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Isolation of a novel LPS-induced component of the ML superfamily in Ciona intestinalis

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ABSTRACT

ML superfamily represents a group of proteins playing important roles in lipid metabolism and innate immune response. In this study, we report the identification of the first component of the ML superfamily in the invertebrate *Ciona intestinalis* by means of a subtractive hybridization strategy. Sequence homology and phylogenetic analysis showed that this protein forms a specific clade with vertebrate components of the Niemann-Pick type C2 protein and, for this reason, it has been named *Ci*-NPC2. The putative *Ci*-NPC2 is a 150 amino acids long protein with a short signal peptide, seven cysteine residues, three putative lipid binding site and a three-dimensional model showing a characteristic β-strand structure. Gene expression analysis demonstrated that the *Ci*-NPC2 protein is positively upregulated after LPS inoculum with a peak of expression 1 h after challenge. Finally, *in-situ* hybridization demonstrated that the *Ci*-NPC2 protein is preferentially expressed in hemocytes inside the vessel lumen.

1. Introduction

Innate immune system represents the first line of defense against pathogens that is immediately activated when microbial components are recognized by specific receptors, which include several molecules such as Toll-Like Receptors (TLRs), C-type lectin receptors and others. These pattern recognition receptors (PRRs) display the ability to recognize different microbial components including lipopolysaccharides (LPS) from gram-negative bacteria which are capable of inducing a strong immune response. In vertebrates, the signal transduction events involved in LPS recognition have been studied in details and several components of the cascade have been already identified. The lipopolysaccharide (LPS) receptor complex consists of two interacting receptors (CD14 and TLR4) and an associated protein (Myeloid Differentiation protein-2, MD-2) which is responsible for the direct binding to LPS. When engaged by LPS, as in gram-negative infection, the CD14/TLR4/MD-2 complex transduces a signal across the membrane resulting in activation of NF-kappaB leading to the expression of several sets of genes involved in the immune response against pathogen (Park et al., 2009). In invertebrates, the mechanisms behind LPS recognition has been investigated in particular in crustaceans where the binding of a β-glucan-binding proteins (bGBPs) to LPS has been described leading to the activation of the proPO system of the freshwater crayfish, *Pacifastacus leniusculus* (Lee et al., 2000). In shrimp, several LPS β-1,3-glucan binding proteins (LGBPs) have been cloned and characterized (Amparyup et al., 2012; Cheng et al., 2005; Du et al., 2007; Roux et al., 2002) but very little is known in Ascidiacea.

The ML (MD-2-related Lipid-recognition) superfamily contains a large set of genes encoding proteins such as MD-1, MD-2, Niemann-Pick type C2 (NPC2) protein, the GM2 activator protein and the mite allergen Der p 2. All these proteins share the so called ML domain characterized by two anti-parallel beta-pleated sheets stabilized by disulphide bonds forming a hydrophobic central cavity which has been predicted to be involved in lipid recognition and metabolism. Members of the ML domain play important role in lipid metabolism (sterol homeostasis and steroid biosynthesis) but also in innate immune signal pathways (LPS induced signalling). In invertebrates, a few ML genes have been identified in *Drosophila melanogaster* (Shi et al., 2012), in the shrimp *Litopenaeus vannamei* (Liao et al., 2011) and worker ant *Camponotus japonicus* (Ishida et al., 2014). These

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proteins may have multiple roles in binding bacterial cell wall components and working in chemical and immune signal pathways.

Ascidians (subphylum Tunicata) occupy a key phylogenetic position in chordate evolution and are retained the sister group of vertebrates (Delsuc et al., 2006; Swalla et al., 2000; Tsagkogeorga et al., 2009). These chordate invertebrates are provided with an innate immune system, and humoral and cellular components have been disclosed (Khalturin et al., 2004). Due to the knowledge of the genome (Dehal et al., 2002), the ascidian *Ciona intestinalis* has become a model to study the evolution of immunity and related genes (Du Pasquier et al., 2004; Iwanaga and Lee, 2005). In particular, a strong inflammatory response can be induced by inoculating LPS into the body wall (tunic and contiguous pharynx) (Parrinello et al., 2008, 2010). In ascidians, the pharynx occupies a large part of the adult body and consists of two epithelial monolayers perforated by dorso-ventrally aligned rows of elongated elliptical, ciliated stigmata (Giacomelli et al., 2012; Martinucci et al., 1988) enclosed in a mesh of vessels (also called transversal and longitudinal bars), where the hemolymph, containing abundant mature and immature hemocytes, flows. In the pharynx vessels and tunic, various inflammatory hemocyte types have been identified including hyaline amoebocytes, morula cells, granular amoebocytes and Unilocular Refractile Granulocytes (Ermak, 1976). In these cells, a set of immune related genes can be upregulated by LPS. Type IX collagen-like (Vizzini et al., 2002, 2008), CAP-like (Bonura et al., 2010), MBL-like (Bonura et al., 2009), galectin-like (Vizzini et al., 2012), peroxinectin (Vizzini et al., 2013b), Interleukin 17 (Vizzini et al., 2015a) and TNFα-like (Parrinello et al., 2008, 2010) gene expression were disclosed. In addition, the activation of a lectin-dependent complement-like system (Giacomelli et al., 2012; Pinto et al., 2003), a proPO-system (Cammarata et al., 2008; Vizzini et al., 2015b) while an increased release of galectin-like lectins with opsonic property (Parrinello et al., 2007) support the hypothesis that pharynx is the primary organ of immune-surveillance. Despite such findings, very little is known about the LPS signalling in the inflammatory responses in *C. intestinalis*.

In this manuscript, we report the first identification of a *C. intestinalis* Niemann-Pick type C2-like protein belonging to the ML superfamily (*Ci*-NCP2). Homology search and phylogenetic tree show that the *Ci*-NCP-2 is homologous to vertebrate NCP2, while real-time PCR analysis and *in situ* hybridization disclose that the gene can be upregulated by LPS challenge. The transcribed mRNA appears to be localized in hemocytes circulating in the pharynx.

