

The *Sp185/333* immune response genes and proteins are expressed in cells dispersed within all major organs of the adult purple sea urchin

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Abstract

Purple sea urchins (*Strongylocentrotus purpuratus*) express a highly variable set of immune genes called *Sp185/333* by two subtypes of coelomocytes: the polygonal and small phagocytes. We report that the *Sp185/333* genes and their encoded proteins are also expressed in all of the major organs in the adult sea urchin, including the axial organ, pharynx, esophagus, intestine and gonads. After immune challenge, there is an increase in the level of *Sp185/333* mRNA in cells associated with the intestine and axial organ. The *Sp185/333* proteins increase in the axial organ, pharynx, esophagus and intestine after challenge. However, the proportion of *Sp185/333*-positive cells only increases in the axial organ, while there is no change in that proportion in the other organs after challenge. The size range of the major *Sp185/333* proteins expressed by organs is broader (5 kDa to > 250 kDa) compared with those in coelomocytes (~40 kDa to < 250 kDa). Images of the different organs do not clarify whether coelomocytes or parenchymal cells express the *Sp185/333* proteins. The increase in levels of *Sp185/333* transcripts, protein expression and *Sp185/333*-positive cells in the axial organ in response to challenge suggests that this organ may have an important role in immunity for this species.

Keywords

Axial organ, coelomocytes, echinoderm, innate, immune diversity

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Introduction

The purple sea urchin (*Strongylocentrotus purpuratus*) has a complex and sophisticated immune system composed of a large repertoire of gene models encoding proteins capable of recognizing and responding to a vast array of pathogens.^{1–6} There are a number of immune gene families in vertebrates that are greatly expanded in the sea urchin. Examples include 253 TLRs (*Homo sapiens* possess ~10), 203 nucleotide-binding oligomerization domain-like (NLR) receptors (*H. sapiens* have 20) and 218 scavenger receptor cysteine-rich (SRCR) domain proteins (*H. sapiens* have about 16).^{1,2,6} Included among the immune response genes is the *Sp185/333* gene family, which was identified originally based on significant up-regulation in coelomocytes (immune cells) responding to immune challenge^{3,7} and significant sequence similarity to an uncharacterized differential display sequence DD185,⁷ and an expressed sequence tag EST333.⁸ Because the deduced proteins have no matches to

other known proteins, the gene family was named for these two unknown sequences and called *185/333*,³ which was revised to *Sp185/333* to indicate the species and genus given that these genes have also been identified in other echinoids.⁹

Estimates of the *Sp185/333* gene family size ranges from 40–60 genes per genome,¹⁰ and each gene is small, with two exons, of which the first encodes a signal sequence and the second encodes the mature protein.^{10–12} The extraordinary sequence diversity among

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the genes and messages derives from mosaic patterns of blocks of sequence called 'elements', in addition to single nucleotide polymorphisms (SNPs) and/or small insertions/deletions (indels) within individual elements.^{11–13} The structure of the second exon is likely the result of gene recombination, duplication, deletion and conversion generating a set of very young extant genes.^{10,14} RNA editing has been inferred¹⁵ based on comparisons between the perfect full-length open reading frames from 170 genes sequenced from three animals¹³ and the cDNA sequences from the same animals,¹¹ of which half encode truncated and missense proteins due to SNPs and small indels that are not encoded by the genes. It is noteworthy that some of the missense amino acid sequences predicted from cDNA sequences can be identified from proteins isolated from coelomocytes,⁹ indicating that edited mRNAs are translated. Overall, the *Sp185/333* system is a family of tightly clustered, similar genes that undergo processes of diversification, and mRNA that is edited to generate a broad array of proteins.

All of the deduced Sp185/333 proteins share overall structural similarities that include a hydrophobic N-terminal signal sequence, a glycine-rich region with an integrin binding motif (RGD), a histidine-rich region and a C-terminal region.^{11,12} Except for the α -helical prediction for the hydrophobic signal sequence, the amino acid sequences of the Sp185/333 proteins do not enable informative predictions of secondary structures or folding patterns, which suggests that they may be intrinsically disordered. The size of the Sp185/333 proteins isolated from coelomocytes range from 30 kDa to > 200 kDa,^{9,15} which is larger than predicted from the cDNA sequences (4–55.3 kDa).¹¹ This appears to be due multimerization, which may be augmented by post-translational modifications and results in as many as 264 distinct proteins in an individual sea urchin.⁹

The Sp185/333 proteins are localized to two subtypes of coelomocytes—polygonal phagocytes and small phagocytes—where they associate with membranes and are localized to perinuclear vesicles, and are present on the cell surface of small phagocytes.^{15,16} Following immune challenge *in vivo*, the proportion of Sp185/333-positive (Sp185/333⁺) cells in the coelomic cavity significantly increases relative to pre-challenge.¹⁵ However, it is not known whether Sp185/333 proteins are present in various organs and tissues throughout the body. Therefore, we evaluated both immunoregulated (immune down-regulated) sea urchins and animals challenged with LPS and found that Sp185/333 proteins are present in the axial organ, pharynx, esophagus, intestine and gonads. *Sp185/333* gene expression increases in intestine, axial organ and coelomocytes after immune challenge compared with immunoregulated animals. Sp185/333⁺ cells are present in all organs, and the proportion of Sp185/333⁺ cells in the axial organ and coelomocytes is higher in immune-

challenged animals compared with non-challenged control animals. Similarly, the Sp185/333 protein content is higher in the pharynx, esophagus, intestine, axial organ and coelomocytes from immune-challenged animals compared with samples from non-challenged control animals. The size range of the major Sp185/333 proteins in organs is < 5 kDa to > 250 kDa, which is broader than that observed previously for coelomocytes.^{9,15} There are no observable changes in the repertoires of Sp185/333 proteins in organs from the immune-challenged animals compared with control animals. Overall, these results indicate that the Sp185/333⁺ cells are not restricted to the coelomic cavity, but are found throughout the organs and tissues. These immune response proteins are either produced by somatic cells within the organs, in addition to being produced by coelomocytes, or the Sp185/333⁺ coelomocytes are present in all tissues throughout the body.

Materials and methods

Sea urchins

Purple sea urchins, *S. purpuratus*, were supplied by Marinus Scientific (Long Beach, CA, USA) and maintained in aquaria as described.¹⁷ Animals were fed weekly with commercial rehydrated kelp (Quickspice, Commerce, CA, USA).

All animals ($n=22$) chosen for the study were either immunoregulated (Iq) through acclimation to the aquaria for > 8 months without significant disturbance,^{17,18} or were not allowed to acclimate to an aquarium [non-acclimated (N-Ac); $n=2$] and used within 2 wk of shipping.

Immunological challenge

A subset of Iq sea urchins ($n=12$) was activated immunologically by 1–4 separate injections of LPS (Sigma-Aldrich, St. Louis, MO, USA) (1.0 $\mu\text{g}/\mu\text{l}$) in artificial coelomic fluid (aCF; see Brockton et al.¹⁵) such that each animal received $\sim 1 \mu\text{g}$ LPS per ml of coelomic fluid according to Smith et al.¹⁹ The initial injection was administered at 0 h, followed by a second injection at 24 h. Some animals ($n=2$) received a third injection at 48 h, and 1 animal received a fourth injection at 60 h (Table 1). Samples were collected 24 h after each injection (see below). Iq animals were redefined as challenged (Ch) after injections. A second subset of Iq animals were never challenged and served as the controls.

Antisera

Three polyclonal rabbit antisera (anti-185-66, –68, and –71) were raised against three conserved synthetic peptides present in the most common *Sp185/333* cDNA

Table 1. Experimental groups, based on analysis performed on sea urchin coelomocyte and organ samples.

Animal	Injections received (h)	Coelomocytes			Organs						
		Pre-injection analysis	Post-injection analysis	Other analysis	Int	Gonad			Ph	Eso	AO
						M	F	U			
Group I											
5	0, 24	IF	IF	qP	IF,qP	IF,qP			IF	IF,qP	IF
6	0, 24	IF	IF	qP	IF	IF			IF	IF	IF
7	0, 24	IF	IF	qP	IF,qP		IF,qP		IF	IF,qP	IF
Group II											
12	0, 24	P	P	qPE	n/d			qPE	qPE	n/d	qPE
13	0, 24	P	P	qPE	n/d	qPE			qPE	n/d	qPE
14	0, 24	P	P	qPE	E			E	E	E	E
15	0, 24	P	P	qPE	E			E	E	E	E
16	0, 24	P	P	qPE	n/d	E			E	n/d	E
Group III											
17	0, 24	P	E	qP	qPE			qPE	qPE	qPE	E
18	0, 24, 48, 60	P	E	qP	qPE			qPE	qPE	qPE	E
19	0, 24, 48	P	E	qP	qPE			qPE	qPE	qPE	qPE
20	0, 24, 48	P	E	qP	qPE	qPE			qPE	qPE	E
Group IV											
21	n/a	n/d	n/a	qP	IF	IF			IF,qP	IF	IF,qP
22	n/a	n/d	n/a	qP	qP			qP	qP	qP	qP
Controls											
1	n/a	n/d	n/a	qP	IF	IF			IF	IF	IF
2	n/a	n/d	n/a	qP	IF	IF,qP			IF,qP	IF,qP	IF
3	n/a	n/d	n/a	qP	IF,qP		IF		IF	IF,qP	IF
4	n/a	n/d	n/a	qP	IF,qP		IF		IF,qP	IF,qP	IF,qP
8	n/a	P	n/a	qPE	E	E			E	E	E
9	n/a	P	n/a	qPE	n/d			qPE	qPE	n/d	qPE
10	n/a	P	n/a	qPE	E			E	E	E	E
11	n/a	P	n/a	qPE	n/d	qPE			qPE	n/d	qPE

IF: immunofluorescence; P: reverse transcription polymerase chain reaction; E: ELISA; qP: qRT-PCR; Int: intestine; Ph: pharynx; Eso: esophagus; AO: axial organ; n/d: not done; n/a: not applicable; M: male; F: female; U: unknown gender.

sequence, as described previously.^{9,15} Anti-185-66 recognizes a sequence near the N-terminus and adjacent to the leader, anti-185-68 recognizes a sequence in the middle of the proteins near the RGD motif and anti-185-71 recognizes a sequence near the C-terminus.^{9,15}

Immunocytology

Coelomocyte collection. Whole coelomic fluid (fluid plus cells; 200–300 µl) was collected 15 min prior to first injection and 24 h after the last LPS injection. Fluid was withdrawn directly from the coelomic cavity by piercing the peristomial membrane with a 23-gauge needle attached to a 1-ml syringe. The syringe was pre-loaded with 0.3–0.5 ml ice-cold calcium and magnesium-free sea water that included 70 mM EDTA and 50 mM imidazole, pH 7.4 (CMFSW-EI).²⁰ After withdrawal, the whole coelomic fluid was mixed with additional CMFSW-EI that was added to the contents in the syringe for a final dilution of whole coelomic fluid

of 1:3 in CMFSW-EI. Syringe contents were expelled into a 1.5-ml microcentrifuge tube on ice. Cells were counted with a hemocytometer.

Coelomocyte fixation. Cells (1×10^5 in 100 µl CMFSW-EI) were spun onto poly-L-lysine-coated slides (Polysciences, Warrington, PA, USA/Newcomer Supply, Middleton, WI, USA) using a cytospin rotor at 600 g (centrifuge model 5403, Eppendorf, Hamburg, Germany) for 5 min at 4°C followed by incubation at 25°C [room temperature (rt)] for 5 min. The fluid was removed gently, replaced with coelomocyte culture medium (CCM²¹), and incubated at rt for 30 min. Coelomocytes were fixed on slides according to Brockton et al.¹⁵ Following fixation, slides were washed 3 times (5 min each) at rt in PBS (0.2 M NaCl, 0.15 M phosphate buffer, pH 7.4).

