Fish & Shellfish Immunology 71 (2017) 136–143

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Molecular and expression analysis of the Allograft inflammatory factor 1 (AIF-1) in the coelomocytes of the common sea urchin *Paracentrotus lividus*

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ARTICLE INFO

Article history:
Received 20 July 2017
Received in revised form
26 September 2017
Accepted 29 September 2017
Available online 3 October 2017

Keywords:
Allograft inflammatory factor 1
Paracentrotus lividus
Coelomocytes
Amoebocytes
Red cells
Bacterial challenge

ABSTRACT

Allograft inflammatory factor 1 (AIF-1) is a highly conserved gene involved in inflammation, cloned and characterized in several evolutionary distant animal species. Here, we report the molecular identification, characterization and expression of AIF-1 from the common sea urchin *Paracentrotus lividus*. In this species, AIF-1 encodes a predicted 151 amino acid protein with high similarity to vertebrate AIF-1 proteins. Immunocytochemical analyses on coelomocytes reveal localization of the AIF-1 protein in amoebocytes (perinuclear cytoplasmic zone) and red sphaerulocytes (inside granules), but not in vibratile cells and colorless sphaerula cells. The significant increase of AIF-1 expression (mRNA and protein) found in the coelomocytes of the sea urchin after Gram + bacterial challenge suggests the involvement of AIF-1 in the inflammatory response. Our analysis on *P. lividus* AIF-1 contributes to elucidate AIF-1 function along the evolutionary scale and consolidate the key evolutionary position of echinoderms throughout metazoans with respect to the common immune paths.

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1. Introduction

A highly complex biological regulatory network protects all invertebrates from pathogens. As in vertebrates, inflammation is the first defense line of such protective network [1]. The inflammation process involves coordinated cellular events and it is induced by tissue injury or microbial infection [2]. In this respect, inflammation plays an important part in wound repair and during microbial infections [3].

Allograft inflammatory factor 1 (AIF-1) is an inflammation-responsive protein that influences the immune system and modulates diverse inflammatory disease states. This highly conserved Ca^{2+}-binding EF-hand cytokine was firstly identified and cloned from activated macrophages of rat heart grafts subject to chronic cardiac rejection [4]. AIF-1-type proteins were later identified in many vertebrates, but also invertebrates, including sponges [5], cnidarians [6], mollusks [7–14], annelids [15,16] and echinoderms [17,18]. About echinoderms, AIF-1 has first been cloned and characterized in the Antarctic sea urchin *Sterechinus neumayeri* [17] and then in the Japanese sea cucumber *Apostichopus japonicus* [18]. All the AIF-1 proteins reveal high degree of similarity, suggesting an evolutionary conservation [1].

The echinoderm immune system lacks the hallmarks of adaptive immunity [19], while it shares with all animals the same innate immune reactions. Recognition is mediated by pattern recognition receptors (PRRs), which lack the discriminative capacity of the immunoglobulin-based receptors of the vertebrate adaptive immune system [20,21]. Notably, sea urchins rapidly clear their coelomic cavity from microbes, foreign cells, and other materials by means of phagocytic coelomocytes [22,23]. It is widely accepted that echinoderm coelomocytes play an important role in immune responses, but their classification is a critical issue, due to variations in cell type and number. Many morphologically distinct classes of

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https://doi.org/10.1016/j.fsi.2017.09.078
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coelomocytes (amoebocytes/phagocytes, red and uncolored sphaerula cells, and vibratile cells) can be distinguished [24]. Amoebocytes/phagocytes are macrophage-like cells that beside their phagocytic activity play a fundamental role in encapsulation and clotting [19,25]. On the other side, sphaerula cells are characterized by the presence of large granules in their cytoplasm and have been associated with antibacterial activity [26], inflammatory responses [27], extracellular matrix remodeling [28] and wound healing [29]. The red sphaerula cells, characterized by the presence of echinochrome A, possess antibacterial activity [30]. Finally, vibratile cells are involved in the response to injury, being generally associated with clotting reactions [31].

Here we report the molecular identification and functional characterization of AIF-1 of the common sea urchin *Paracentrotus lividus* and its localization in a sub-set of coelomocytes. Moreover, we report that AIF-1 expression varies in sea urchin coelomocytes after Gram + bacterial challenge.

2. Materials and methods

2.1. Animal collection and samples preparation

Adult specimens of *P. lividus* were collected (SCUBA diving) in the coastal area of Porto Cesareo (Northern Ionian Sea, Lecce, Italy) at a depth of 5–10 m. The animals were immediately transferred in laboratory at the Department of Biological and Environmental Sciences and Technologies, University of Salento, and maintained in aquaria filled with circulating filtered (0.22 μm) seawater (FSW) and under controlled conditions for 24 h before use.