2. Material and methods

2.1. Ascidians, LPS inoculation and sample preparation

Ascidians were gathered from Termini Imerese marinas (Italy), maintained in aerated sea water at 15 °C and fed every second day with a marine invertebrate diet (Coraliquid, Sera Heinsberg, Germany). According to a previous published paper (Vizzini et al., 2008), 100 μg LPS (*Escherichia coli* 055:B5, LPS, Sigma–Aldrich, Germany) in 100 μl of sterile marine solution *per* specimen (MS: 12 mM $CaCl_2$, 11 mM KCl, 26 mM $MgCl_2$, 43 mM Tris HCl, 0.4 M NaCl, pH 8.0), were inoculated into the median region of the body wall just under the tunic. Ascidians, either untreated (naïve) or injected with 100 μl MS (sham), were used as a control. The ascidian tunic surface was cleaned and sterilized with ethyl alcohol. A suitable amount (200 mg/ascidian) of pharynx tissue was excised at various time-points p.i. (1–72 h) from the injection region of the body wall, immediately soaked in RNAlater Tissue collection (Ambion, Austin, TX), and stored at −80 °C.

2.2. Total RNA extraction and cDNA synthesis

Total RNA was isolated from pharynx tissue by using a RNAqueous™-Midi Kit purification system (Ambion, Italy) and reverse-transcribed by the Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen, Milan, Italy).

2.3. Subtractive hybridization

Subtractive hybridization was performed with the PCR-Selected cDNA Subtraction Kit (Clontech Laboratories, USA) according to the manufacture's instruction. This strategy is based on a PCR-based methods for the selective amplification of differentially expressed sequences, thereby allowing the isolation of transcripts from activated tissue (pharynx). Briefly, 2 μg of mRNA poly(A)$^+$ from non-injected (driver) and injected (tester) animals were retro-transcribed. The tester and the driver cDNAs were digested with the Rsa I restriction enzyme to yield blunt ends. The tester cDNA was then subdivided into two parts, and each one was ligated with a different cDNA adaptor (Adaptor 1 and Adaptor 2) according to manufacturer's instructions. The end of the adaptors did not contain a phosphate group, and so only one strand of each adaptor attached to the 5′ ends of the cDNA. Two hybridizations were then performed. In the first run, an excess of driver was added to each sample of tester. The samples were heat-denatured and then annealed. In the second run, the two primary hybridization samples were mixed together without a previous denaturation to allow the subtracted single-strand tester cDNAs to re-associate. These new hybrids were molecules with different ends corresponding to the sequences of the two adaptors. After filling in the ends by DNA polymerase, the differentially expressed sequences displayed different annealing sites for the nested primers on their 5′ and 3′ ends. The entire population of molecules was then subjected to PCR in order to amplify the differentially expressed sequences by using the following primers (Nested PCR Primer 1 and Nested PCR Primer 2) and PCR conditions (94 °C for 30 s, 68 °C for 30 s, 72 °C for 90 s; 12 cycles).

2.4. Full-length cDNA sequences identification and analysis

Differentially expressed cDNA were cloned in the pCR4-TOPO vector (Invitrogen, USA) and sequenced. Sequence analysis showed a cDNA fragment of 156 nucleotides. Similarity searches performed by using FASTA algorithm (http://www.ebi.ac.uk/Tools/sss/fasta/) showed a high degree of homology with some expressed sequence clones from mixed embryonic stages (Egg to Neurula) of *C. intestinalis* (data not shown). The full-length cDNA sequence was identified by Gene RACE (Rapid Amplification cDNA Ends) technology, using the GeneRacer kit (Invitrogen, USA). The kit allows only the amplification of full-length transcripts and the elimination of truncated mRNA from the amplification process; the 5′ Race was performed by PCR (94 °C for 60 s, 50 °C for 30 s, 72 °C for 60 s, 35 cycles) with the GeneRacer 5′ forward primer and the gene specific 5′raceNPC2 Reverse primer. The PCR product was diluted 1:100 and a nested PCR was performed with the GeneRacer 5′ Nested primer and the 5′nested NPC2 reverse primer a fragment of 332 bp was obtained. 3′ RACE (94 °C for 60 s, 52 °C for 30 s, 72 °C for 60 s; 35 cycles) was performed with the gene specific oligonucleotide 3′NPC2 forward and the GeneRacer 3′ reverse oligonucleotide. The amplification product was diluted 1:100 and a nested PCR was performed with the 3′NCP2 Nested Forward and the GeneRacer 3′ Nested primer. A product of 493 bp was identified. Fragments were purified, ligated in the pCR4-TOPO vector and sequenced. See Fig. 1 for details for the position of the oligonucleotides on the cDNA sequence and Table 1 for primer sequences.