Tissue preparation. Whole organs or parts of organs (axial organ, pharynx, esophagus, intestine and

gonad) were removed from N-Ac ($n=1$), Ch ($n=3$) and control ($n=4$) sea urchins, and placed immediately in prefix (4% paraformaldehyde; 0.000025% glutaraldehyde in CCM) with gentle rocking at rt for approximately 8 h, followed by stationary incubation at 4°C overnight (16–21 h). The primary fixative was removed and replaced with secondary fixative (1% formaldehyde; 0.000025% glutaraldehyde; 0.25% Triton X-100 in AC320 buffer) for 3 h at rt with gentle rocking.¹⁵ Specimens were washed in AC320 buffer 3 times (15–30 min each) at rt with gentle rocking, followed by freezing on dry ice in optimal cutting temperature medium (Tissue-Tek®, Sakura Finetek USA, Torrance, CA, USA) and stored at -70°C .

Tissue sections. A cryostat (Hacker-Bright Safecut 7000 or Microm HM 505E; Zeiss, Oberkochen, Germany) was used to cut 3–10- μm frozen sections of organs according to machine specifications. Both longitudinal and transverse sections were obtained for pharynx, intestine and axial organ, and longitudinal sections were cut for esophagus and gonads. Sections were bound to Fisherbrand® Superfrost®/Plus microscope slides (Thermo Fisher Scientific, San Jose, CA, USA) by incubation at 25–35°C for 10–20 min, and stored at -70°C .

Coelomocyte and tissue staining. Fixed coelomocytes and tissue sections were incubated with blocking solution (2% v/v normal goat serum and 1% BSA in PBS) in a humid chamber at rt. Cells or sections were incubated with an equal mixture of anti-185-66, -68 and -71 antisera (1:4000 dilution in blocking solution) plus mouse monoclonal anti-actin Ab (1:600 dilution; MP Biomedicals, Solon, OH, USA) for 1 h at rt in a humid chamber. Slides were washed with PBS as described above and incubated with a mixture of goat anti-rabbit-Ig (G α R-Ig) conjugated to AlexaFluor 568 (1:400 dilution; Pierce Biotechnology, Rockford, IL, USA) and donkey anti-mouse-Ig (D α M-Ig) conjugated to AlexaFluor 488 (1:200 dilution; Pierce Biotechnology) for 1 h at rt in a humid chamber. Negative controls included normal rabbit serum substituted for the primary antisera using the same dilution in blocking buffer followed by the secondary Ab. Samples were washed with PBS as described above and mounted with ProLong® Gold Antifade with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA, USA).

Microscopy. Whole fixed organs or freshly dissected unfixed organs were viewed and digital images were recorded using a Wild M5 dissecting microscope. Coelomocytes and tissue sections were examined with an Axioplan fluorescence microscope (Carl Zeiss Microscopy, Jena, Germany). Cells were examined with either a 10 \times NA 0.25 plan phase, 20 \times NA 0.5 or 40 \times NA 0.75 planapochromatic phase contrast

objective lens connected to a CCD camera (Hitachi, Tokyo, Japan). The Axioplan microscope was used to collect epifluorescent images of stained cells. Images were also recorded for ovary and axial organ on an Axio Examiner upright spectral confocal laser-scanning microscope (CLSM, Zeiss LSM 710). Images were captured using either 20 \times /0.8 M27 D=0.55 or 63 \times /1.40 Oil DIC plan-apochromatic phase contrast objective lens.

Sp185/333⁺ cell quantitation. The Olympus MicroSuite™ B3SV software program was used to quantify the number of Sp185/333⁺ cells compared to the total number of cells. Cells in 5–13 fields were counted for each slide. Between 1 and 3 sections were analyzed for each tissue.

RT-PCR

Sample preparation and RNA isolation. Coelomocytes were collected as described above, counted on a hemocytometer, and approximately 10⁵–10⁷ cells were transferred to 1.5-ml microcentrifuge tubes and centrifuged at 10,000 *g* for 1–2 min at 4°C. Pelleted cells were stored in RNAlater (Ambion Diagnostics, Austin, TX, USA) at -20°C . Axial organ, pharynx, esophagus, intestine and gonads were dissected from animals and placed immediately in RNaprotect® (Qiagen, Valencia, CA, USA) and stored at -20°C . Total RNA was extracted using an RNeasy Mini or Micro Kit (Qiagen) according to manufacturer's protocols. Contaminating DNA was removed by treatment with RNase-free DNase according to the manufacturer's protocols (Promega, Madison, WI, USA).

Reverse transcription. Total RNA samples were reverse transcribed using SuperScript™ III reverse transcriptase (Invitrogen) with 7- μM random hexamer primers (Operon Technologies, Huntsville, AL, USA) according to the manufacturer's instructions. An initial PCR was performed with SpL8 primers (SpL8F: CAG CGT AAG GGA GCG GGA AGC GTC TT; SpL8R: GTT TGC CGC AGA AGA TGA ACT GTC CCG TGT A) that amplified sea urchin SpL8 (encodes a homologue of the human ribosomal L8 protein; GenBank accession number R62029) to examine the cDNA quality and to evaluate the presence of genomic DNA (the primers were positioned on either side of a small intron). Sp185/333 gene expression was evaluated with a pair of primers (F5; GGA ACY GAR GAM GGA TCT C and 3'UTR; AAA TTC TAC ACC TCG GCG AC) that amplified a 200-base pair fragment, which corresponded to the 3' end of the coding region plus part of the 3' untranslated region (3'UTR).¹² PCR reactions employed 0.4 μM of each primer, 0.2 mM of each deoxynucleotide, 2 mM MgCl₂, 1 \times company supplied buffer, 0.1 U of Taq DNA polymerase (Thermo

Fisher Scientific), plus 2 μ l of the reverse transcriptase reaction (cDNA template) in a total volume of 12.5 μ l. Reactions were performed in either an iCycler IQ in non-quantitative mode (Bio-Rad Laboratories, Hercules, CA, USA), a PTC-200 Peltier Thermo Cycler (MJ Research, Ramsey, MA, USA) or a GeneAmp PCR System 9600 (PerkinElmer, Waltham, MA, USA). The PCR program was 95°C for 5 min followed by 25 cycles of 95°C for 1 min, 61°C (*SpL8* primers) or 56°C (*Sp185/333* primers) for 1 min, 72°C for 2 min, with a final extension of 72°C for 7 min and a hold at 4°C. Amplicons were electrophoresed through 1% agarose (Promega) in 0.5 \times TBE buffer (0.045 M Tris-borate, 0.001 M EDTA). Images were captured on a DC120 digital camera (Eastman Kodak, Rochester, NY, USA) with Digital Science1D software v. 3.0.0 (Eastman Kodak).

Quantitative RT-PCR

Quantitative RT-PCR (qPCR) was performed on the same cDNA samples used for RT-PCR. *SpL8* cDNA was amplified with SL8F (CAC AAC AAG CAC AGG AAG GGA) and ASL8 (AGC GTA GTC GAT GGA TCG GAG T), and a region of the *Sp185/333* sequence was amplified using the primers F5 and 3'UTR (see above). Each qPCR reaction contained 1 \times Absolute QPCR SYBR Fluorescein Mix (ABgene, Surrey, UK), 0.5 μ M of each primer, and 1–2 ng of cDNA in a total volume of 20 μ l. A standard curve was generated using five 10-fold serial dilutions (10^3 – 10^7 plasmids/sample) of a *Sp185/333* gene cloned from coelomocyte genomic DNA (GenBank accession number EF607663.1). qPCR reactions were performed on an iCycler IQ (Bio-Rad Laboratories) in duplicate using the following program: 95°C *Taq* activation for 12 min, followed by 40 cycles of 95°C for 15 s, 59°C for 45 s and 72°C for 30 s. The amount of fluorescence was collected during the annealing step of amplification. A melt curve was performed for each sample to document amplicons of a single size. Data was collected and analyzed using iCycler software (Bio-Rad Laboratories).

ELISA

Sample preparation. The axial organ, pharynx, esophagus, intestine and gonads were dissected from Iq ($n=4$) and N-Ac ($n=9$) animals. Each sample of whole coelomic fluid was centrifuged (see above), the cell-free coelomic fluid was decanted and the pelleted cells were frozen in liquid nitrogen. Tissue samples were placed directly into 1.5-ml microcentrifuge tubes and frozen immediately in liquid nitrogen. All samples were stored at -70°C . Samples were thawed on ice after being overlaid with 200–300 μ l of 20 mM Tris, pH 7.4, containing 10% protease inhibitor cocktail

(Cat. No. P8465, Sigma-Aldrich). Coelomocyte samples were homogenized with a sonic dismembrator (Model 500, Thermo Fisher Scientific) 1–3 times on ice for 10 s each in 20 mM Tris, pH 7.4 with 10% protease inhibitor cocktail. Tissue samples were sonicated 5–7 times on ice for 10 s each in 20 mM Tris, pH 7.4 with 10% or 30% protease inhibitor cocktail. Samples were centrifuged at 10,000 g for 5 min at 4°C. Samples and aliquots to determine total protein concentration (see below) and for Western blot analysis (see below) were stored at -70°C .

Protein content analysis and ELISA. Total protein content in each duplicated sample was determined using a Micro BCATM Protein Assay Kit (Pierce Biotechnology). The second aliquot (see above) for each sample was thawed, adjusted to 0.136 M NaCl by adding distilled water and samples were distributed in duplicate into 96-well ELISA plates (Corning, Corning, NY, USA). Samples of 10 μ g, 5 μ g and 2.5 μ g of total protein were distributed into wells containing TBS (20 mM Tris, 0.136 M NaCl, pH 7.4) for a final volume of 100 μ l per well. Proteins were bound to EIA/RIA plates (Corning) at rt with gentle agitation for 2 h followed by 3 washes of 5 min each with 150 μ l per well of TBST (TBS containing 0.5% Tween-20). Plates were blocked with 4% BSA in TBS at rt with gentle agitation for 1.5 h followed by 3 washes with TBST (5 min each). Plates were incubated with 100 μ l of anti-185 antisera (1:1000 dilution of each anti-185-66, -68 and -71 in TBS) per well at rt for 1.5 h with gentle agitation. Plates were washed and incubated with G α R-Ig conjugated to alkaline phosphatase (1:15,000 dilution in TBS; Sigma-Aldrich) at rt for 1.5 h with gentle agitation. Samples were washed again and incubated with 50 μ l of *p*-nitrophenyl phosphate (1 mg/ml in 1 M diethanolamine, pH 9.8; Sigma-Aldrich) for 10 min in the dark. The reaction was stopped by adding 50 μ l of 3 M NaOH and absorbance was read at 405 nm on a Spectra MAX microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Controls substituted buffer for coelomic fluid proteins or the primary antisera were substituted with normal rabbit serum (1:1000 dilution in TBS).

Western blots

Sample preparation. Freshly collected samples were frozen and stored at -70°C . For analysis, they were thawed, homogenized and the total protein concentration was determined with the Micro BCATM Protein Assay Kit (Bio-Rad Laboratories). Protein samples were diluted to the appropriate concentration and analyzed by Western blot. Some samples were thawed twice from the original homogenized sample that was prepared for ELISA (see above) and used for Western blot analysis.

Protein separation and transfer. Samples (1–50 µg/sample) were heated to 70°C for 10 min in NuPAGE® LDS Sample Buffer (Invitrogen) containing NuPAGE® reducing agent or 0.5 M DTT before loading onto NuPAGE® Novex® pre-casted Bis-Tris 4–20% gradient mini-gels with NuPAGE® MOPS SDS running buffer (Invitrogen). Proteins were transferred onto a polyvinylidene fluoride membrane (Immobilon-PSQ Transfer Membrane; Millipore, Bedford, MA, USA) by semi-dry electroblotting with a Trans-Blot® SD semi-dry transfer cell (Bio-Rad Laboratories) at constant 100 mA for 1.5 h. Transfer buffer was NuPAGE® Transfer Buffer with 10% methanol and 0.1% NuPAGE® antioxidant. After transfer, membranes were stained with 0.1% Ponceau S (Sigma-Aldrich) in 5% acetic acid and de-stained in 5% acetic acid to visualize bands and mark the positions of the standards. Membranes were washed twice in Tris-NaCl (TN; 25 mM Tris pH 7.4, 0.5 M NaCl) and once in Tris-NaCl-Tween (TNT; TN containing 0.1% Tween 20) prior to gentle rocking overnight in 'blotto' (5% milk proteins in TNT).