Coelomocytes were recovered from sea urchins by cutting the peristomal membrane and harvesting the coelomic fluid. From each individual, an aliquot of coelomic fluid was fixed for immunocytochemistry (formalin 4% in FSW) after evaluation of cell viability by Trypan blue and cell counting. For downstream molecular analyses, the remaining coelomic fluid was centrifuged at 400 x *g* for 10 min (4 °C). The pellets containing the coelomocytes were collected together, resuspended in RNALater (Ambion) and sonicated on ice with an ultra-sound probe (Sonifer sonicator Model 250/240, Brain Ultrasonic Corporation), for 4 min at 50% duty cycles.

2.2. Total RNA and protein extraction

After storage in RNALater, coelomocytes were processed for RNA and protein extraction by using the AllPrep DNA/RNA/Protein mini kit (Qiagen) protocol and reagents, according to the manufacturer's instructions. The protocol was implemented with the on-column DNase digestion protocol to avoid genomic DNA interference during the following PCR assays. At the end of the extraction protocol, RNA aliquots were stored in RNase-free conditions at -80 °C until use. RNA concentrations were calculated by spectrophotometry, and the $\lambda_{260}/\lambda_{280}$ ratios were calculated to evaluate RNA purity. All the RNA extractions were tested by loading RNA samples onto agarose gels. Protein concentrations in extracts were calculated by the Bicinchoninic Acid Kit for Protein Determination (BCA1 kit; Sigma Aldrich), according to the manufacturer's protocol.

2.3. Primer design and qPCR assays

The reference sequence of AIF-1 mRNA (Acc. no. FJ824731.2) of *Sterechinus neumayeri* (the Antarctic sea urchin) was used to select two oligonucleotide sequences as primer pair (forward primer 5′-3′ sequence AAGTGGAGACCTCGACGACA; reverse primer 5′-3′ TTCTTTGGAGGTGGCCCAAC) for consequent RT-PCR assays on RNA extracted from *P. lividus* coelomocytes. To validate primer sequences, the AmplifX software version 1.5.4 was used (http://ifrjr.nord.univ-mrs.fr/AmplifX) to analyze GC content, end stability and self/cross-dimer formation. Oligonucleotides were purchased from Eurofins Genomics (Germany). Sequencing of the PCR product (240 bp) obtained by using the selected primers was performed and a partial cDNA sequence was submitted to the GenBank database (www.ncbi.nlm.nih.gov/genbank/; GenBank Submission ID 1952522).

Reverse transcriptions on the extracted RNA samples were performed on 0.25–1 μg RNA using the Bio-Rad iScript Select cDNA Synthesis kit (Bio-Rad) according to the manufacturer's instructions and in the presence of random primers. Before qPCR analysis, primer pairs were tested for efficiency, according to the amplification efficiency parameters for genes of interest and internal controls proposed by Schmittgen and Livak [32]. qPCR was performed using the iQ SYBR Green Supermix protocol (Bio-Rad) with a Rotor-Gene 3000 (Corbett Research, St. Neots, UK) real-time thermal cycler. To assess gene expression quantitatively, the *P. lividus* 18S RNA gene was used as internal control to normalize mRNA amplifications (18S forward primer 5′-3′ sequence CCTGCCAGTAGTCATATGCTT; reverse primer 5′-3′ CTCGATCCAATGAACCAAACT). Gene expression relative quantification was calculated by analyzing the output threshold values (Ct) by the comparative Ct method, as previously described [32], and qPCR data were shown as $2^{-\Delta CT}$ values. Δ_{Ct} values (Δ_{Ct} = target gene Ct − standard gene Ct) were obtained from three different rounds of qPCR for both the target mRNA and the 18S internal control, of three biological replicates. Statistical analysis was performed after the $2^{-\Delta Ct}$ transformation [32].

2.4. Discontinuous density gradient

The coelomic fluid from healthy sea urchins was harvested directly in an isosmotic anticoagulant solution (20 mM Tris, 0.5 M NaCl, 70 mM EDTA, pH 7.5) (ISO–EDTA) by cutting the peristomal membrane. After centrifugation (900 x *g* for 10 min at 4 °C), the coelomocytes were washed two times in ISO–EDTA and resuspended at 5 x 10^6 cells/ml in ISO–EDTA. Coelomocyte number was calculated by a haemocytometer chamber and cell viability evaluated by Trypan blue exclusion test. Four milliliter coelomocytes suspension (1.5 x 10^6/ml) were layered on the top of an Iodixinol (Optiprep; Nycomed Oslo, Norway) discontinuous density gradient. The gradient was prepared as follows: 1 mL of 10, 20, 30 and 70% (v/v) iodixinol stock solutions in 0.5 M NaCl containing 10 mM EDTA were layered into a 15 ml centrifuge tube. The gradient was then centrifuged in a swing-out rotor (800 x *g* for 30 min at 7 °C). The resulting cell populations on the top of 10, 20, 30 and 70% Iodixinol layer, referred to as bands P1, P2, P3 and P4, respectively, and containing the coelomocytes were collected. Each cell population was gently removed, washed two times with ISO–EDTA and identified according to Smith [33].