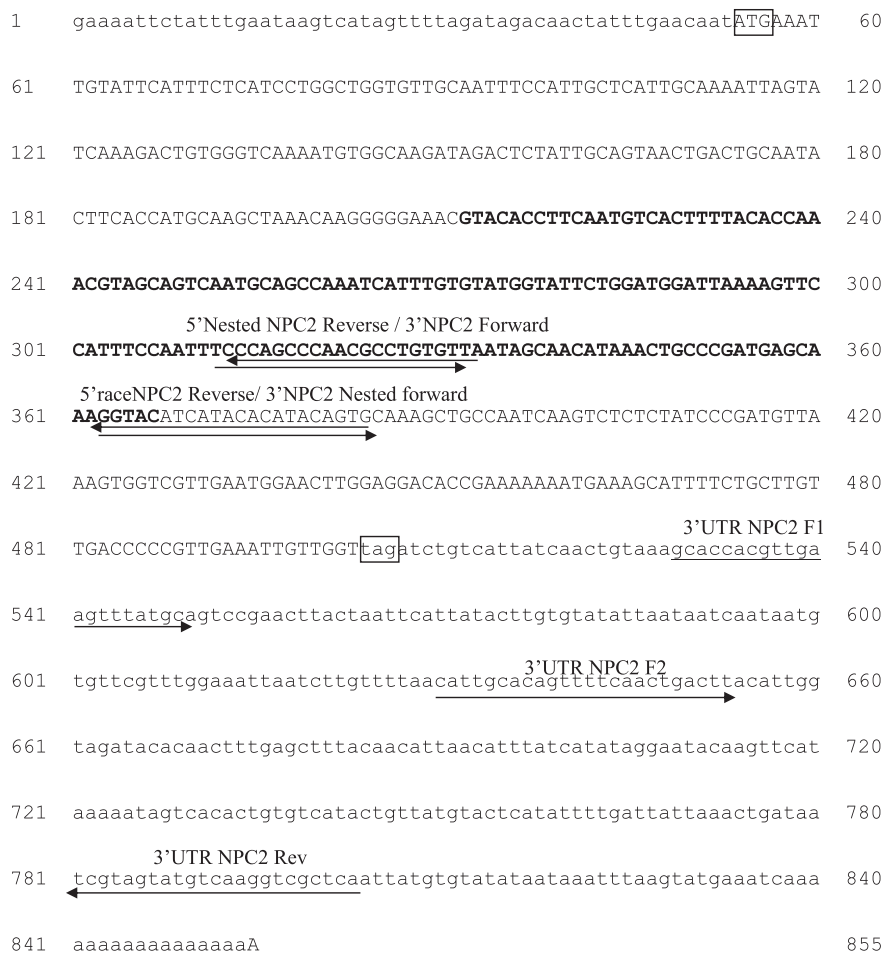

Fig. 1. Nucleotide sequence of the full length *Ciona intestinalis Ci*-NPC2 cDNA. The 5′ and 3′ untraslated regions are shown in lower case letters. Upper case letters indicate the coding region; in the boxes are reported the first ATG and the Stop codon. In bold is highlighted the 156 bp fragment identified by subtractive hybridization strategy. The arrows indicate the oligonucleotides used for GENE RACE, Real time PCR (3′UTR NPC-2 F2, 3′UTR NPC-2 Rev) and for preparation of *in situ* hybridization probes (3′UTR NPC-2 F1, 3′UTR NPC-2 Rev).

2.5. In silico analysis

Similarity structures were obtained by using the services of the Swiss-Model Protein Modelling server (http://swissmodel.expasy.org) using as a template the structure of the bovine NPC2 (entry 2hka.2.A). Predicted secondary structure was analysed using the web based predict protein algorithm (https://www.predictprotein.org/).

Table 1
Primers used for cloning and gene expression analysis.

Primers	Sequence
Nested PCR Primer 1	5′-TCGAGCGGCCGCCCGGGCAGGT-3′
Nested PCR Primer 2	5′- AGCGTGGTCGCGGCCGAGGT-3′
Gene Racer 5′ Forward	5′-CGACTGGAGCACGAGGACACTGA-3′
Gene Racer 5′ Nested	5′-GGACACTGACATGGACTGAAGGAGTA-3′
Gene Racer 3′Nested	5′-CGCTACGTAACGGCATGACAGTG-3′
Gene Racer 3′reverse	5′-GCTGTCAACGATACGCTACGTAACG-3′
Race NPC2 5′Reverse	5′-CACTGTATGTGTATGATGTACC-3′
5′ Nested NPC2 Reverse	5′-AACACAGGCGTTGGGCTGG-3′
3′ NPC2 Forward	5′-TCCCAGCCCAACGCCTGTG-3′
3′ NPC2 Nested Forward	5′-GGTACATCATACACATACAGTG-3′
3′ UTR NPC2 F1	5′-GCACCACGTTGAAGTTTATGC-3′
3′ UTR NPC2 F2	5′-CATTGCACAGTTTTCAACTGACT-3′
3′ UTR NPC2 Rev	5′-TGAGCGACCTTGACATACTACG-3′
Actin forward	5′-TGATGTTGCCGCACTCGTA-3′
Actin reverse	5′-TCGACAATGGATCCGGT-3′

2.6. Real-time PCR analysis

Tissue expression of the Ci-*NCP2* gene was examined by real-time PCR analysis with the Sybr-Green method (Applied Biosystems 7500 real-time PCR system), as previously described (Vizzini et al., 2013a). Primers were designed by using Custom Primers OligoPerfect Designers software (https://tools.lifetechnologies.com/content.cfm?pageid=9716) and synthesized commercially (Eurofins MWG Operon, Ebersberg, Germany). Tissue expression was performed in a 25-μl PCR containing 2 μl cDNA converted from 250 ng total RNA, 300 nM 3′ UTR NPC2 F2 and 3′ UTR NPC2 Rev primers, 300 nM actin forward and actin reverse primers, and 12.5 μl Power Sybr-Green PCR Master Mix (Applied Biosystems). The 50 cycles of the two-step PCR program consisted of initial polymerase activation for 3 min at 95 °C followed by a denaturing step at 95 °C for 15 s, and then annealing/extension was carried out at 60 °C for 45 s when the fluorescent signal was detected. Each set of samples was run three times, and each plate contained quadruplicate cDNA samples and negative controls. The specificity of amplification was tested by real-time PCR melting analysis. To obtain sample quantification, the $2-\Delta\Delta Ct$ method was used, and the relative changes in gene expression were analysed as described in the Applied Biosystems Use Bulletin N.2 (P/N 4303859). The amount of *Ci*-NPC2 transcript from the various tissues was normalized to actin in order to compensate for variations

in input RNA amounts. Relative *Ci*-NPC2 expression was determined by dividing the normalized value of the target gene in each tissue by the normalized value obtained from the untreated tissue. See Fig. 1 for details for the position of the oligonucleotides on the cDNA sequence and Table 1 for primer sequences.

2.7. Phylogenetic analysis

A phylogenetic tree was made by the Neighbor- Joining method (NJ) after 1000 bootstrap iterations by using MEGA 6 (Tamura et al., 2013). The accession numbers are as follows: The accession numbers are as follows: JAB90059.1 (*Ceratitis capitata* NPC2), AAN13595.1 (*D. melanogaster* NPC2Fruit fly), AAF55013.1 (*D. melanogaster* NPC2bA), ABD65303.1 (*Litopenaeus vannamei* LvML), AAH80500.1 (*Xenopus tropicalis* NPC2, Xenopus), AAH45895.1 (*Danio rerio* NPC2, Zebrafish), NP_006423.1 (*Homo sapiens* NCP2), NP_075898.1 (*Mus musculus* NCP2), NP_775141.2 (*Rattus norvegicus* NCP2), AIB53030.1 (*Apis cerana* NCP2), EFX86463.1 (*Daphnia pulex* NCP2), NP_004262.1 (*H. sapiens* MD1), NP_034875.1 (*M. musculus* MD1), BAG55275.1(*H. sapiens* MD2), AAX73194.1 (*R. norvegicus* MD2), ABC47879.1 (*Bos taurus* MD2), CAJ18497.1 (*M. musculus* GM2A), AAD25741.1(*H. sapiens* GM2A), XP_003379182.1 (*Trichinella spiralis* GM2A).