Ab staining. Membranes were incubated with an equal mixture of anti-185-66, -68 and -71, or pre-bleed serum (1:15,000 dilution for each antiserum in blotto) while rocking at rt for 1.5 h followed by 3 washes with TNT. Membranes were incubated with GαR-Ig conjugated with HRP (GαR-Ig-HRP; 1:30,000 dilution in blotto; Pierce Biotechnology) while rocking for 1.5 h at rt. Membranes were washed twice in TNT and once in TN, followed by 5 min in either Super Signal West Pico Chemiluminescent Substrate System (Pierce Biotechnology) or Western Lightning™ Chemiluminescence Reagent (PerkinElmer). Membranes were exposed to Classic Blue Autoradiography film (MidSci, St. Louis, MO, USA).

Statistical analysis

Statistical analysis of the results from tissue and coelomocyte samples were performed using SAS v. 9.1.3 (SAS, Cary, NC, USA). The relative gene expression ratio (R) for qPCR was calculated using the equation $(E_{\text{target}})^{\Delta C_{\text{P}}^{\text{target}}(\text{control-sample})} / (E_{\text{ref}})^{\Delta C_{\text{P}}^{\text{ref}}(\text{control-sample})}$, where target refers to the *Sp185/333* target sequence and ref refers to the *SpL8* reference sequence.²² Data sets of raw measurements for immunofluorescence (IF) and ELISA, or R values for qPCR, were log₁₀-transformed to approximate the normal overall distributions and statistical significance was assessed by ANOVA in general linear models regression procedures. Bonferroni correction was applied to the pairwise means comparison in order to contrast groups of values, such as different treatments, organs and individual animals, and

the significance levels shown are post-Bonferroni. Experimental error was addressed by including replicate measurements among model parameters.

Results

Preliminary analysis of immune activation and definitions of experimental groups

Individual sea urchins were assessed for their level of immune activation by evaluating small samples of coelomocytes. Results from the preliminary analyses were used to select animals for a more extensive analysis of coelomocytes and organs for *Sp185/333* gene expression and protein content that are reported below. The preliminary analysis of the coelomocytes employed three different approaches prior to and after challenge with LPS, and were designed to detect changes in (i) the percentage of coelomocytes that were *Sp185/333*⁺, (ii) the level of *Sp185/333* transcripts, and/or (iii) the *Sp185/333* protein content. Animals were categorized into four experimental groups (I–IV) based on the combination of method(s) used in the preliminary analysis of coelomocytes to determine whether the animals had responded to the LPS challenge (Figure 1; Table 1).

Preliminary analysis of coelomocytes from animals in group I was based on IF assessment of coelomocytes to determine the percentage of cells that were *Sp185/333*⁺ before immune challenge and 24 h after a second injection of LPS (Figure 1). Animals showing approximately a twofold increase in the percentage of *Sp185/333*⁺ coelomocytes after challenge compared with the pre-challenge state of immunoquiescence (3 of 7 animals; Table 1, animals #5–7) were chosen for further analysis. Coelomocytes from animals in group II were evaluated for *Sp185/333* transcript level before and 24 h after immune challenge using RT-PCR (Figure 1). Gel images of the amplicons were used to estimate the change in *Sp185/333* transcript level in coelomocytes induced to Ch from Iq animals. Those animals that showed a substantial increase in *Sp185/333* transcript level in response to immune challenge were chosen for further study (5 of 7 animals; Table 1, animals #12–16). Coelomocytes from animals in group III were collected 24 h after 2 or more injections of LPS and evaluated using 2 methods (Figure 1). RT-PCR was used to estimate the *Sp185/333* transcript level prior to immune challenge and ELISA was used to evaluate the *Sp185/333* protein content in coelomocytes after immune challenge. Animals in group III that had a lower level of *Sp185/333* transcripts prior to immune challenge were assumed to have a lower level of *Sp185/333* protein content compared to post-challenge. Immune-challenged animals that had a twofold higher *Sp185/333* protein content in the coelomocytes over the internal control background levels were chosen for

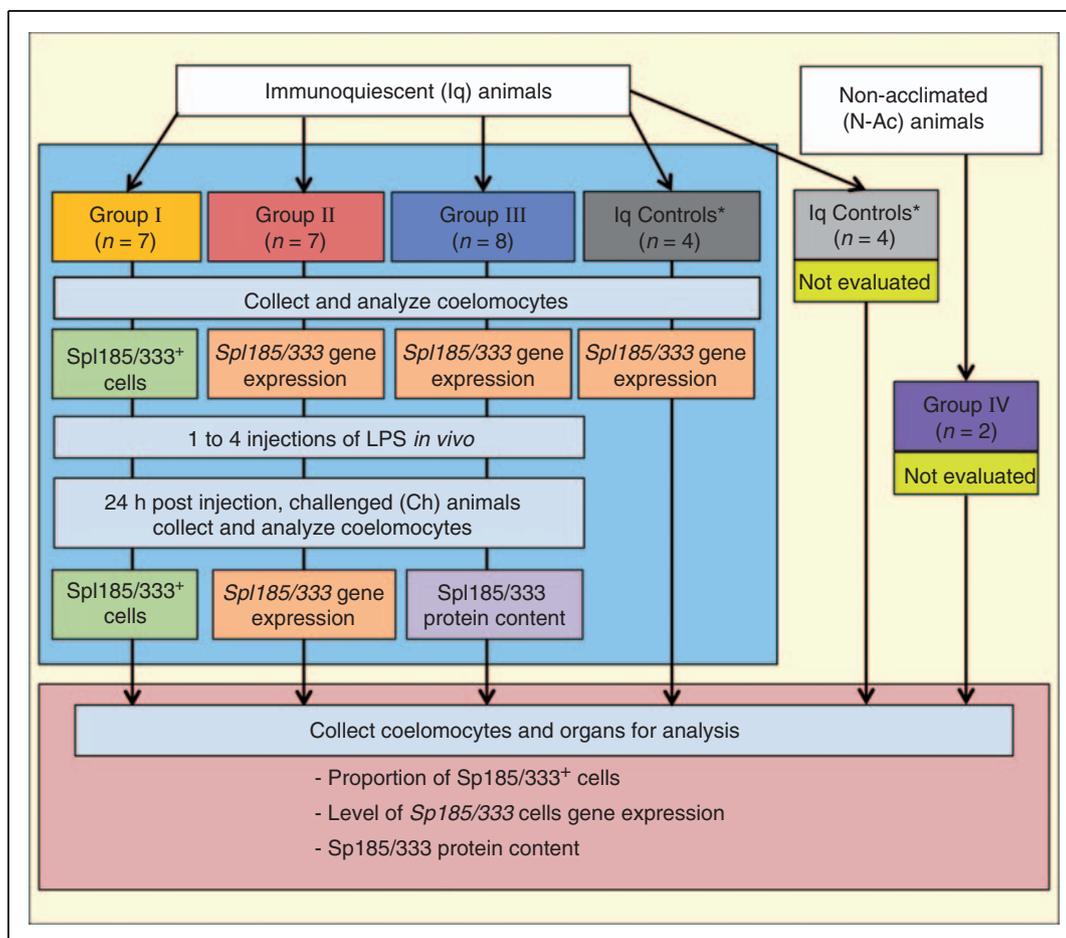


Figure 1. Flow chart of preliminary experimental analysis of coelomocytes and organs. The immune status of the sea urchins that were chosen for analysis is indicated as either immunoquiescent (Iq) or as not acclimated to the aquaria (non-acclimated; N-Ac) with unknown immune status. The area bounded by the light blue box indicates preliminary analysis that was done on coelomocytes to determine whether sea urchins had responded to LPS and had become immune challenged (Ch). Coelomocytes were evaluated using different combinations of analyses (groups I–III; indicated in bold colors), including (i) an evaluation of the number of *Spl185/333*⁺ cells (light green), (ii) an evaluation of *Spl185/333* gene expression (light orange) (iii) and an evaluation of the *Spl185/333* protein content (lavender). Three groups of controls were employed: Iq animals that were evaluated for *Spl185/333* gene expression (dark gray), another group of Iq animals that were not evaluated (light gray), and a third group of animals that were N-Ac and were not evaluated (dark purple). After the preliminary analyses were completed on coelomocytes, selected sea urchins were sacrificed and the organs and coelomocytes were collected for further evaluation (area bounded by the pink box). Results from the post-challenge samples are reported in this article. Colors of the boxes indicating the groups and controls are also used in Figures 2, 5, 9 and 10. *Indicate that data from control animals were combined for some analyses (see Table 1).

dissection and analysis of the organs (4 of 8 animals; Table 1, animals #17–20). Group IV animals were N-Ac animals and assumed to be immune activated upon delivery to the laboratory. Consequently, immune activation through LPS injection was not administered and samples for preliminary evaluation were not collected (Figure 1, Table 1, animals #21–22). Coelomocytes from a subgroup of the controls were analyzed for *Spl185/333* transcripts using RT-PCR to demonstrate no expression prior to organ collection (Figure 1; Table 1, animals #8–11). The remaining control animals were assumed to be Iq and no preliminary analysis was done on coelomocytes prior to organ collection (Figure 1; Table 1, animals #1–4).

Spl185/333 transcripts increase in intestine, axial organ and coelomocytes after immune challenge with LPS

Increases in *Spl185/333* gene expression has been demonstrated previously by non-quantitative methods;^{3,7,11} consequently, we repeated the evaluation of gene expression in coelomocytes using quantitative methods, and found that Ch animals in groups I–III showed a significant increase in the relative *Spl185/333* transcript level after immune challenge compared with the same animals before challenge ($P < 0.05$; Figure 2). However, results for group IV did not show increased

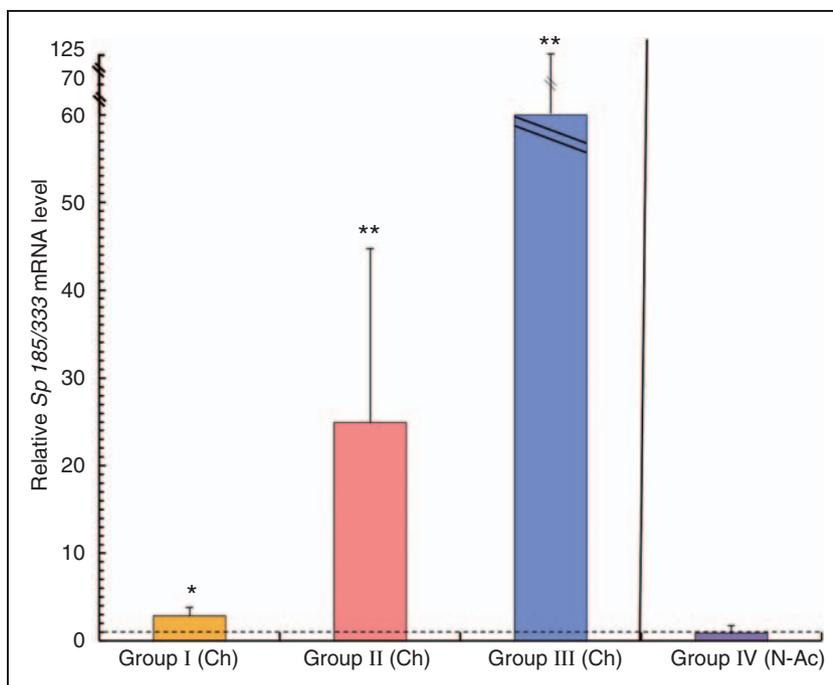


Figure 2. *Sp185/333* gene expression increases in coelomocytes in sea urchins after immune challenge. qPCR was used to determine the *Sp185/333* gene expression in coelomocyte samples relative to *SpL8* expression. Animals are grouped by treatment, which includes post-immune-challenged animals (Ch; bars to the left of the vertical dotted line), non-acclimated animals (N-Ac; bar to the right of the vertical solid line) and control animals. The combined results for coelomocytes collected from lq plus control animals (light and dark gray as in Figure 1) were set to 1 and are represented by the horizontal dotted line. Ch animals are separated into Groups I–III, and N-Ac animals are in Group IV (see Table 1; group colors as in Figure 1). Significant differences (* $P < 0.05$ or ** $P < 0.0001$) are in comparison to *Sp185/333* expression in coelomocytes from respective lq or control animals. SEM are shown.