2.5. Immunocytochemistry

For immunocytochemistry, coelomocytes from each population were resuspended in PBS and allowed to settle on a cover glass for 30 min before fixation in 4% paraformaldehyde. Cells were washed with PBS (3 times for 5 min), pre-incubated for 30 min with PBS containing 1% (w/v) bovine serum albumin (BSA) and 1% (v/v) normal donkey serum, then incubated overnight at 4 °C in primary rabbit polyclonal antibody (Proteogenix, France) raised against the C-terminal region of human AIF-1 (antigenic sequence: TGPPAKKAISELP) (dilution 1:5000). An aliquot of rabbit pre-immune serum was collected from the animal prior to begin the polyclonal serum production. The washed specimens were incubated for 1 h at room temperature with the goat anti-rabbit fluorescein isothiocyanate (FITC)-conjugated antibody (λ_{ex} 493 nm, λ_{em} 518 nm) secondary

antibody diluted 1:200 (Abcam, Cambridge, UK).

Nuclei were counterstained by incubating cells for 15 min with 4,6-diamidino-2-phenylindole (DAPI, 0.1 mg/ml in PBS, λ_{ex} 340 nm, λ_{em} 488 nm). Coverslips were mounted in Citifluor (Citifluor, London, UK) on glass slides and examined with a confocal laser microscope (LSM 710 Zeiss). In controls, primary polyclonal anti-human AIF-1 antibody was substituted with rabbit pre-immune serum (1:20,000) or primary antibody was omitted and cells were treated with BSA-containing PBS and incubated only with the secondary antibody.

2.6. Western blotting

Following electrophoresis on 4-15% polyacrylamide gels, proteins were electro-transferred to nitrocellulose membrane (*Trans*-Blot Turbo Mini Nitrocellulose Transfer packs; Bio-Rad) using a *Trans*-Blot Turbo Transfer System (Bio-Rad). After protein transfer, the membranes were blocked for 1 h in 5% (w/v) milk in PBS at room temperature, and then immunoblotted in 5% milk and 0.1% (w/v) Tween-20 in PBS with the rabbit polyclonal anti-human AIF-1 primary antibody (dilution 1:10,000); in control experiments, rabbit pre-immune serum (1:10,000) was adopted. After rinsing, blots were incubated with HRP-conjugated goat anti-rabbit secondary antibody (Sigma Aldrich), and immune reactive bands were detected using an enhanced chemiluminescence method (ECL kit; BioRad). Densitometric quantitative analysis was carried out by using the ImageJ software (v 1.48) (National Institutes of Health, Bethesda, MD, USA). The pixel intensity for each region was analyzed, the background was subtracted and the protein expression results were normalized with respect to protein concentration values of the loaded samples.

2.7. In silico sequence search and phylogenetic analysis

The nucleotide sequence of the partial cDNA (mRNA) of *P. lividus* was blasted (https://blast.ncbi.nlm.nih.gov/Blast.cgi) in the GenBank database according to standard procedures. Four *P. lividus* ESTs (Expressed sequence Tags; GenBank Acc. Nos. AM551156.1, AM587832.1, AM561595.1, AM187178.1) and one TSA (Transcribed GeneBank Acc. No. GCZS01073201.1) inferred by BLAST were submitted to multiple sequence alignment by ClustalO (http://www.ebi.ac.uk/Tools/msa/clustalo/). The amino acid sequence prediction was performed by ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/).

To identify orthologs of the *P. lividus* predicted protein product, we performed a BLAST analysis against the genome of several species using the ENSEMBL (http://www.ensembl.org/index.htmL) and NCBI (http://www.ncbi.nlm.nih.gov) databases. Sequences with high blast scores were considered, alignments were performed on ortholog proteins using ClustalO, and a neighbor joining (NJ) tree was built using MEGA7 (http://www.megasoftware.net). Conserved motifs have been searched by Simple Modular Architecture Research Tool 7 (SMART 7) (http://smart.embl-heidelberg.de/).

2.8. Bacterial challenge

To stimulate the immune response, experimental bacterial challenges were conducted with 30 sea urchins by injecting a suspension (200 μl per urchin; 1 x 10^6 bacteria/ml FSW) of heat-inactivated *Micrococcus lysodeikticus* (Gram+) into the coelomic cavity. A second group of 30 animals was injected with FSW (200 μl per urchin) and used as a control.