2.8. In situ hybridization assay (ISH)

The tunic surface was cleaned and sterilized with ethyl alcohol. Body wall fragments (200 mg), containing both tunic and pharynx tissue, were excised from the injection site at different time points after the LPS inoculation. For *in situ* hybridization studies, fragments were fixed in Bouin's fluid (saturated picric acid: formaldehyde:acetic acid 15:5:1) for 24 h, paraffin embedded, and serially cut at 6 μm (Leica RM2035 microtome, Solms, Germany).

To examine gene expression in tissue sections, ISH was carried out with digoxigenin-11-UTP-labelled riboprobes (1 μg/ml final concentration). The *Ci*-NCP2 probe was generated by PCR amplifying a cDNA fragment of 275 bp covering the region from nucleotide 529 to nucleotide 804 of the isolated cDNA using the 3′ UTR NPC2 F1 oligonucleotide and the 3′ UTR NPC2 Rev oligonucleotides. The digoxigenin-11-UTP-labelled riboprobes was carried out according to manufacturer's instructions (Roche Diagnostics). The re-hydrated histological sections were digested with proteinase K (10 μg/ml) in PBS for 5 min, washed with PBS-T, and treated for hybridization with 50% formamide, 5X SSC (1X SSC: 0.15 M NaCl/0.015 M sodium citrate, pH 7), 50 μg/ml heparin, 500 μg/ml yeast tRNA, and 0.1% Tween 20, at 37 °C overnight. After exhaustive washing in PBS-T and 4XSSC (twice for 10 min), the sections were incubated for 1hr with anti-DIG-Fab-AP conjugate (Roche Diagnostics) diluted 1:500 and washed in PBS-T. Finally, the sections were incubated in the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium liquid substrate system (Sigma–Aldrich, Germany). Colour development was stopped after 30 min at room temperature.

2.9. Statistical methods

Multiple comparisons were performed with one-way analysis of variance (ANOVA), and different groups were compared by using Tukey's t-test. Standard deviations were calculated on four experiments. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Identification of a NPC2 protein (Ci-NPC2)

By means of a PCR-based subtractive hybridization strategy performed with mRNA from naive and LPS challenged pharynx of *C. intestinalis*, a 156 bp cDNA encoding for a member of ML-superfamily protein was identified. The full-length mRNA was isolated performing a 5′ and 3′ RACE strategy. Sequence analysis showed an 855 bp full-length cDNA with 5′ and 3′ untranslated regions of 53 bp and 349 bp respectively (see Fig. 1). The cDNA encodes for a putative 150 amino acids long protein with a 19 amino acids long signal peptide capable of expressing a mature 131 amino acids protein (Theoretical pI/Mw: 7.48/12961.90) (Fig. 2).

A search in Ensembl genome browser identified a gene (ENSCING00000021770) localized on Chromosome 2:6,262,025-6,262,483. This analysis identified a unique transcript (ENSCINT00000032764) for this gene. *In silico* analysis using the Delta-Blast algorithm highlighted the presence of putative structural domains; *Ci*-NPC2, as other members of ML-superfamily contains a characteristic signal peptide, the ML/MD2 related lipid recognition domain (28–146 a.a.), the putative cholesterol/lipid binding site (Phe84, Ile115, Tyr119), six conserved cysteine residues (Cys26, Cys41, Cys46, Cys92, Cys99, Cys141) and two hypothetical glycosylation sites (N57 and N136) (details are reported in Figs. 2 and 3). A 3D model was build up using the ProMod Version 3.70 showing a β-sheet structure similar to other component of the ML superfamily (QMEAN4 -1.89) (Fig. 4).

3.2. Phylogenetic analysis

Similarity search showed a significant homology of *Ci*-NCP2 with several components of the Niemann-Pick type C2/epididimal secretory family of proteins. Identity search performed with the Clustal W algorithm showed that *Ci*-NPC2 display a certain degree of homology to orthologues genes from human (50% identity), Xenopus, rat and mouse (45% identity), Bovine (43% identity), zebrafish (40% identity), fruit fly (33% identity) and shrimp LvML (34% identity).

By using MEGA6 program, *Ci*-NPC2 was aligned with vertebrate and invertebrate members of ML superfamily proteins: MD-1, MD-2 from vertebrate (*R. norvegicus*, *B. taurus*, *H. sapiens*, *M. musculus*), NPC2 from chordate (*H. sapiens*, *D. rerio*, *M. musculus*, *R. norvegicus*, *X. tropicalis*, *C. intestinalis*) and invertebrates (*C. capitata D. melanogaster*, *A. cerana*, *D. pulex*) and GM2A from *H. sapiens*, *M. musculus*, *T. spiralis*, and the phylogenetic tree was constructed by the neighbour-joining method showing four main clusters (Fig. 5). The first (grey box) includes MD-1 and MD-2 from vertebrate (*R. norvegicus*, *B. taurus*, *H. sapiens*, *M. musculus*), the second (cyan box) includes GM2A (*H. sapiens*, *M. musculus*, *T. spiralis*). The third one (red box) includes NPC2 from *D. melanogaster*, *A. cerana*, *D. pulex*, *C. capitata and* LvML *from L. vannamei*. The fourth one (green box) include NPC2 from chordate (*R. norvegicus*, *H. sapiens*, *M. musculus*, *X. tropicalis*, *D. rerio* and the *Ci*-NCP2 from *C. intestinalis*).