transcript level, which indicated that although these N-Ac animals were assumed to be immune activated, the coelomocytes did not contain an elevated level of *Sp185/333* transcripts compared with coelomocytes from animals in groups I–III that had received LPS. When the relative *Sp185/333* mRNA levels in coelomocytes collected prior to immune challenge in groups I–III were compared with coelomocytes from the control group, no differences in the relative transcript levels were observed (data not shown). These results demonstrated that qPCR could be used to evaluate *Sp185/333* transcript content in coelomocytes and that the results were in agreement with previous reports.^{3,7,11}

Although *Sp185/333* gene expression in coelomocytes has been well documented (reviewed in Ghosh et al.²³ and Smith²⁴) it is not known whether these genes are expressed by cells in organs. Therefore, we undertook a preliminary analysis of organ samples from *S. purpuratus* and found that every organ showed *Sp185/333* gene expression (Figure 3). Because *Sp185/333* transcripts increase in coelomocytes after exposure to bacteria or to several different pathogen-associated molecular patterns,^{3,7,9,11} we measured whether *Sp185/333* transcript level changed in organs after LPS injections compared with organs from control animals. Quantitative analyses of the relative *Sp185/333* transcript level in organ samples for Ch

animals in groups I–III, as well as N-Ac animals in group IV (Table 1), were compared with the control samples. Results showed an increase in the relative *Sp185/333* transcript levels in the intestine for groups III and IV animals after challenge ($P < 0.05$; Figure 4). There was also an increase in the relative *Sp185/333* transcript level in the axial organ for group III compared with controls ($P < 0.05$; Figure 4), but elevated transcripts were not observed for samples from other groups. No significant changes in the relative *Sp185/333* transcript level after immune challenge were observed in gonad, esophagus or pharynx for any experimental group. Overall, these results provided initial evidence that *Sp185/333* expression in intestine and axial organ in Ch and N-Ac animals was responsive to immune challenge.

Sp185/333 proteins increase in coelomocytes, intestine, esophagus, pharynx and axial organ after immune challenge with LPS

Previous work demonstrates that *Sp185/333* proteins in coelomocytes increase significantly with a ~twofold increase in protein content between 24 and 48 h after LPS injection compared with control animals that were sham injected.⁹ Because *Sp185/333* gene expression was observed in all major organs, and the level of mRNA

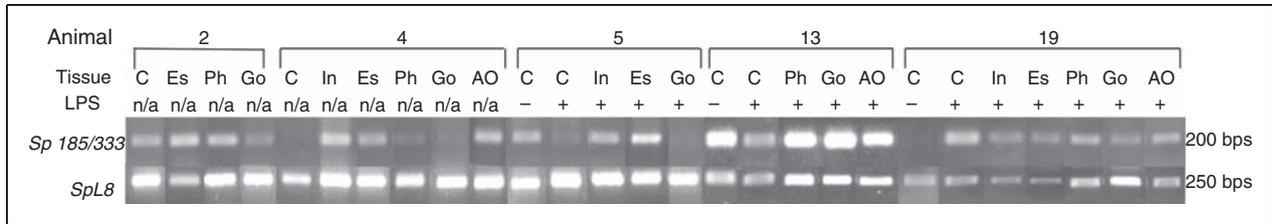


Figure 3. *Sp185/333* genes are expressed in organs. RT-PCR was used to analyze gene expression in organs collected 24 h after LPS injection. cDNA samples were amplified with a pair of primers (F5 and 3'UTR) that target a portion of the full-length *Sp185/333* transcript, and a different pair of primers that target the *SpL8* transcript (encodes a homologue of the human ribosomal L8 protein), which was used to assess sample quality. C: coelomocyte; Es: esophagus; Ph: pharynx; Go: gonad; In: intestine; AO: axial organ; +: Ch animal that was challenged with LPS; -: lq animal; n/a: not applicable.

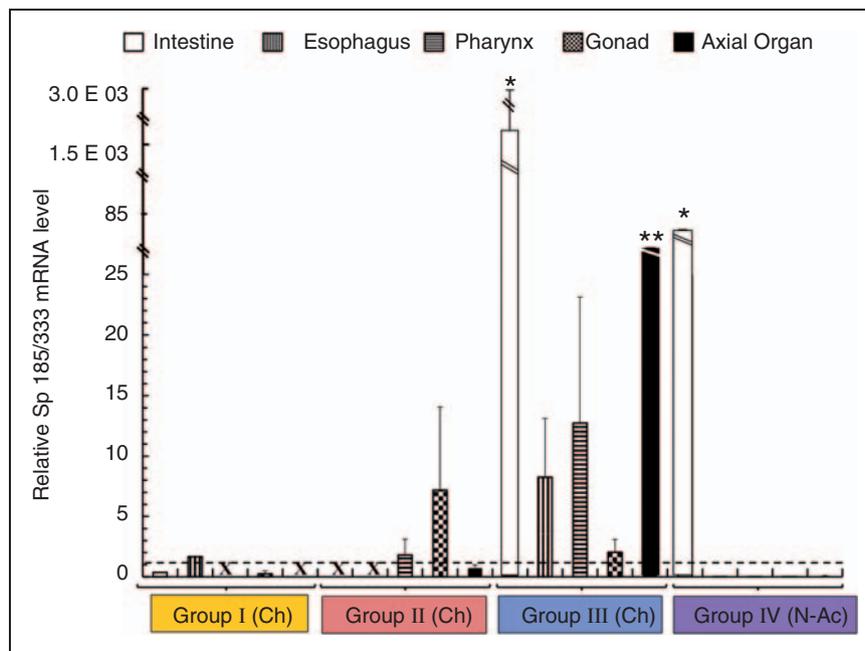


Figure 4. *Sp185/333* gene expression increases in organs of sea urchins after immune challenge. qPCR was used to determine the relative *Sp185/333* gene expression in coelomocytes and organ samples relative to *SpL8* expression. Animals are grouped by treatment, which includes Ch and N-Ac animals (Groups I–IV, colors as in Figure 1) and control animals (light and dark gray as in Figure 1; see also Table 1) which were set to 1 and represented by the horizontal dotted line. Significant differences in *Sp185/333* gene expression (* $P < 0.05$ or ** $P < 0.0001$) are relative to the expression in the same organs from the control animals. X indicates missing data. SEM are shown.

significantly increased in some of these organs after immune challenge (see above), we assumed that there would also be an increase in the level of Sp185/333 proteins in organs from Ch animals. Therefore, we analyzed the Sp185/333 protein content in organ samples from animals that received LPS injections compared with samples from control animals. We also compared the Sp185/333 protein content in coelomocytes from the Ch animals before and after challenge to confirm that the immune response was activated in these animals. Results for coelomocytes from animals in group III (Figure 1; Table 1) showed an increase in the level of

Sp185/333 proteins in response to LPS challenge compared with the pre-challenge time point ($P < 0.01$); however, group II animals did not show a similar increase (Figure 5). We also evaluated the level of Sp185/333 proteins in organ samples for groups II and III after injections and compared them to the protein level in organ samples from control animals. Results indicated that group III had an overall increase in Sp185/333 proteins in the pharynx ($P < 0.05$), esophagus, intestine and axial organ ($P < 0.01$); however, there was no change in the level in testes for Ch animals compared with control animals for this group

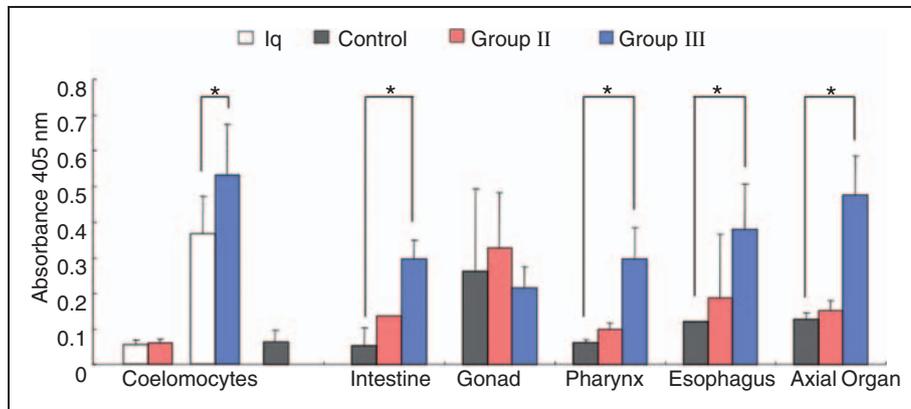


Figure 5. Sp185/333 protein content increases in coelomocytes, intestine, esophagus, pharynx and axial organ in sea urchins after immune challenge. Treatment groups include animals prior to challenge with LPS (Iq, white bars), post-immune challenge (Ch, group colors as in Figure 1), and controls (gray bars as in Figure 1). Significant differences (brackets with asterisk, $P < 0.05$) are compared to protein content from control animals. SEM are shown.

(Figure 5). Animals in group II showed no significant change in Sp185/333 protein content in any organ after immune challenge compared with the controls, similar to the results from coelomocytes for that group. Overall results from immune-challenged animals for coelomocytes and organs for group III animals showed an increase in the level of Sp185/333 proteins for both coelomocytes and cells associated with the organs, while the level of Sp185/333 proteins from group II animals did not change after immune challenge. These results reflect the preliminary analyses of coelomocytes in response to immune challenge that were done prior to organ collection. Coelomocytes from group II were elevated for *Sp185/333* mRNA level, while coelomocytes from group III were evaluated for Sp185/333 protein content (Figure 1; Table 1). Preliminary evaluation of coelomocytes in group III showed an increase in Sp185/333 protein content and these changes were also observed in the organs in the subsequent experimental analysis (Figure 5). However, it was assumed that because the level of *Sp185/333* transcripts had increased in coelomocytes from group II after immune challenge, there would be a correlated increase in protein content for the organs. However, results for the organs from group II animals did not reflect these changes. Furthermore, all sea urchins in group II received two injections of LPS, while most of the animals in group III received three or more injections prior to organ collection (Table 1). The preliminary evaluation of coelomocytes for the level of *Sp185/333* mRNA may not be a good predictor of protein levels in the organs. However, three injections of LPS that were administered to animals in group III may have been required to increase the level of Sp185/333 proteins above the pre-injection controls. This is consistent with the level Sp185/333 proteins in group II animals

that did not change in response to two injections of LPS.