One, 3 and 24 h after bacterial injection, coelomocytes were recovered from stimulated and control groups (10 animals per time) as described above and processed for RNA extraction.

2.9. Statistical analysis

All experiments were repeated three times for each treatment condition. Unless otherwise stated, all data were expressed as mean value ± S.E.M.; data were analyzed by a two-tailed unpaired Student's *t*-test and significant differences were considered at $P < 0.05$.

3. Results

3.1. Detection and identification of AIF-1 gene expression in P. lividus coelomocytes

Based on *Sterechinus neumayeri* (the Antarctic sea urchin) AIF-1 mRNA, oligonucleotide sequences were selected as primer pair to be used in subsequent RT-PCR analysis to identify an AIF-1-related mRNA on total RNA extracted from *P. lividus* coelomocytes. Sequencing of the derived cDNA amplicon (expected size 240 bp) revealed 89.58% identity with the corresponding sequence region of *S. neumayeri* AIF-1 mRNA (see Supplemental Table S1).

3.2. In silico identification of the putative P. lividus AIF-1 nucleotide coding and amino acid sequences

The nucleotide sequence of the partial cDNA (mRNA) of *P. lividus* was used for a BLAST mining in the GenBank database. This led to the identification of a few *P. lividus* sequences, namely four ESTs (GenBank Acc. Nos. AM551156.1, AM587832.1, AM561595.1, AM187178.1) and one TSA (Transcriptome Shotgun Assembly; limited by echinoderms [taxid: 7586]; Genbank Acc. No. GCZS01073201.1) inferred by BLAST. Sequence alignment by ClustalO led to the identification of a complete nucleotide contig containing a putative coding sequence (cds) 456 bp long. Consequent ORF analysis predicted a peptide sequence of 152 aminoo acids (Fig. 1) for the putative *P. lividus* AIF-1.

3.3. Phylogeny and comparative analysis of P. lividus AIF-1

The evolutionary relation of *P. lividus* AIF-1 was studied with respect to other metazoans. By multiple sequence alignment, the predicted AIF-1 sequence of *P. lividus* was compared to a conspicuous number of sequences of (orthologous) AIF-1- and (closest paralogous) AIF-1l-type proteins. The optimal tree from the alignment was generated (see Supplemental Fig. S1 and Supplemental Table 1), indicating that the putative *P. lividus* AIF-1 is comprised in the echinoderm cluster of the AIF-1-type proteins and that the echinoderm composes, with the hemichordate and the chordate, the root branch from which vertebrate AIF-1- and AIF-1l-type groups have diverged by duplication (Fig. 2A).

The multiple alignment among *P. lividus* AIF-1, human AIF-1 and human AIF-1l amino acid sequences, as obtained by ClustalO using default parameters (for comparison see Supplemental Fig. S1), is reported in Fig. 2B. Along the aligned sequences, the conserved EF-hand calcium binding motifs in *P. lividus* AIF-1, as identified by SMART analysis, are indicated.

3.4. AIF-1 in P. lividus coelomocytes

The Aif-1-related protein product in *P. lividus* coelomocytes was revealed by immunocytochemistry assays, using a rabbit polyclonal anti-human AIF-1 antibody.