3.3. Ci-NPC2 gene expression is upregulated by LPS

Real time PCR analysis of the inflamed ascidian pharynx showed enhanced *Ci*-NPC2 mRNA levels as an effect of the LPS challenge (Fig. 6). To examine the time course of the response, four ascidians in three distinct experiments were examined at increasing post-inoculation time points (1, 2, 4, 8, 12, 24, 48, 72 h). At each time point, four sham ascidians were used as non-treated control animals. The *Ci-NPC2* gene expression significantly boosted at 1–2 h ($p < 0.001$), decreased at 4–24 h and increased again at 48–72 h ($p < 0.001$). The response by sham ascidians indicates that the inoculation procedure did not significantly modulate the mRNA expression (Fig. 6).

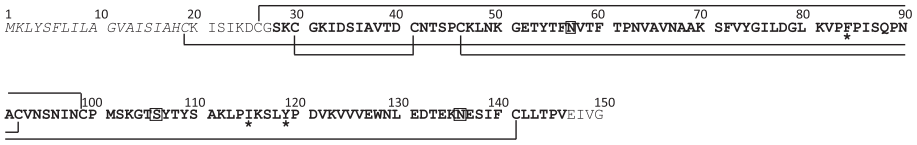

Fig. 2. cDNA deduced amino acid sequence of the *Ci*-NPC2-like protein. Locations of putative structural domains were based on the Delta-Blast algorithm. The signal peptide is shown in italic letters (1–19 a.a.); the ML domain/MD2 related lipid recognition domain is highlighted in bold letters (28–146 a.a.). The putative cholesterol/lipid binding sites are indicated with asterisk (Phe84, Ile115, Tyr119). Lines indicate the predicted disulphide bonds pairing (19–92, 26–99, 30–41, 46–141). Boxes indicate the hypothetical glycosylation sites.

3.4. *In situ hybridization assay (ISH)*

Histological sections of the pharynx were examined at 1 h following medium (Fig. 7 panel B) or LPS inoculation (Fig. 7 panel C and D). Fig. 7 panels C and D shows that the *Ci*NCP2 transcript signal is present in tightly packed hemocytes clusters within the vessel lumen. Fig. 7 panel D shows higher magnification of the *Ci*NCP2 expressing hemocytes, and reveal that compartment/cells (CC) and signet ring cells (SRC) were mainly involved. In addition, a comparison between LPS- and medium-induced animals showed an increase in the number of cells for each cell clamp (25–35%), but not differences are revealed in the involvement of different cell types (Fig. 7 panel C and D). Controls with the sense strand probe were negative (Fig. 7 panel A).

4. Discussion

ML is a conserved domain identified in at least four groups of proteins comprising the human MD-1 and MD-2, the NPC2 and mite major allergens, the Phosphatidylglycerol/phosphatidylinositol transfer protein (PG/PI-TP) and the human GM2A which are involved in lipid metabolisms and innate immune responses.

In vertebrate, the intracellular cholesterol transport is achieved by the presence of the NPC genes encoded by two independent families of proteins, NPC1 (a lysosomal membrane protein) and NPC2 (a soluble protein present in the lysosomal lumen) through a mechanisms which seems to be evolutionary conserved. In particular, human NPC2 binds cholesterol that has been released from low-density lipoproteins in the lumen of late endosomes/lysosomes and accelerate the rate of cholesterol transfer to NPC1. Furthermore, the NPC2 protein seems to possess a wider specificity for a subset of other sterols. In human, mutations in one of the two NPC1 and NPC2 genes result in an atypical lysosomal storage disease leading to a neurovisceral genetically inherited disorder (Vanier, 2015).

In invertebrates, components of the NPC2 family have been identified in *D. melanogaster* (Shi et al., 2012), insect (Ishida et al., 2014) and shrimp (Liao et al., 2011). However, little is known