Sp185/333⁺ cells in the axial organ and Sp185/333⁺ coelomocytes increase after immune challenge with LPS

Because the Sp185/333 proteins were detected in all of the organs, we next evaluated the location of the Sp185/333⁺ cells in organ samples using IF (Table 1). Sp185/333⁺ cells within the axial organ (Figure 6A) were dispersed throughout the organ, lining various canaliculi or lacunae, and, in some cases, were located within these spaces (Figure 6B–D). In the intestine (Figure 7A) and esophagus (Figure 7B), Sp185/333⁺ cells were present within the columnar epithelium lining the lumen of the intestine and along the coelomic epithelium covering the gastro-intestinal (GI) tract and in contact with the coelomic fluid (Figure 7C, D). Sp185/333⁺ cells were not observed in the lumen of the GI tract (pharynx, esophagus and intestine). The pharynx (Figure 7E–G) is a complex structure with columnar epithelium lining the lumen of the mouth cavity and connective tissue connecting the pharynx to the lantern structure.²⁵ Here, Sp185/333⁺ cells were dispersed throughout the pharynx in both the peripheral connective tissue (Figure 7H) and the columnar epithelium (Figure 7I). Sp185/333⁺ cells were scattered throughout the testes (Figure 8A shows a mature gonad), both in the center near mature sperm and along the coelomic epithelium (Figure 8B, C). Sp185/333 proteins were found within the ovaries; however, the patterns of the Sp185/333 proteins were associated with ring structures localized to the periphery along the outer rim of mature eggs (Figure 8D, E, yellow arrow). In the oocytes, the Sp185/333 proteins were present as

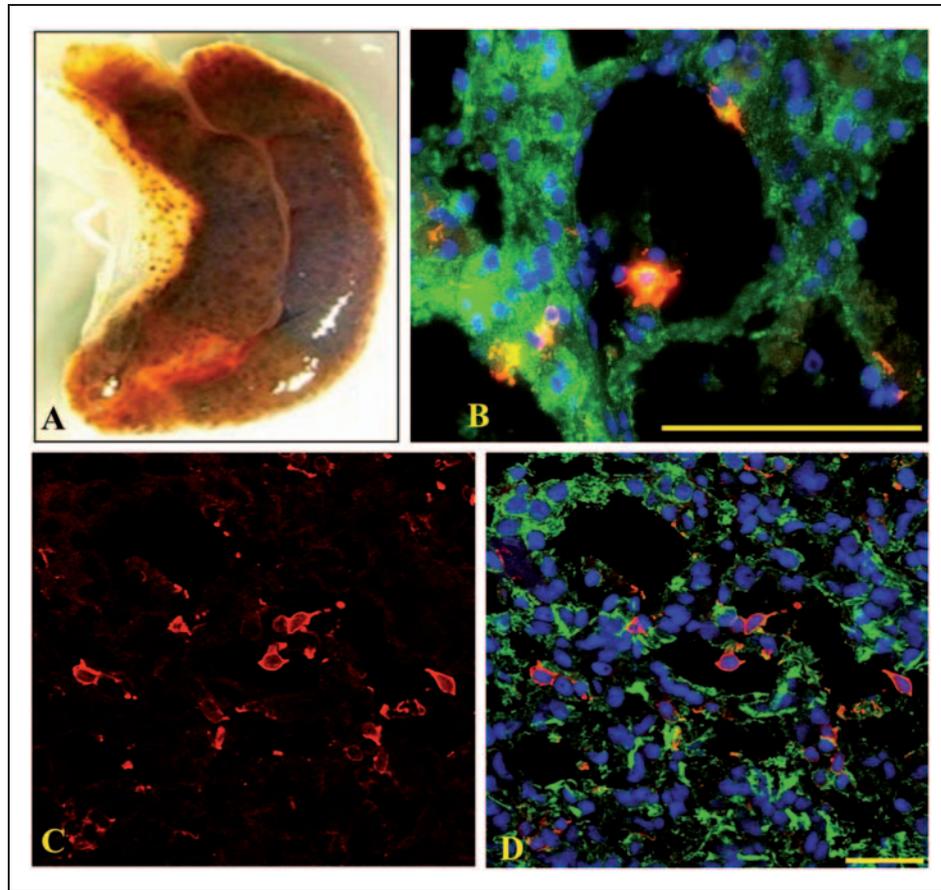


Figure 6. Sp185/333⁺ cells are present in axial organ. (A) A whole fixed axial organ. (B–D) Sp185/333⁺ cells are present within the various spaces and dispersed throughout the axial organ. Sp185/333⁺ cells are red, actin is green and DNA is blue. Image A was recorded on a Wild M5 dissecting microscope. Image B was recorded on an Axioplan fluorescence microscope (Zeiss). (C), (D) Paired images recorded on a spectral CLSM (LSM 510 META, Zeiss). Images B–D are transverse sections of the organ. Bar is 100 μ m.

ring structures scattered about the cytoplasm (Figure 8D, red arrows; Figure 8F, yellow arrow) in a pattern consistent with cortical granules. The Sp185/333 proteins may also be present in follicular cells that surround the eggs and oocytes.^{26–28}

Because Sp185/333⁺ cells were present in all organs that were examined, we next evaluated whether there were changes in the percentages of Sp185/333⁺ cells in the organs in response to immune challenge (Table 1). Results for group I Ch animals compared with controls showed a significant increase in the percentages of Sp185/333⁺ cells in the axial organ after challenge ($P < 0.0001$); however, there were no detectable changes in pharynx, esophagus, intestine and testes compared with the controls (Figure 9). Similar results were obtained for the group IV N-Ac animals, which had a higher percentage of Sp185/333⁺ cells in the axial organ, pharynx and esophagus compared with controls ($P < 0.05$; data not shown). There was no change in the percentage of Sp185/333⁺ cells in the intestine and testes from animal 21 (group IV). The locations of Sp185/333⁺ cells within some of the organs were also evaluated. Within the esophagus and intestine there

was a higher percentage of Sp185/333⁺ cells in the coelomic epithelium in Ch animals compared with controls ($P < 0.05$), whereas there was no difference in the percentage of Sp185/333⁺ cells in the columnar epithelium from Ch animals compared with controls (Figure 10). When the location of the Sp185/333⁺ cells within pharyngeal tissues was evaluated without regard to the treatment (i.e. Ch animals and controls were analyzed together given that there was no difference between them), the percentage of Sp185/333⁺ cells was higher in the connective tissue at the periphery of the pharynx compared to the columnar epithelium that lines the mouth ($P < 0.05$; Figure 10). There were no differences in the location of Sp185/333⁺ cells in the testes (center vs periphery), which did not change with respect to immune challenge. The Sp185/333⁺ cells were distributed uniformly throughout the axial organ, including sections from the end regions and the middle of the organ. In summary, Sp185/333⁺ cells were observed in all organs. There was as an overall increase in the percentage of Sp185/333⁺ cells in axial organ in Ch animals, whereas the increase in Sp185/333⁺ cells was variable among the other organs from Ch animals.

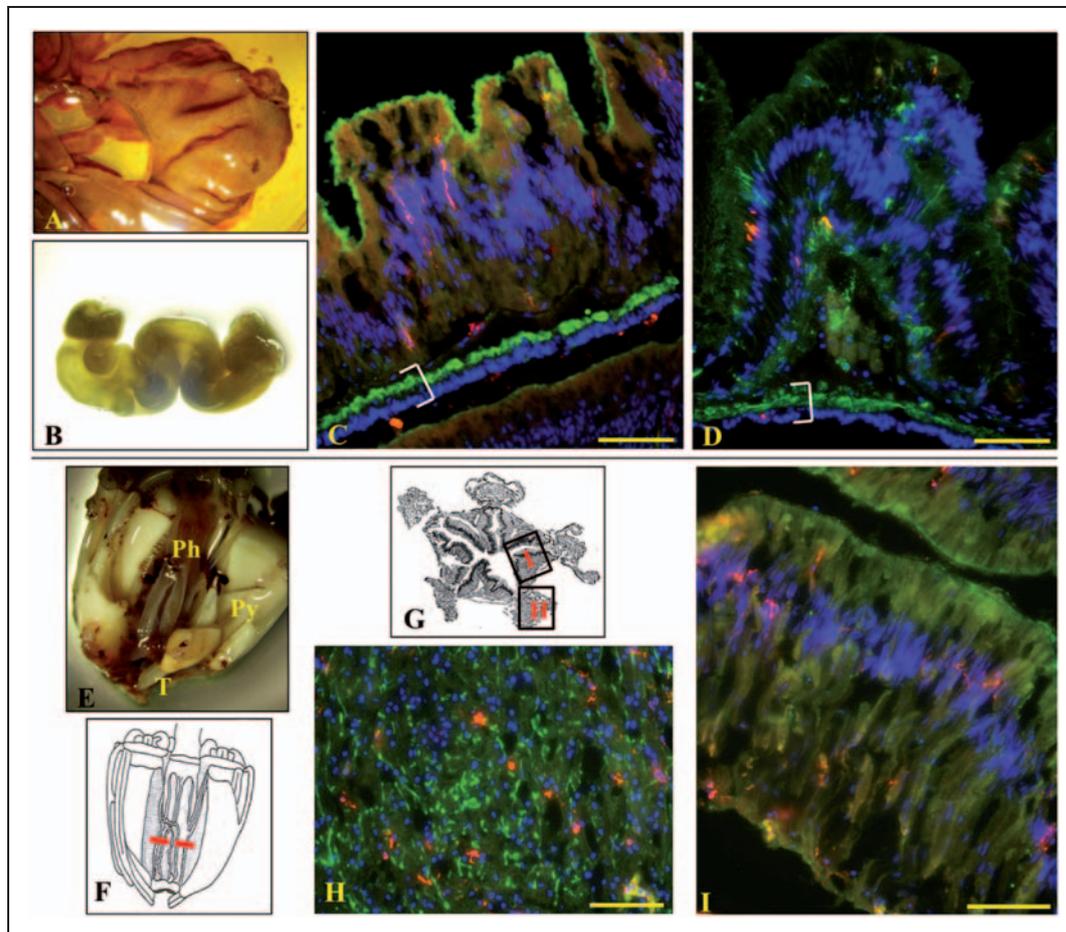


Figure 7. Sp185/333⁺ cells are present in the pharynx, esophagus and intestine. (A) A fixed portion of the intestine. (B) Whole fixed esophagus. Longitudinal sections are shown for the intestine (C) and the esophagus (D). Sp185/333⁺ cells (red) are present throughout columnar epithelium (green, actin; blue, DNA). White brackets enclose the region containing two layers of muscle fibers and the coelomic epithelium. (E) Whole fixed Aristotle's Lantern (Py: pyramid; T: tooth) with a portion cut away to show the tubular pharynx (Ph). (F) Drawing of the Aristotle's Lantern in which the red horizontal dotted line indicates the location of transverse sections that were evaluated by IF. (G) Drawing of a transverse section through the pharynx. (H) and (I) correspond to images H and I. Sp185/333⁺ cells are located throughout the connective tissue (H) and columnar epithelium (I) of the pharynx. Images A, B and E were recorded a Wild M5 dissecting microscope. Images C and D and H–I were recorded on an Axioplan fluorescence microscope (Zeiss). Bar is 100 μ m.

Furthermore, the percentages of Sp185/333⁺ cells within the esophagus and intestine in Ch animals responding to LPS showed an increase in the percentage of Sp185/333⁺ cells within the coelomic epithelium, which was not observed for cells within the columnar epithelium.

Sp185/333 protein repertoires vary in coelomocytes and organ samples within and among sea urchins

Sp185/333 protein repertoire in coelomocytes is known to be highly variable showing a broad range of size and pI among different animals.^{9,15} However, the protein repertoire has not been investigated in sea urchin organs. Western blot analyses for 12 animals that included samples from Ch animals ($n=9$) and controls ($n=4$) showed that the repertoires of Sp185/333

proteins from organs varied among animals and ranged in size from < 5 kDa to > 250 kDa (a representative blot is shown in Figure 11). The most common small bands, which may be monomers, were ~40 kDa in organs and 52 kDa in coelomocytes. The major bands in coelomocytes ranged from ~90 kDa to < 250 kDa, which was similar to previous reports.^{9,15} In immune-challenged animals, additional bands were observed compared with the Sp185/333 protein bands in the same animals before immune challenge (arrows in Figure 11).