From discontinuous Iodixinol gradient, four distinct cell populations (P1, P2, P3, P4) with enrichment in a specific cell type were obtained (Fig. 3A). In sea urchins, about 75% of circulating cells showed a positive reaction to this antibody. Cell population P1 was

```
  1 ATG CCT CGT ACA ACT TTC GAC CGT ACA AAC GTG CAG GGT GGG AAA
  1  M   P   R   T   T   F   D   R   T   N   V   Q   G   G   K

 46 GAT TGG GGG AAA GCA AAG CAA CGC CAA ACA GAG CAG ATA GAT GAT
 16  D   W   G   K   A   K   Q   R   Q   T   E   Q   I   D   D

 91 GAA ATC GAG GAC ATT ATC ACA AAA AAC ACA TAC CCA GAG GTT GAG
 31  E   I   E   D   I   I   T   K   N   T   Y   P   E   V   E

136 GAT TTA GAC GAG AAA CTG ACA GCA TAC AAA GAT CAA TTT ATT ACG
 46  D   L   D   E   K   L   T   A   Y   K   D   Q   F   I   T

181 TAC GAC CTC GAC GGA AGT GGA GAC CTC GAC GAC AAT GAC GTT CGA
 61  Y   D   L   D   G   S   G   D   L   D   D   N   D   V   R

226 GTC ATG ATG GAG AAA CTA GGC CAA CCC AAG AAC CAT ATA GAA ATC
 76  V   M   M   E   K   L   G   Q   P   K   N   H   I   E   I

271 AGG AAA ATG ATC AAA GAG ATT GAC CTC AAC GGA AGT GGA ACC ATC
 91  R   K   M   I   K   E   I   D   L   N   G   S   G   T   I

316 AAC TTC CGG GAG TTC GTC CAG ATG ATG CTT GGA GGC AAG ACT AGC
106  N   F   R   E   F   V   Q   M   M   L   G   G   K   T   S

361 ATT ATG AGA ATG ATT CTC ATG TTC GAA GAA AAG AAT AAA GAA AAG
121  I   M   R   M   I   L   M   F   E   E   K   N   K   E   K

406 GAA AAG CCG GTT GGG CCA CCT CCA AAG AAA AGC TTC TCC GAT TTG
136  E   K   P   V   G   P   P   P   K   K   S   F   S   D   L

451 CCT TGA
151  P  ***
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Fig. 1. Nucleotide coding sequence (cds) and predicted amino acid sequence of the putative *Paracentrotus lividus* AIF-1 inferred by ORF finder. Numbers on the left refer to the nucleotide (upper row) and amino acid (lower row) positions. Nucleotides are numbered, starting from the first putative ATG initiation codon. Asterisks indicate the stop codon. The nucleotide sequence corresponding to the cloned and sequenced amplicon is underlined.

mainly composed of amoebocytes (91 ± 3.2%), P2 of vibratile cells (83.5 ± 1.8%) P3 of colorless sphaerulocytes (89.3 ± 4.6%) and P4 of red cells (95.2 ± 7.4%). The immunocytochemical analysis of AIF-1, performed on the four cell populations, revealed a positive reaction in cells from P1 and P4 (Fig. 3B). The characteristics of expression changed between the two cell types, being the protein localized around the nucleus in amoebocytes and preferentially expressed in the granules in red sphaerula cells (Fig. 3B). The protein was completely absent in vibratile cells and rarely expressed in colorless sphaerulocytes.

3.5. AIF-1 expression in P. lividus coelomocytes after bacterial challenge

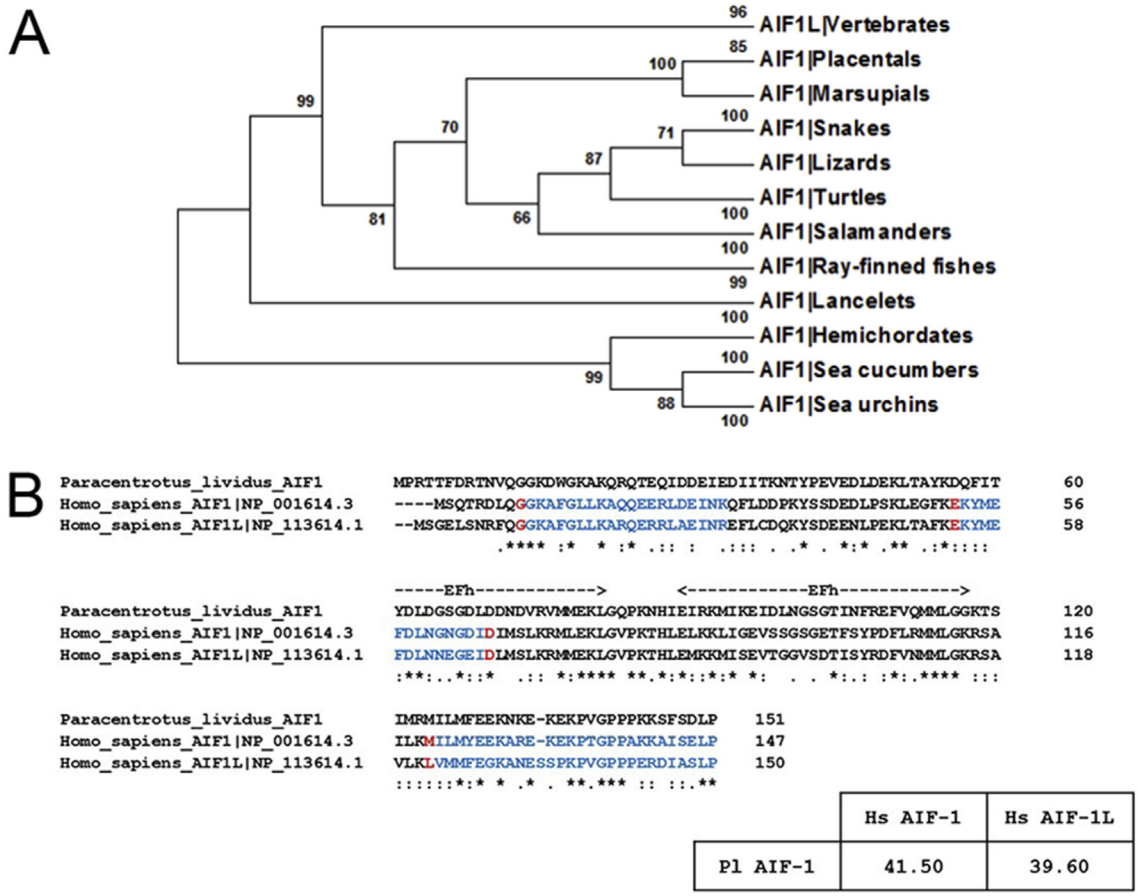
P. lividus specimens underwent immune challenge by coelomic injection of *M. lysodeikticus*. In sea urchins exposed to the bacterial injection, no significant changes ($P > 0.05$) in the total amount of living coelomocytes were evidenced after 1, 3 and 24 h (*data not shown*). AIF-1 mRNA expression was quantitatively evaluated by qPCR in control (**Ctrl**) and treated specimens (**T**) at 1, 3 (short-term) and 24 h (long-term) time point after injection. AIF-1 mRNA levels were found to significantly increase in the challenged coelomocytes 24 h after injection (~10-fold *vs.* control) (Fig. 4A), while no significant changes were detected 1 and 3 h after injection. Western blot analysis performed using the anti-human AIF-1 antibody confirmed a strong up-regulation also at the protein level (Fig. 4B). No immune reactive band were detected in the control immunoblot performed with rabbit pre-immune serum.

