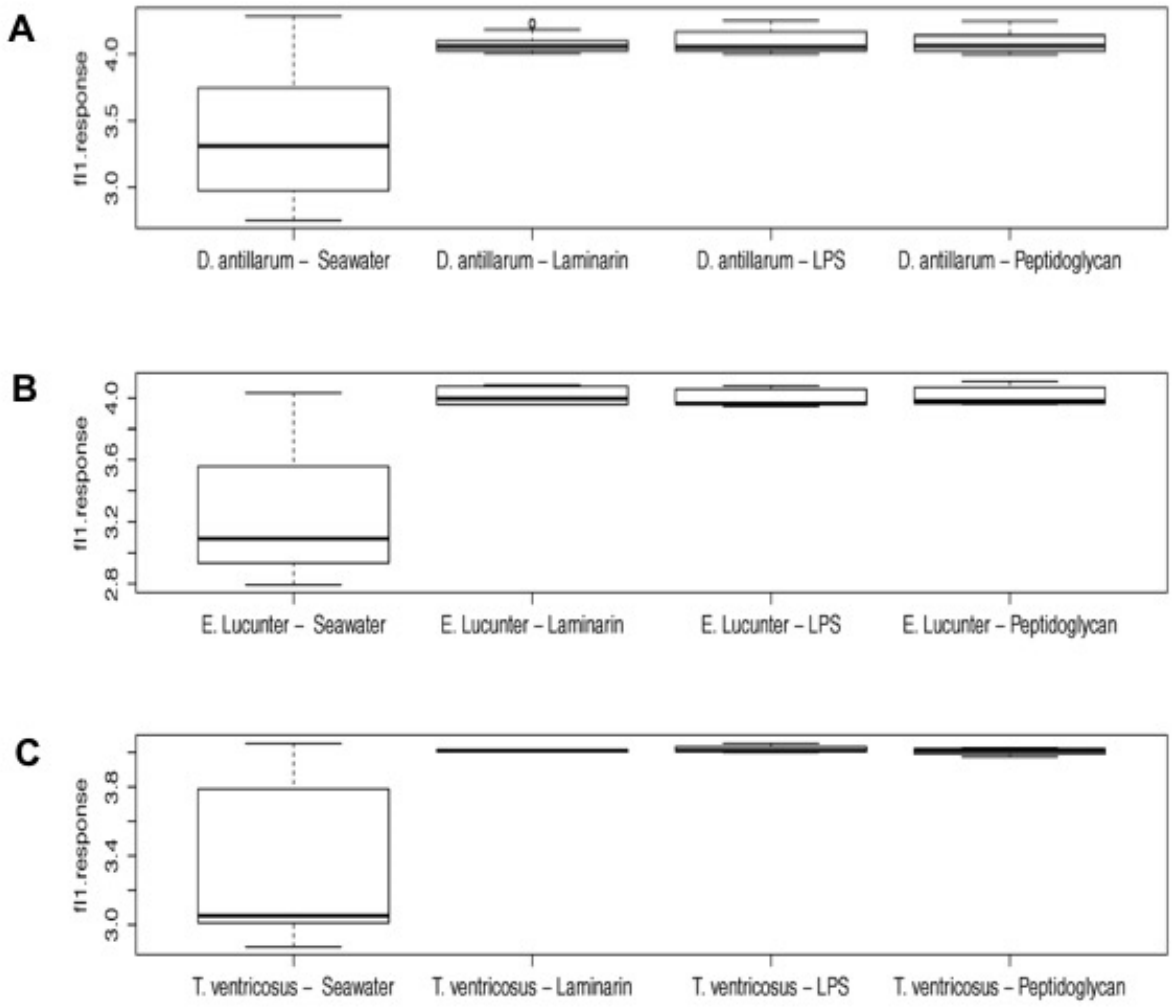


Figure 5. Percent specific cytotoxicity of sea urchin coelomocytes. Coelomocytes were incubated with sRBC target cells. *D. antillarum*, *E. lucunter*, and *T. ventricosus* coelomocytes were treated with, from left to right, seawater, LPS, or zymosan. After 4 hr the supernatants were removed and scored as described in the *Material and Methods* section. Data are presented as Mean ± SEM of percent specific cytotoxicity from six experiments. **P* < 0.01 as compared to the seawater control, ***P* < 0.001 as compared to the seawater control.