The size range for the major Sp185/333 bands was expanded in the organs compared with the size range in coelomocytes. There was one major pattern of bands in the gonads, which was common among the animals (Figure 11; Table 1). The patterns of Sp185/333 bands in pharynx and esophagus were very similar within and

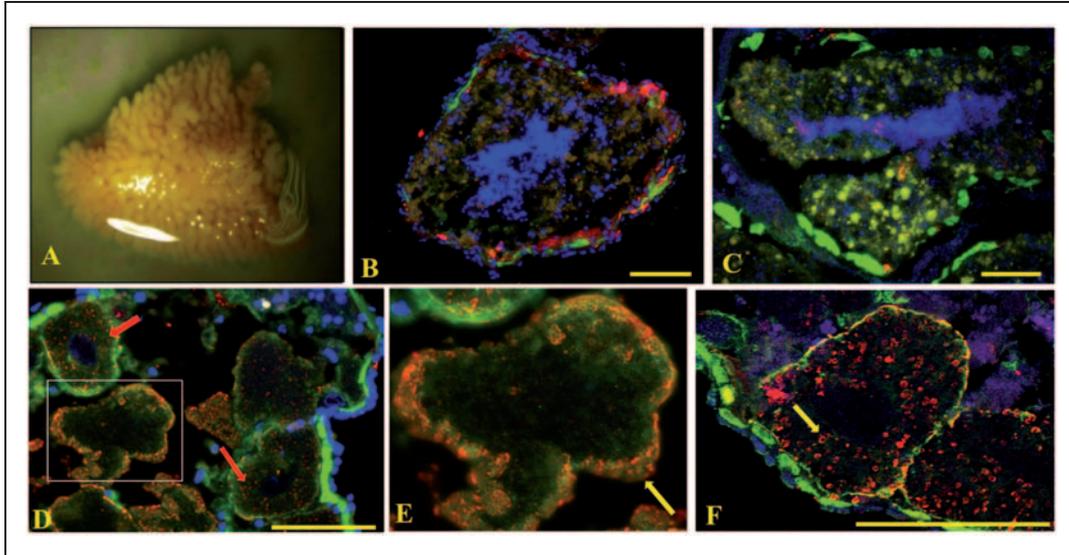


Figure 8. Sp185/333⁺ cells are present in the gonad. (A) A segment of a gonad. (B, C) Testes. Sp185/333⁺ cells (red) are present in association with the coelomic epithelium (B) surrounding the testes and within the testes with the mature sperm (C) (green, actin; blue, DNA). (D–F) Ovary. Sp185/333 proteins appear in a scattered pattern throughout the oocyte (red arrows), but localized to the outer portion of the egg (E; yellow arrow), perhaps in cortical granules and follicular cells. An enlarged portion of image D is shown in E. (F) In the oocytes (yellow arrow), some of the Sp185/333 staining pattern is crescent-shaped and similar in shape to cortical granules. Image A was recorded with a Wild M5 dissecting microscope. Images B–E were recorded with an Axioplan fluorescence microscope (Zeiss). Image F was recorded with a spectral CLSM (LSM 510 META, Zeiss). Longitudinal sections of IF images are shown. Bar is 100 μ m.

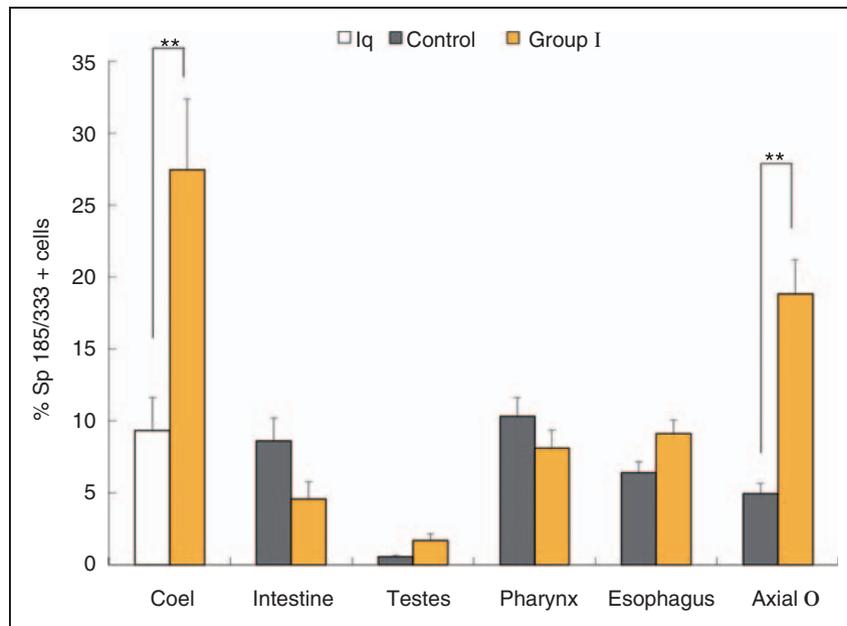


Figure 9. The percentage of Sp185/333⁺ coelomocytes and Sp185/333⁺ cells in the axial organ increases after immune challenge. The percentages of Sp185/333⁺ cells versus the total number of cells were collected for individual animals using IF and grouped by similar treatment and organ. Coelomocytes (coel) were collected from animals in group I ($n = 3$) 0 h before (white bar; lq) and 24 h after (yellow bar, Ch) LPS injection. Tissues were collected from group I animals (yellow bars as in Figure 1) after challenge, and were the same animals that donated coelomocytes ($n = 3$; $n = 2$ for testes). Additional organs were collected from lq control animals ($n = 4$; $n = 2$ for testes; dark gray bars as in Figure 1). The percentages of Sp185/333⁺ coelomocytes increase in challenged animals compared with pre-injection samples (lq), and the percentages of Sp185/333⁺ cells in axial organ (axial o) are also higher in Ch animals compared with controls (bars with asterisk; $P < 0.0001$). No differences in the percentage of Sp185/333⁺ cells in intestine, testes, pharynx or esophagus were observed between Ch and control animals. SEM are shown.

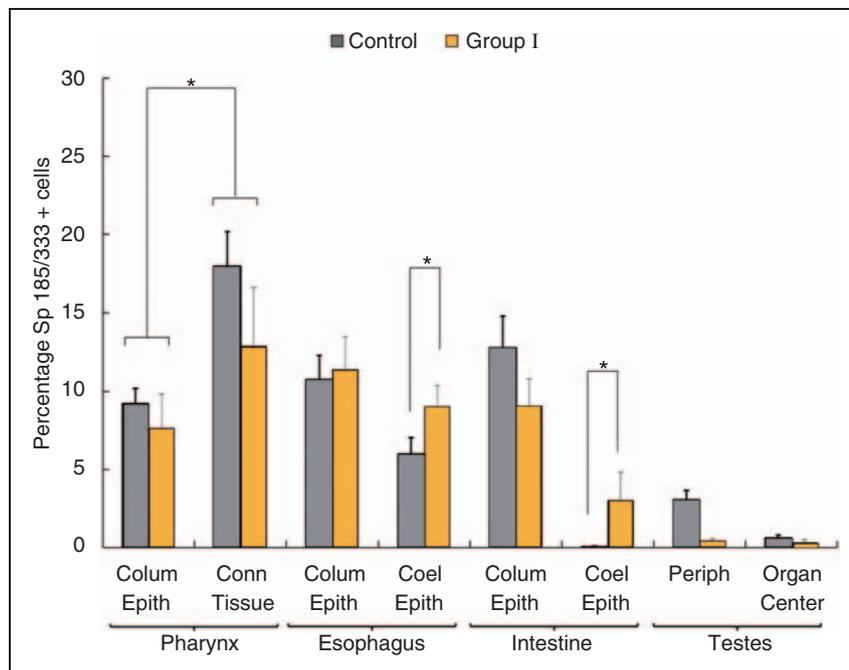


Figure 10. The percentage of Sp185/333⁺ cells in the coelomic epithelium increases in esophagus and intestine after immune challenge compared with the controls. The percentages of Sp185/333⁺ cells versus the total number of cells were evaluated for individual animals using IF and grouped by treatment and tissue. Tissues were collected from controls animals (dark gray bars as in Figure 1; $n = 4$; $n = 2$ for testes) and Ch animals in group I (yellow bars as in Figure 1; $n = 3$; $n = 2$ for testes) 24 h after LPS injection. Percentages of Sp185/333⁺ cells in the coelomic epithelium of the esophagus and intestine are higher in group I Ch animals compared with the same samples in controls (brackets with asterisk; $P < 0.05$). The percentage of Sp185/333⁺ cells in the connective tissue is higher than in the columnar epithelium of the pharynx, when treatment (i.e. Ch vs control) is not taken into consideration (brackets with asterisks; $P < 0.05$). SEM are shown.

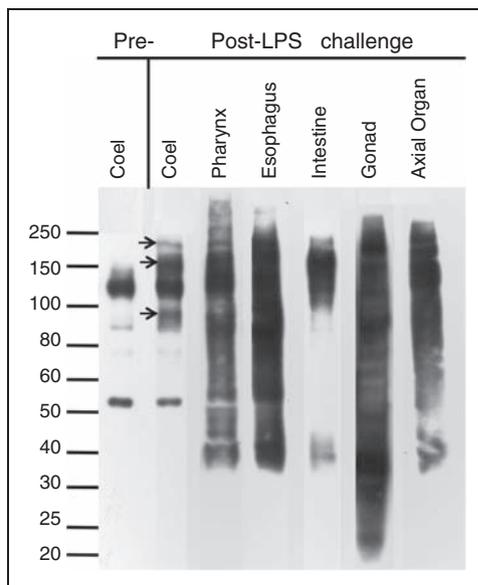


Figure 11. The Sp185/333⁺ banding patterns are different in coelomocytes and organs. Western blot results are shown for coelomocytes (coel), pharynx, esophagus, intestine, gonad (unknown gender) and axial organ for animal #15. Coelomocytes were collected pre- and post-immune challenge with LPS, and organ samples were collected post-immune challenge. Standards are shown on the left. Units are in kDa.

among different animals; however, the patterns in the intestine varied in the number and size of bands among different animals. The Sp185/333 band sizes in the axial organ were markedly similar to both pharynx and esophagus both within and among animals. However, in general, there were fewer bands in the axial organ, which were similar to the overall pattern of bands in coelomocytes after activation with LPS. There were no obvious changes in patterns of Sp185/333 bands in organs from Ch animals after LPS challenge compared with the same organs from controls (not shown). Overall, there was diversity among Sp185/333 protein repertoires among the different organs and coelomocytes within and among animals. In immune-challenged animals there was an expansion in the sizes of the Sp185/333 bands in coelomocytes compared with coelomocytes from unchallenged animals, but there were no major shifts in the repertoire of these proteins in organs from Ch versus controls.