4. Discussion

The echinoderm immune system involves humoral and cellular components [39]. In these organisms, coelomocytes, a heterogeneous cell population that moves freely in all coelomic spaces, are the immune competent cells. As main effectors of the immune response, coelomocytes are able to discriminate self from non-self and to act as primary mediators of allograft rejection [24,40]. Due to their role in immune processes, echinoderm circulating cells functionally resemble vertebrate macrophages [40] and are responsible for the production of pro-inflammatory factors [41]. In the presence of bacteria, allogenic stimulation and injury, sea urchin coelomocytes produce a typical gene activation response [42]. One gene family that is highly up-regulated in response to immune challenge is e.g. the Sp185/333 family [43,44]. In *Strongylocentrotus purpuratus* these genes respond to marine bacteria showing significantly elevated expression in three subpopulations of phagocytes [45].

In this paper, we have described the constitutive expression of the AIF-1 gene in the *P. lividus* sea urchin coelomocytes, and its modulation upon Gram + bacterial challenge.

The AIF-1 of the sea urchin *P. lividus* has been identified and characterized by means of a suitable combination of wet (experimental) and dry (*in silico*) molecular approaches. Namely, a reverse transcription-polymerase chain reaction molecular approach first allowed generation of a cDNA that represented the partial sequence of an AIF-1 transcript in *P. lividus* coelomocytes. Then, this cDNA fragment sequence was used successfully as a mining probe to blast into the nucleotide databases, which led to detection of five



	Hs AIF-1	Hs AIF-1L
Pl AIF-1	41.50	39.60

Fig. 2. (A) Evolutionary relationships of taxa. The evolutionary history was inferred using the Neighbor-Joining method [34]. The optimal tree is shown for AIF-1- and AIF-1L-type (sum of branch length = 4.79430556) proteins. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [35]. The evolutionary distances were computed using the Poisson correction method [36] and are in the units of the number of amino acid substitutions per site. The analysis involved 20 and 23 aminoo acid sequences for AIF-1- and AIF-1L-type proteins, respectively. All positions containing gaps and missing data were eliminated. There were a total of 136 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [37]. (B) Multiple alignment among common urchin AIF-1 and human AIF-1 and AIF-1L amino acid sequences was obtained by ClustalO using default parameters. The pairwise scores (Clustal 2.1) for common urchin *Paracentrotus lividus* AIF-1 *vs.* human AIF-1 and human AIF-1L sequences are representatively shown in the table. Amino acids from coding exons (exons 1 to 6 if one refers to human AIF-1 and to human AIF-1L) are alternately indicated by black and blue letters. Residue overlap splice sites are indicated by red letters. Along the aligned sequences, EFh indicates the EF-hand, calcium binding motif, as assessed by SMART 7 [38]. Hs, *Homo sapiens*; Pl, *Paracentrotus lividus*. Further details are given in Supplemental Figure S1 and Supplemental Table 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