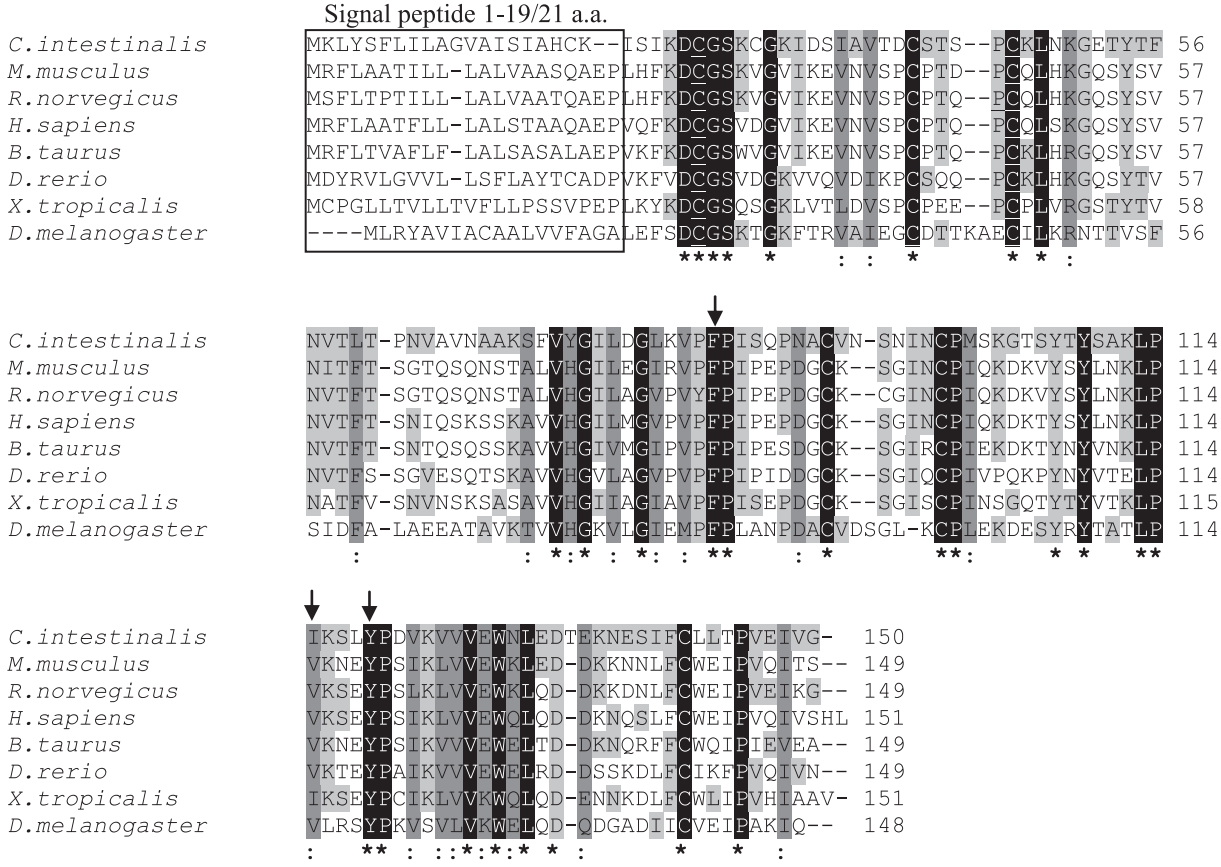

Fig. 3. Sequence alignment of the *Ci*-NPC2 with vertebrate and invertebrate members of NPC2 family of proteins. The conservation of amino acids are indicated in black boxes; dark grey indicates conserved residues; light grey boxes show at least one conserved amino acids between *C. intestinalis* and other species; arrows display lipid binding site; colon identifies conserved amino acids; cysteine residues are underscored; boxed amino acids represent the signal peptide sequences.

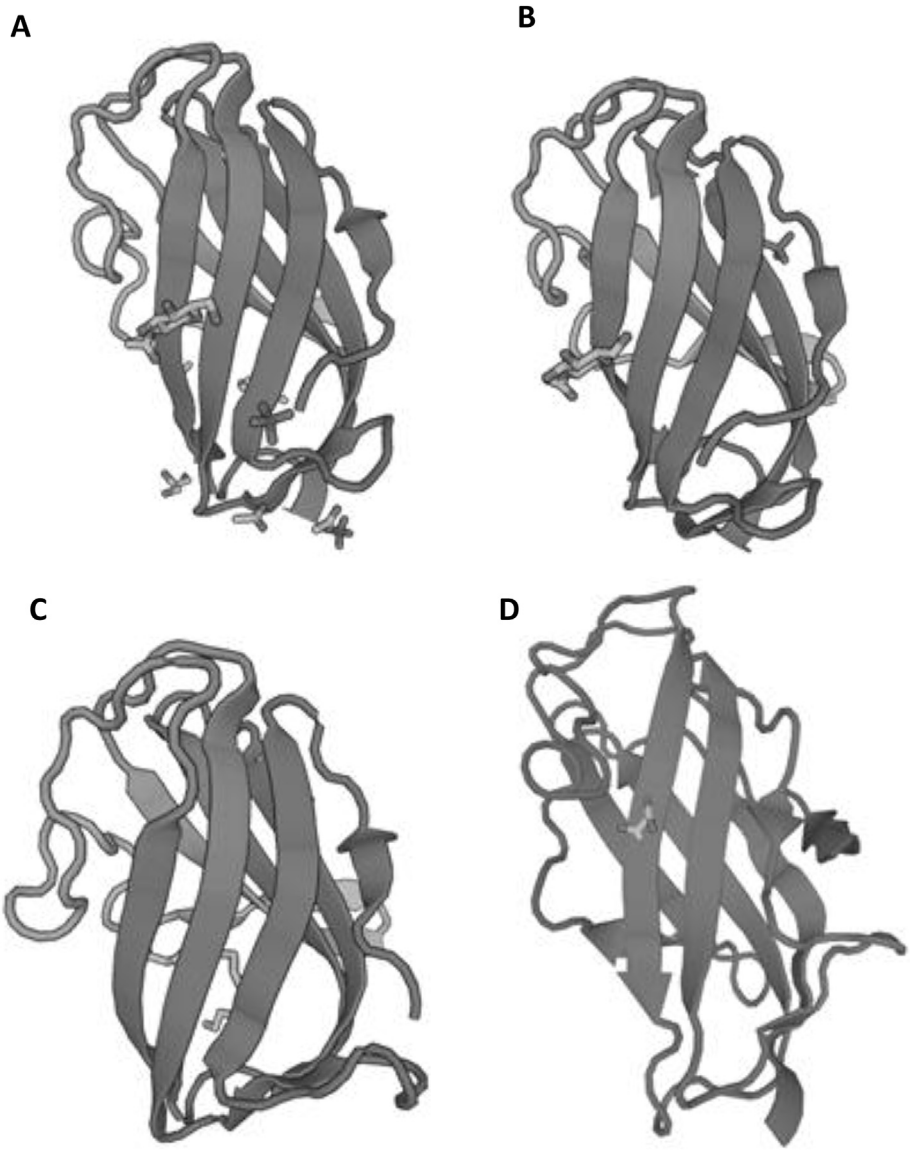

Fig. 4. Backbone ribbon representation of the *Ci*NCP2 (panel D) determined by using the services of the Swiss-Model Protein Modelling Server using the bovine NPC2 structure as a template (PDB entry 2hka.2.A) (panel A). Panels B–C display the X-ray structure of the human (1nep.1.A) and insect (3wea.1.A),respectively. Panel D shows the 3D model of the *Ci*-NPC2 protein.

about the function of these genes, which can express for a variety of biological functions which seem not to be restricted to the lipid metabolism.

The use of a subtractive hybridization strategy to identify LPS differentially expressed sequences allowed us the first identification of a *Ci*-NPC2 mRNA derived from the transcription of the (ENSCING00000021770) annotated gene, localized on Chromosome 2:6,262,025–6,262,483 in *C. intestinalis* genome. Sequence analysis showed a significant homology of the *Ci*-NCP2 with several component of the ML superfamily, with the highest similarity (71%) and identity (50%) to human NCP2. The generation of a phylogenetic tree demonstrated that the *Ci*-NPC2 forms a specific clade with several vertebrate NPC-2 proteins, supporting a conserved evolution of the NCP2 protein suggesting an evolutionary model based on gene duplication and sequence diversification of ML family components. On the other hand, Liao and coworkers (Liao et al., 2011) showed that LvML from the invertebrate *L. vannamei*, clustered together with other invertebrate NPC2 proteins.