Discussion

We show here that the diverse family of *Sp185/333* immune genes and the proteins that they encode are expressed by cells dispersed in the major organs of *S. purpuratus*, in addition to expression by the

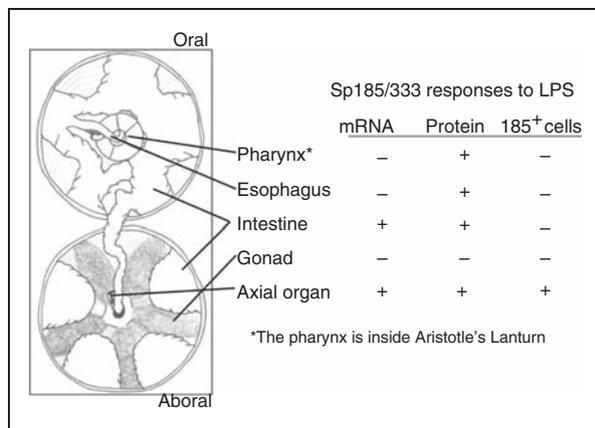


Figure 12. The axial organ and intestine respond to LPS challenge. The internal anatomy of an adult sea urchin that has been cut in half and opened is shown on the left. The table to the right indicates the responses to LPS in Ch animals. The axial organ shows increases in *Sp185/333* mRNA, *Sp185/333* protein and *Sp185/333*⁺ cells after challenge with LPS. The intestine shows increases in *Sp185/333* mRNA and *Sp185/333* protein. Responses by the pharynx and esophagus are consistent with the production of additional *Sp185/333* protein, perhaps from existing mRNA. Neither testis nor ovary show changes in response to LPS. +: samples that show significantly increased levels of *Sp185/333* mRNA, *Sp185/333* protein or *Sp185/333*⁺ cells compared with controls; -: samples that did not show differences from the controls.

coelomocytes that has been reported previously.^{9,15} The *Sp185/333* transcript level and *Sp185/333* protein content in coelomocytes increases after immune challenge, and these changes are also observed in the intestine and the axial organ (Figure 12). While the *Sp185/333* protein content increases significantly after LPS injection in pharynx and esophagus, there is no change in the level of *Sp185/333* transcripts or the proportion of *Sp185/333*⁺ cells in these organs (Figure 12). This suggests that rather than an influx of *Sp185/333*⁺ cells to the pharynx and esophagus, there may be an increase in protein production from pre-existing *Sp185/333* transcripts. Finally, an increase in the proportion of *Sp185/333*⁺ cells was only observed in the axial organ after immune challenge (Figure 12). The increase in *Sp185/333* transcript level, protein content and the proportion of *Sp185/333*⁺ cells after LPS injection in the axial organ suggests that either existing *Sp185/333*-negative (*Sp185/333*⁻) cells may be induced to produce the proteins and/or there may be an influx of *Sp185/333*⁺ cells to the axial organ from elsewhere. In addition to the assessment of *Sp185/333* transcript expression and *Sp185/333* protein content, the range of commonly observed *Sp185/333* protein sizes in the organs (< 5 kDa to >250 kDa) is broader than that detected for the most common *Sp185/333* proteins in coelomocytes (~40–250 kDa). This suggests that these

proteins may either be expressed by non-coelomocytes or that the proteins are multimerized and/or processed differently in organs irrespective of the cell type that expresses them. Post-translational processing may be the major mechanism for generating variations in the *Sp185/333* proteins because the size range of mRNAs is very similar among organs and coelomocytes (Supplementary File 1). In general, the *Sp185/333* proteins are present in the major organs of the sea urchin and this, perhaps, extends throughout all of the tissues.

Sp185/333 in coelomocytes

In response to immune challenge, almost twice as many coelomocytes appear in the coelomic cavity compared with pre-challenge for Iq sea urchins.¹⁵ This includes a significant increase in the total number of *Sp185/333*⁺ cells, which are composed of small phagocytes and polygonal phagocytes. The additional small phagocytes that appear post-challenge include cells that are *Sp185/333*⁻ and *Sp185/333*⁺, suggesting that both appear in the coelomic fluid as a result of either proliferation or release of pre-existing marginated cells.¹⁵ However, there is no overall change in the number of polygonal phagocytes in the coelomic fluid after immune challenge, although there is an increase in the number of this cell type that is *Sp185/333*⁺. This indicates that some of the existing *Sp185/333*⁻ polygonal phagocytes may be induced to produce *Sp185/333* proteins. Our results for coelomocytes are in agreement with these previously published data.¹⁵

Sp185/333 in the intestine

He185/333 proteins have been isolated from in another sea urchin species, *Heliocidaris erythrogramma*, where they are present in cells associated with the intestine and are localized to the coelomic epithelium in contact with the coelomic fluid.¹⁶ This is the same location as reported here for *Sp185/333*⁺ cells in *S. purpuratus*, which are also associated with the coelomic epithelium that covers the outer surface of the intestine. However, *Sp185/333*⁺ cells present in the columnar epithelium of the intestine in *S. purpuratus* were not reported in *H. erythrogramma*.¹⁶ The intestinal localization of the *Sp185/333*⁺ cells within the columnar epithelium is strikingly similar to the variable region-containing chitin-binding proteins (VCBPs) expressed in intestine-associated cells in amphioxus, *Branchiostoma floridae*, and in the solitary tunicate, *Ciona intestinalis*.^{29,30} It is noteworthy that both the *Sp185/333* and the *VCBP* gene families have been described as highly diverse,^{12,13,29–35} that the VCBPs bind to bacteria and augment phagocytosis by hemocytes in *C. intestinalis*³⁰ and that a recombinant *Sp185/333* protein binds to marine Gram-negative bacteria (Lun, Schrankel, Sacchi and Smith, unpublished). The *Sp185/333*⁺

cells in the columnar epithelium of the intestine may be intestine cells, while the cells associated with the coelomic epithelium surrounding the intestine in contact with the coelomic fluid may be coelomocytes. The increased *Sp185/333* transcript level and elevated *Sp185/333* protein content in the intestine in response to immune challenge may be an indication that these cells are involved in immune functions or specialized intestine-associated immunity and that they may all be coelomocytes. Because the GI tract contains ingested and resident/commensal microbes, an active mucosal immune response as predicted from the large numbers of NLR gene models in the genome would be required to control or combat these microbes.^{2,36} This mucosal immune system would likely include cells expressing proteins with antimicrobial function. Overall, the *Sp185/333* protein expression pattern in the intestine is similar to the *He185/333* proteins in the coelomic epithelium, as well as to the staining pattern of VCBPs in the GI tract of a urochordate and a cephalochordate. This suggests that the *Sp185/333* proteins may have immune effector function(s) in organs, either through parenchymal cells of the organs or wandering coelomocytes.

Sp185/333 in the axial organ

The axial organ lies at the central axis of the spheroid echinoid body, and is positioned at the conjunction of the perivisceral coelom, and the water vascular and haemal systems.^{25,37} It is suspended in these cavities through attachment to the stone canal near the esophagus, anus and madreporite.³⁸ It has been described as a porous complex with sinuses or haemal spaces called lacunae and canaliculi.³⁷ Coelomocytes are described as being present in the haemal spaces of the axial organ,^{25,37} but this has not been verified using modern molecular or cellular methods owing to the unavailability of markers that are specific for coelomocytes. Proposed functions of the axial organ include that of an immune organ,^{39–42} or the site of coelomocyte and cell production⁴³ or cell degradation.⁴⁴ It has also been described as an excretory organ,^{45,46} a contractile vessel,³⁷ a primitive heart^{47,48} and a secretory gland.^{48,49} However, owing to the lack of convincing experimental evidence, the functions of this organ remain unknown.

The axial organ is the only organ that shows significant increases in all parameters that were measured: *Sp185/333* transcript levels, *Sp185/333* protein content, and the proportion of *Sp185/333*⁺ cells in response to immune challenge (Figure 12). Although, we could not determine definitively which cell type(s) express the *Sp185/333* proteins in any of the organs, there are several possible explanations for the increased proportion of *Sp185/333*⁺ cells in the axial organ after immune challenge: (i) coelomocytes and/or parenchymal cells

present in the axial organ may respond to immune challenge by inducing *Sp185/333* gene expression and protein production; (ii) *Sp185/333*⁺ cells in the axial organ may proliferate; (iii) *Sp185/333*⁺ cells may enter the organ from the coelomic fluid. Any combination of these possibilities may also occur. The axial organ may be a site of coelomocyte proliferation, including the subsets of phagocytes that express the *Sp185/333* genes and produce the encoded proteins. This hypothesis is consistent with the expression of *SpTie1/2* in the axial organ, which increases in response to immune challenge and is a homologue of vertebrate *Tie* genes that are involved in hematopoiesis.⁵⁰ Recent evidence suggests that cells proliferate in the axial organ, coelomic epithelium and Tiedemann bodies of the sea star, *Asterias rubens*, in response to immune challenge.⁵¹ If axial organ parenchymal cells express the *Sp185/333* proteins, this may indicate that the axial organ plays a role in immunity for the animal, perhaps similar to that of the fat body in insects.⁵² However, if the accumulation of *Sp185/333*⁺ cells is a result of cells migrating to the organ, these may be senescent cells that accumulate in the axial organ to be removed from the body either via the stone canal and madreporite, by transfer to the rectal tissues and removal through the anus, and/or otherwise undergo apoptosis. While we present here the first characterization of *Sp185/333* protein expression in the organs of the purple sea urchin, the identity of the *Sp185/333*⁺ cells in tissues remains speculative. However, the morphology of *Sp185/333*⁺ cells in the axial organ is very similar to small phagocytes, and we speculate that this organ may be a source of *Sp185/333*⁺ cells and coelomocytes in general.

Sp185/333 in the ovaries

Apart from the axial organ, the identification of the *Sp185/333*⁺ cells associated with pharynx, esophagus, intestine and testes could not be established with certainty based on cell morphology. However, within the ovary, the pattern of the *Sp185/333* proteins in oocytes and mature eggs is very similar to the pattern of proteins associated with cortical granules (Gary Wessel, personal communication).^{26,28} Cortical granules in oocytes are scattered throughout the cytoplasm, and in the mature egg they are localized to the cortex near the plasma membrane. The fertilization reaction induces the granules to undergo exocytosis and release their contents into the perivitelline space, which results in the proteolysis of the sperm receptor,⁵³ transformation of the outer soft vitelline envelope layer into a thickened fertilization envelope⁵⁴ and blocks polyspermy. It is noteworthy that a sea urchin integrin is also localized to cortical granules with the same pattern as described here for *Sp185/333* proteins.⁵⁵ Upon fertilization, the integrin is integrated into the fertilization

envelope and putative interaction with the Sp185/333 proteins, perhaps via the RGD or other conserved sequence for integrin binding, would position the Sp185/333 proteins on the surface of the fertilized egg. It is therefore intriguing to speculate that the Sp185/333 proteins may function to protect the egg from bacterial colonization or other foreign invasion.

There are a number of reports and reviews of echinoid diseases (e.g. Jangoux⁵⁶), of which most focus on skin infections, their outcomes and their effect on sea urchin aquaculture. Spotted gonad disease, reported for *Strongylocentrotus intermedius*, shows an accumulation of red spherule cells (and likely other types of coelomocytes) in the gonad, which surround metacercaria of parasitic digenetic trematode worms.^{57,58} Red spherule cells are sometimes released with eggs during spawning (LCS, personal observation) and these cells may infiltrate gonadal tissues in response to an infection. Although there is no description of how microbes and parasites might enter the gonads of otherwise healthy animals, migration down the gonopore into the gonadal tissue is likely. The expression of Sp185/333 proteins by ovarian follicular cells and perhaps by phagocytes in ovary and testicular tissue may be important for deterring or blocking potential infections to these organs.