unclassified expressed sequences from *P. lividus*. Alignment of these sequences revealed the complete cds and, consequently, the predicted amino acid sequence of *P. lividus* AIF-1. That the newly-identified *P. lividus* is an AIF-1-type protein is further corroborated by the following pieces of evidence: a) it clusters with AIF-1 sequences from sea urchins (*S. neumayeri* and *S. purpuratus*) and sea cucumbers (*A. japonicas*, *Sclerodactyla briareus* and *Parastichopus californicus*); b) together with the other Echinoderms, Hemichordate and Chordate AIF-1 composes the root branch to the vertebrate orthologous AIF-1 and paralogous AIF-1L groups of genes (*alias* Ionized calcium-binding adapter molecule 1, Iba1, and 2, *Iba*2) that result from gene duplication process; c) it shares higher similarity with vertebrate AIF-1 than with vertebrate AIF-1l proteins (for details, see Supplemental Table 1). The use of an anti-human AIF-1 antibody on proteins extracted from *P. lividus* coelomocytes reveals the presence of a ~17 KDa protein product in Western blot assays, which is coherent with a predicted 151 aminoo acid long protein. Moreover, as it emerges from the immunocytochemistry assays, there is a remarkable difference in the localization of the immunodetected protein, according to the cell type. Notably, the anti-AIF-1 signal was found in amoebocytes, where it is located at the perinuclear cytoplasm, and in red cells, where it appears confined in vesicles/granules. The involvement of amoebocytes in inflammatory processes has been suggested by Mangiaterra and Silva [46] after their observation on the cells ability to recognize non-self particles (i.e. yeast cells) and to phagocytize them. Our data, evidencing the presence of the AIF-1 in the cytoplasm of amoebocytes, agrees and confirms this hypothesis. Also, red cells, that are known to be the echinochrome producers [47], are positive to the immunoassays with the anti-AIF-1 antibody. In this case, the signal is confined in discrete sub-cellular inclusions. Noteworthy, sea urchin red cells, characterized by the presence of the red pigment, are able to inhibit bacterial growth [30], and their involvement in inflammatory-like reactions has been underlined in previous studies describing their presence in green sea urchin (*Strongylocentrotus droebachiensis*) from Nova Scotia that were affected by a disease causing mass mortality [48]. The absence of response in the two other cell populations (i.e. vibratile cells and colorless sphaerula cells) further suggests that amoebocytes and red sphaerula cells are directly and competently involved in the inflammatory process.

The classification of the circulating cells of the echinoderms usually results from morphological observations [19]. Indeed, with the lack of a clear identification of specific epitopes expressed on

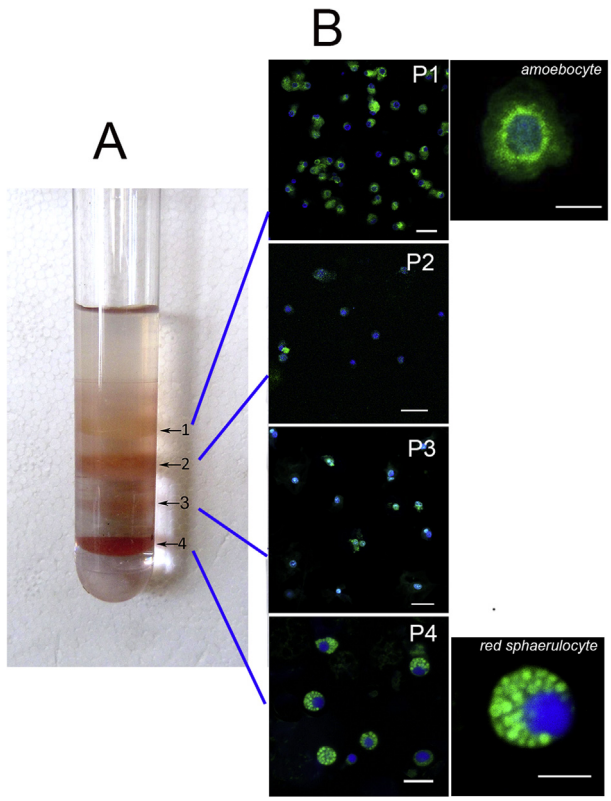


Fig. 3. Separation of *Paracentrotus lividus* coelomocytes and AIF-1 protein expression. (A) The result of an Iodixinol discontinuous gradient; (B) Cells from the four bands, named P1, P2, P3, and P4, respectively). Bar = 20 μm for P1, P2, P3; 10 μm for P4; 5 μm for amoebocytes and red sphaerulocytes). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the cell membranes, the distinction among the various circulating cell types remains rather confusing. However, some studies have shown that in the sea urchin the identification and characterization of a phagocytes sub-population is made possible by NK cell surface markers [49], against which monoclonal antibodies have been generated [50]. Also in sea cucumber, the production of monoclonal antibodies allows the identification of coelomocytes sub-groups [19]. Furthermore, in the purple sea urchin *S. purpuratus* the sub-populations of discoidal and polygonal phagocytes have been characterized by the expression of the sea urchin complement homolog SpC3 [51]. Similarly, Brockton and coworkers [52] evidenced that there are sub-populations of phagocyte morphotypes that express 185/333 proteins (see above; for review see also Smith [53]. In this view, the AIF-1 cell-specific expression in the different coelomocytes can represent a novel marker for monitoring immune dynamics upon inflammatory and/or infectious states.