Furthermore, sequence alignment of *Ci*-NPC2 with its orthologues showed the presence of a signal peptide of about 20 amino acids and several stretches of highly conserved amino acids. In particular, we observed that *Ci*-NPC2 contains six cysteine residues in the mature processed protein and an additional cysteine in the signal peptide sequence. Sequence alignment demonstrated that the number of such amino acids is not always conserved among the components of the NPC2 family as shown for a cysteine residue in the signal peptide region of *D. melanogaster* NPC2 protein and within the rat mature NPC2 protein. In addition, we found highly conserved functional domains such as the putative lipid binding site corresponding to Phe84, Ile115 and Tyr119 amino acids. A three-dimensional structure model was built on the basis of the crystal structure of bovine NCP2 showing a β-strand structure similar to all the members of ML superfamily.

The involvement of the *Ci*-NPC2 in the *C. intestinalis* innate immunity responses was disclosed by Real Time PCR analysis comparing RNA expressed in sham and LPS challenged pharynx.

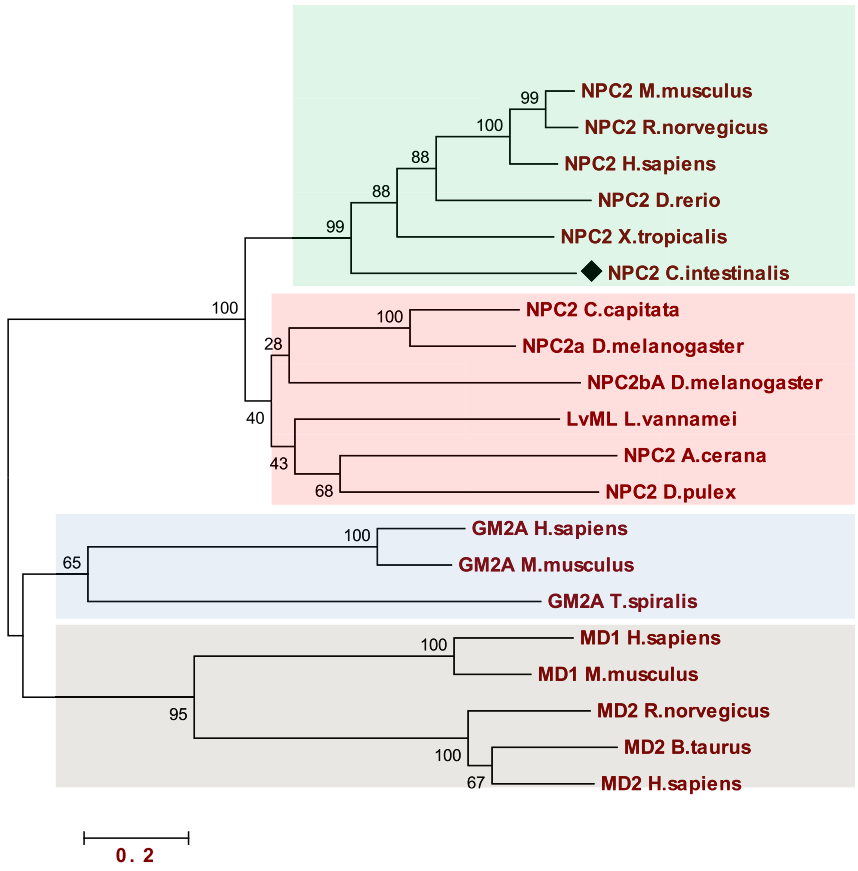

Fig. 5. Phylogenetic tree of ML superfamily proteins (MD-1, MD-2, NPC2,GM2A).The tree was constructed by the neighbour-joining method and bootstrap analysis. Bootstrap value indicates the number (%) particular node occurrences in 1000 trees generated by bootstrapping the sequences. Bar indicate the number of amino acid residues substitutions for site.

LPS inoculation enhances the expression of the *Ci*-NPC2 mRNA at 1 h after the inoculation, followed by a further increase at 48 h. A similar pattern has been already observed for other immune-related genes: CAP-like (Bonura et al., 2010), MBL-like (Bonura et al., 2009), galectin-like (Vizzini et al., 2012), Interleukin 17 (Vizzini et al., 2015a), proPO-sistem (Vizzini et al., 2015b) showing that LPS injection in the body wall of the ascidians induces an early immune response followed by a second wave of activation of gene expression. These findings suggest putative different mechanisms

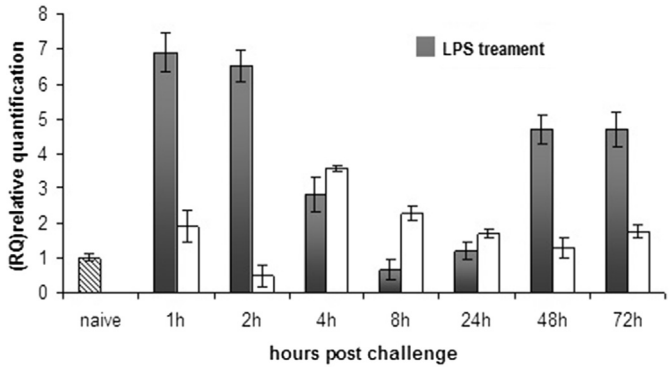

Fig. 6. Real-time PCR analysis. Time-course of *Ci*-NPC2 gene expression in C. *intestinalis* pharynx after inoculation of 100 μg of bacterial lipopolysaccharide (LPS) in 100 μl of marine solution (MS). Relative quantification of the transcript was evaluated comparing RNA from LPS-treated ascidians with those from ascidians inoculated with 100 μl MS (sham ascidians), while values from sham ascidians were compared with those from naive ascidians. $^{**}p < 0.01$, $^{***}p < 0.001$.