Conclusions

The evidence presented in this study suggests that cells expressing Sp185/333 proteins may not be confined to coelomocytes in the coelomic fluid of the purple sea urchin. The Sp185/333 transcripts and proteins are present in cells in the major organs and tissues throughout the body, including the axial organ, pharynx, esophagus, intestine and gonads. It is feasible that the Sp185/333⁺ cells in the organs are wandering phagocytes and that they may have a variety of functions in the organs, including immune defense. Highly diverse arrays of 185/333 proteins are expressed by two species of sea urchins (*S. purpuratus* and *H. erythrogramma*), and the genes have been identified in the genomes of three additional species, *Strongylocentrotus franciscanus*, *Allocentrotus fragilis*²³ and *Lytechinus pictus* (Buckley and Smith, unpublished). The extraordinary sequence diversity of the encoded proteins is likely a reflection of the arms race between host and pathogen.^{59,60} Sea urchins are in constant contact with microbes that share their seawater and ocean substrate habitat.⁶¹ Infection from this constant exposure to microbes is likely held in check by a variety of immune mechanisms, including the activities of the coelomocytes and the sequence diversity that is present a variety of the sea urchin immune gene families,^{1,2,4,5} including the *Sp185/333* genes.²⁴

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References

- Buckley KM and Rast JP. Dynamic evolution of toll-like receptor multigene families in echinoderms. *Front Immunol* 2012; 3: 136.
- Hibino T, Loza-Coll M, Messier C, Majeske AJ, Cohen A, Terwilliger DP, et al. The immune gene repertoire encoded in the purple sea urchin genome. *Develop Biol* 2006; 300: 349–365.
- Nair SV, Del Valle H, Gross PS, Terwilliger DP and Smith LC. Macroarray analysis of coelomocyte gene expression in response to LPS in the sea urchin. Identification of unexpected immune diversity in an invertebrate. *Physiol Genomics* 2005; 22: 33–47.
- Rast JP and Messier-Solek C. Marine invertebrate genome sequences and our evolving understanding of animal immunity. *Biol Bull* 2008; 214: 274–283.
- Rast JP, Smith LC, Loza-Coll M, Hibino T and Litman GW. Genomic insights into the immune system of the sea urchin. *Science* 2006; 314: 952–956.
- Sodergren E, Weinstock GM, Davidson EH, Cameron RA, Gibbs RA, Angerer RC, et al. The genome of the sea urchin, *Strongylocentrotus purpuratus*. *Science* 2006; 314: 941–952.
- Rast JP, Pancer Z and Davidson EH. New approaches towards an understanding of deuterostome immunity. *Curr Top Microbiol Immunol* 1999; 248: 3–16.
- Smith LC, Chang L, Britten RJ and Davidson EH. Sea urchin genes expressed in activated coelomocytes are identified by expressed sequence tags. Complement homologues and other putative immune response genes suggest immune system homology within the deuterostomes. *J Immunol* 1996; 156: 593–602.
- Dheilly N, Nair S, Smith LC and Raftos D. Highly variable immune-response proteins (185/333) from the sea urchin *Strongylocentrotus purpuratus*: Proteomic analysis identifies diversity within and between individuals. *J Immunol* 2009; 182: 2203–2212.
- Buckley KM, Munshaw S, Kepler TB and Smith LC. The 185/333 gene family is a rapidly diversifying host-defense gene cluster in the purple sea urchin *Strongylocentrotus purpuratus*. *J Mol Biol* 2008; 379: 912–928.
- Terwilliger DP, Buckley KM, Brockton V, Ritter NJ and Smith LC. Distinctive expression patterns of 185/333 genes in the purple sea urchin, *Strongylocentrotus purpuratus*: An unexpectedly diverse family of transcripts in response to LPS, beta-1,3-glucan, and dsRNA. *BMC Mol Biol* 2007; 8: 16.
- Terwilliger DP, Buckley KM, Mehta D, Moorjani PG and Smith LC. Unexpected diversity displayed in cDNAs expressed by the

- immune cells of the purple sea urchin, *Strongylocentrotus purpuratus*. *Physiol Genomics* 2006; 26: 134–144.
13. Buckley KM and Smith LC. Extraordinary diversity among members of the large gene family, 185/333, from the purple sea urchin, *Strongylocentrotus purpuratus*. *BMC Mol Biol* 2007; 8: 68.
 14. Smith LC, Ghosh J, Buckley KM, Clow LA, Dheilly NM, Huang T, et al. Echinoderm immunity. *Adv Exp Med Biol* 2010; 708: 260–301.
 15. Brockton V, Henson JH, Raftos DA, Majeske AJ, Kim YO and Smith LC. Localization and diversity of 185/333 proteins from the purple sea urchin – unexpected protein-size range and protein expression in a new coelomocyte type. *J Cell Sci* 2008; 121: 339–348.
 16. Dheilly NM, Birch D, Nair SV and Raftos DA. Ultrastructural localization of highly variable 185/333 immune response proteins in the coelomocytes of the sea urchin, *Heliocidaris erythrogramma*. *Immunol Cell Biol* 2011; 89: 1–9.
 17. Gross PS, Clow LA and Smith LC. SpC3, the complement homologue from the purple sea urchin, *Strongylocentrotus purpuratus*, is expressed in two subpopulations of the phagocytic coelomocytes. *Immunogenetics* 2000; 51: 1034–1044.
 18. Clow LA, Gross PS, Shih C-S and Smith LC. Expression of SpC3, the sea urchin complement component, in response to lipopolysaccharide. *Immunogenetics* 2000; 51: 1021–1033.
 19. Smith LC, Britten RJ and Davidson EH. Lipopolysaccharide activates the sea urchin immune system. *Dev Comp Immunol* 1995; 19: 217–224.
 20. Gross PS, Al-Sharif WZ, Clow LA and Smith LC. Echinoderm immunity and the evolution of the complement system. *Dev Comp Immunol* 1999; 23: 429–442.
 21. Henson JH, Svitkina TM, Burns AR, Hughes HE, MacPartland KJ, Nazarian R and Borisy GG. Two components of actin-based retrograde flow in sea urchin coelomocytes. *Mol Biol Cell* 1999; 10: 4075–4090.
 22. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001; 29: e45.
 23. Ghosh J, Buckley K, Nair S, Raftos D, Miller C, Majeske A, et al. Sp185/333: A novel family of genes and proteins involved in the purple sea urchin immune response. *Dev Comp Immunol* 2010; 34: 235–245.
 24. Smith LC. Innate immune complexity in the purple sea urchin: Diversity of the Sp185/333 system. *Front Immunol* 2012; 3: 1–14.
 25. Hyman LH. *The invertebrates: Echinodermata, the coelomate bilateria*. New York: McGraw-Hill, 1955.
 26. Berg L and Wessel G. Cortical granules of the sea urchin translocate early in oocyte maturation. *Development* 1997; 124: 1845–1850.
 27. Herath S, Williams E, Lilly S, Gilbert R, Dobson H, Bryant C and Sheldon I. Ovarian follicular cells have innate immune capabilities that modulate their endocrine function. *Reproduction* 2007; 134: 683–693.
 28. Laidlaw M and Wessel G. Cortical granule biogenesis is active throughout oogenesis in sea urchins. *Development* 1994; 120: 1325–1333.
 29. Cannon JP, Haire RN and Litman GW. Identification of diversified genes that contain immunoglobulin-like variable regions in a protochordate. *Nat Immunol* 2002; 3: 1200–1207.
 30. Dishaw L, Giacomelli S, Melillo D, Zucchetti I, Haire R, Natale L, et al. A role for variable region-containing chitin-binding proteins (VCBPs) in host Gut-bacteria interactions. *Proc Natl Acad Sci USA* 2011; 108: 16747–16752.
 31. Azumi K, De Santis R, De Tomaso A, Rigoutsos I, Yoshizaki F, Pinto MR, et al. Genomic analysis of immunity in a Urochordate and the emergence of the vertebrate immune system: “Waiting for Godot”. *Immunogenetics* 2003; 55: 570–581.
 32. Cannon JP, Haire RN, Schnitker N, Mueller MG and Litman GW. Individual protochordates have unique immune-type receptor repertoires. *Curr Biol* 2004; 14: R465–R466.
 33. Dishaw L, Mueller M, Gwatney N, Cannon J, Haire R, Litman GW, et al. Genomic complexity of the variable region-containing chitin-binding proteins in amphioxus. *BMC Genetics* 2008; 9: 1–16.
 34. Dishaw L, Ota T, Mueller M, Cannon J, Haire R, Gwatney N, et al. The basis for haplotype complexity in VCBPs, an immune-type receptor in amphioxus. *Immunogenetics* 2010; 62: 623–631.
 35. Litman GW, Dishaw L, Cannon J, Haire R and Rast JP. Alternative mechanisms of immune receptor diversity. *Curr Opin Immunol* 2007; 19: 526–534.
 36. Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG, et al. The sequence of the human genome. *Science* 2001; 291: 1304–1351.
 37. Millot N and Vevers H. The morphology and histochemistry of the echinoid axial organ. *Phil Trans R Soc Lon Ser B Biol* 1968; 253: 201–230.
 38. Ziegler A, Faber C and Bartolomaeus T. Comparative morphology of the axial complex and interdependence of internal organ systems in sea urchins (Echinodermata: Echinoidea). *Front Zool* 2009; 6: 1–31.
 39. Larson M and Bayne C. Evolution of immunity: Potential immunocompetence of the echinoid axial organ. *J Exp Zool* 1994; 270: 474–485.
 40. Millot N. A possible function for the axial organ of echinoids. *Nature* 1966; 209: 549–596.
 41. Millot N. The axial organ of echinoids, re-interpretation of its structure and function. *Symp Zool Soc London* 1967; 20: 53–63.
 42. Millot N. Injury and the axial organ of echinoids. *Experientia (Basel)* 1969; 25: 756–757.
 43. Carpenter P. Notes on echinoderm morphology, no. IX. On the vascular system of the urchins. *Q J Microscop Sci* 1885; 25: 139–155.
 44. Bachmann S and Goldschmid A. The echinoid axial complex and Tiedmann bodies - the different pathways and accumulation sites of coelomocytes with regard to waste disposal in the organism. In: Jangoux M (ed.) *Echinoderms: Present & Past*. Rotterdam: Balkema, 1980, pp.255–257.
 45. Hartog M. The true nature of the madreporic system of echinodermata, with remarks on nephridia. *Annual Mag Nat History* 1887; 20: 321–326.
 46. Welsch U and Rehkämper G. Podocytes in the axial organ of echinoderms. *J Zool* 1987; 213: 45–50.
 47. Booloootian R, Bell A and Campbell J. Fine structure of the “heart” of *Strongylocentrotus purpuratus* and *Strongylocentrotus drobachiensis*. *Am Zool* 1965; 5: 667A.
 48. Booloootian R and Campbell J. A primitive heart in the echinoid *Strongylocentrotus purpuratus*. *Science* 1964; 145: 173–175.
 49. Millot N and Vevers H. Axial organ and fluid circulation in echinoids. *Nature* 1964; 204: 1216–1217.
 50. Stevens M, Dhillon J, Miller C, Messier-Solek C, Majeske AJ, Zuelke D, et al. SpTie1/2 is expressed in coelomocytes, axial organ and embryos of the sea urchin *Strongylocentrotus purpuratus*, and is an orthologue of vertebrate Tie1 and Tie2. *Dev Comp Immunol* 2010; 34: 884–895.
 51. Holm K, Dupont S, Skold H, Stenius A, Thorndyke M and Hernroth B. Induced cell proliferation in putative haematopoietic tissues of the sea star, *Asterias rubens* (L.). *J Exp Biol* 2008; 211: 2551–2558.
 52. Hoffmann J. The immune response of *Drosophila*. *Nature* 2003; 426: 33–38.
 53. Foltz K and Lennarz W. The molecular basis of sea urchin gamete interactions at the egg plasma membrane. *Dev Biol* 1993; 158: 46–61.
 54. Shapiro B, Somers C and Weidman P. Extracellular remodeling during fertilization. In: Schatten H and Schatten G (eds) *Cell*

- biology of fertilization*. San Diego: Academic Press, 1989, pp.251–276.
55. Murray G, Reed C, Marsden M, Rise M, Wang D and Burke RD. The alphaBbetaC integrin is expressed on the surface of the sea urchin egg and removed at fertilization. *Dev Biol* 2000; 227: 633–647.
 56. Jangoux M. Diseases of echinodermata. I. Agents, microorganisms and protists. *Dis Aquat Organ* 1987; 2: 147–162.
 57. Shimizu M. A metacercaria of a digenetic trematode of the genus *Proctoeces* (Fellodistomidae) parasitic to the sea urchin, *Strongylocentrotus intermedius*. *Zool Mag (Tokyo)* 1979; 88: 318–320.
 58. Shimizu M. Histopathological investigation of the spotted gonad disease in the sea urchin, *Strongylocentrotus intermedius*. *J Invertebr Pathol* 1994; 63: 182–187.
 59. Miller CA, Buckley KM, Easley RL and Smith LC. An *Sp185/333* gene cluster from the purple sea urchin and putative microsatellite-mediated gene diversification. *BMC Genomics* 2010; 11: 575.
 60. Smith LC. Diversification of innate immune genes: Lessons from the purple sea urchin. *Dis Model Mech* 2010; 3: 274–279.
 61. Smith LC. Host responses to bacteria: Innate immunity in invertebrates. *Adv Mol Cell Microbiol* 2005; 10: 293–320.