As already stated, among echinoderms, AIF-1 has firstly been cloned and characterized in the Antarctic sea urchin *S. neumayeri* [17]. In this species, coelomocytes show increased expression of AIF-1 after bacterial stimulation (injection of a mixture of heat-killed bacterial strains into the coelomic cavity) with highest expression measured 24 h post-injection. Later, also in the sea cucumber *A. japonicus* [18] a significant increase of the expression levels of AIF-1 transcripts has been detected in coelomocytes after bacterial challenge (injection of a living bacterial strain into the coelomic cavity); but, this immune challenge results in a peak of expression 4 h post-injection. In this work, *P. lividus* sea urchins were exposed to heat-inactivated *M. lysodeikticus* bacteria in the coelomic cavity, which induces strong up-regulation of the AIF-1-related mRNA and protein product(s) in coelomocytes 24 h post-injection. Our finding fully overlaps the results obtained in *S. neumayeri* coelomocytes, suggesting a common time course in terms of immune response to a bacterial challenge. In our opinion, the peak of expression observed in a shorter time in *A. japonicus* can be due to the type of challenge (heat-inactivated *vs.* living bacterial cells). This idea is supported by observations in mollusk species: in disk abalone [7], in pearl oyster [10] and in Zhikong scallop [11] the injection of living bacteria induced an expression peak around 12 h post-injection.

5. Conclusions

Here, we report for the first time the molecular identification of the AIF-1 gene in the common sea urchin *P. lividus*, which is structurally related to the AIF-1 protein family. Novel insights into AIF-1 protein localization in the different immune coelomocyte types were also evidenced. Furthermore, we demonstrate AIF-1 gene expression variations due to a bacterial (*M. lysodeikticus*) challenge. These findings give novel hints to fully assess the role of

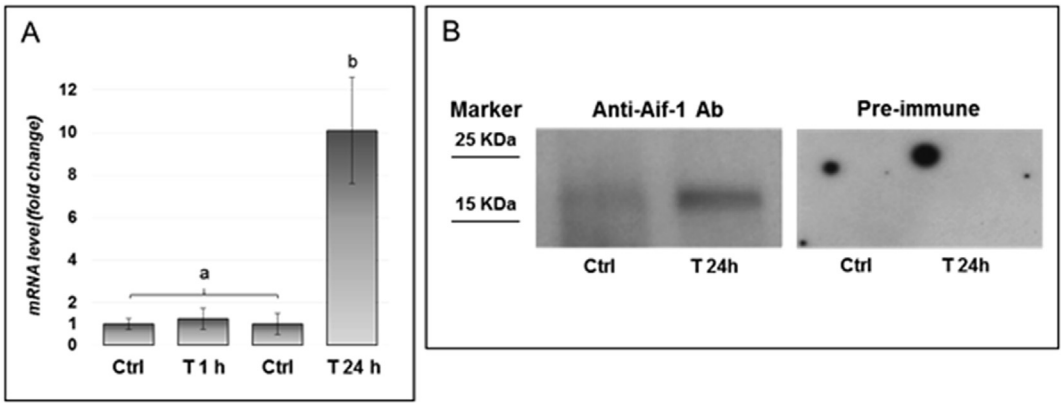


Fig. 4. Quantitative analysis of the expression of AIF-1 gene products in *Paracentrotus lividus* coelomocytes after bacterial challenge. (A) Transcriptional effects on AIF-1 mRNA. Quantitative expression analysis by qPCR was performed on RNA extracted from control and treated coelomocytes 1 and 24 h after bacterial challenge. Expression of the target mRNA is represented as $2^{-\Delta Ct}$ function value, obtained from amplification data (Ct, Threshold cycle), normalized by the related 18S RNA values (internal control for calibration; Schmittgen and Livak [32]). $2^{-\Delta Ct}$ values are relative to the control mean value (Ctrl = 1) and are expressed as fold change on the Y axis. Statistical analysis between datasets: ONE-WAY ANOVA and Bonferroni's post-hoc test (different letters indicate significantly different values; $P < 0.05$). Mean values derive from n = 3 independent assays. (B) AIF-1 detection by Western Blot assay on protein extracts from control and treated coelomocytes 24 h after bacterial challenge. The immunoreactive bands detected by the anti-human AIF-1 antibody (expected molecular weight ~17 KDa) are reported.

AIF-1 in the defense processes within Echinoderms and across animal species, and to fully elucidate the key evolutionary position of Echinoderms in the development of the common immune paths of metazoans.

Founding sources

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fsi.2017.09.078.

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