such as a delayed inflammatory response in pharynx with the recruitment of additional hemocytes into the inflamed tissues and/or a new transcriptional event due to the effect of a second set of signals. Tissue localization performed by means of ISH on sections from sham and inflamed pharynx showed that the expression of the *Ci*-NPC2 gene is restricted to compartment cells and signet ring cells inside the vessel lumen. Previously published data suggest that, in the *C. intestinalis* inflamed tissues, LPS inoculation enhanced the density of hemocytes expressing immune-related genes such as: Type IX collagen-like (Vizzini et al., 2002, 2008), CAP-like (Bonura et al., 2010), galectin-like (Vizzini et al., 2012), peroxinectin (Vizzini et al., 2013b), Interleukin 17 (Vizzini et al., 2015a), proPO-system (Cammarata et al., 2008; Vizzini et al., 2015b) which express a TLR-triggered cytokine (TNFα) (Parrinello et al., 2008) and a mannose-binding lectin (collectin) (Bonura et al., 2009) that are indicative of an LPS-responsive system. However, these findings open the question about the mechanisms of the signalling cascade induced by LPS inoculum and whether endotoxin signalling in Ascidian is mediated by a homolog of the vertebrate MD-2/TLR-4 system. However, recent reports demonstrated that Ci-TLRs are devoid of any response to LPS (Sasaki et al., 2009) and that no MD-2 homolog is present in the *C. intestinalis* genome (Azumi et al., 2003). These and other observations can support the hypothesis that the recognition of LPS by TLR4 through the MD2 binding may have been acquired during the evolution of vertebrates and that *C. intestinalis* may respond to LPS through a complex with other associated proteins (i.e the *Ci*-NCP2 protein) before the differentiation of the MD-2 protein. In fact, in invertebrate, NPC-2 gene function seems to be more broader that in

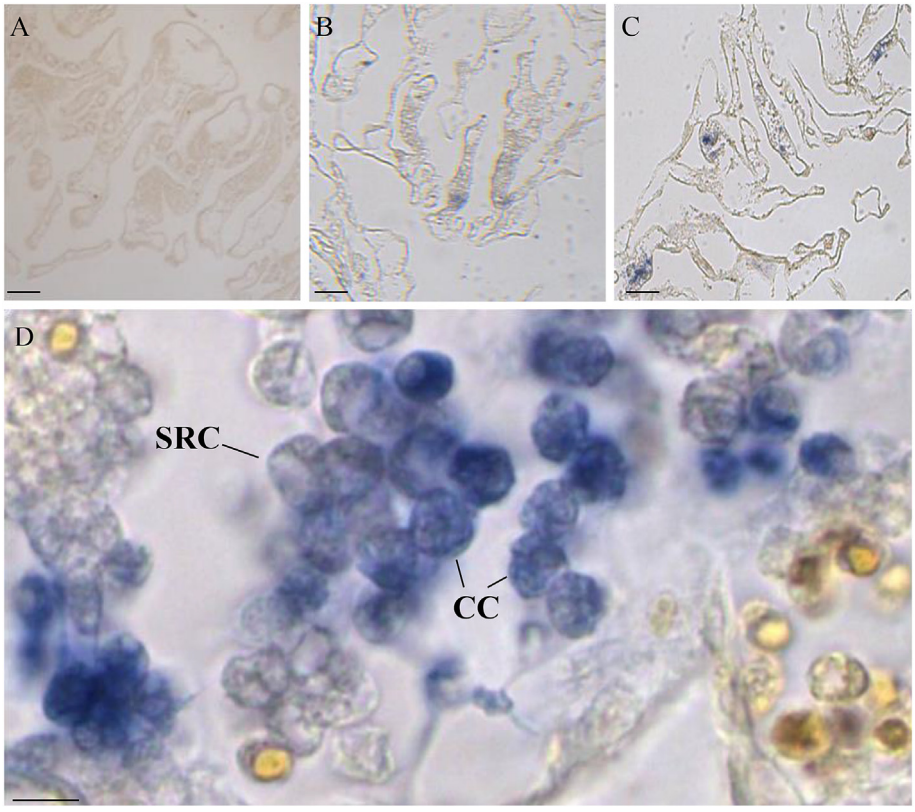

Fig. 7. Histological sections of *C. intestinalis* pharynx. *In situ* hybridization with the *Ci*-NCP2 riboprobe: sham ascidian (B) and ascidian at 1 h after LPS inoculation (C,D). Vessel (A–D); compartment cells (CC) and signet ring cells (SRC). Bars size: 50 μm (A, B,C); 5 μm (D). Controls with the sense strand probe (A).

vertebrate such as mediating chemical communication in the worker ant (Ishida et al., 2014) or binding LPS (Liao et al., 2011; Shi et al., 2012) suggesting that the specific role of this protein is still a matter of discussion in invertebrate.

5. Conclusion

In this paper we described the first identification of a component of the ML superfamily in the protochordate *C. intestinalis*. Evolutionary, the *Ci*-NPC2 protein forms a specific clade in the phylogenic tree together with vertebrate NPC2. Gene expression and tissue localization studies showed that this protein is upregulated after LPS challenge with a peak of expression in the hemocytes flowing in the pharynx suggesting its possible involvement in the inflammation response in *C. intestinalis*.

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References

Amparyup, P., Sutthangkul, J., Charoensapsri, W., Tassanakajon, A., 2012. Pattern recognition protein binds to lipopolysaccharide and beta-1,3-glucan and activates shrimp prophenoloxidase system. J. Biol. Chem. 287, 10060–10069.

Azumi, K., De Santis, R., De Tomaso, A., Rigoutsos, I., Yoshizaki, F., Pinto, M.R., Marino, R., Shida, K., Ikeda, M., Ikeda, M., Arai, M., Inoue, Y., Shimizu, T., Satoh, N., Rokhsar, D.S., Du Pasquier, L., Kasahara, M., Satake, M., Nonaka, M., 2003. Genomic analysis of immunity in a urochordate and the emergence of the vertebrate immune system: "waiting for Godot. Immunogenetics 55, 570–581.

Bonura, A., Vizzini, A., Salerno, G., Parrinello, D., Parrinello, N., Longo, V., Montana, G., Colombo, P., 2010. Cloning and expression of a novel component of the CAP superfamily enhanced in the inflammatory response to LPS of the ascidian Ciona intestinalis. Cell Tissue Res. 342, 411–421.

Bonura, A., Vizzini, A., Salerno, G., Parrinello, N., Longo, V., Colombo, P., 2009. Isolation and expression of a novel MBL-like collectin cDNA enhanced by LPS injection in the body wall of the ascidian Ciona intestinalis. Mol. Immunol. 46, 2389–2394.

Cammarata, M., Arizza, V., Cianciolo, C., Parrinello, D., Vazzana, M., Vizzini, A., Salerno, G., Parrinello, N., 2008. The prophenoloxidase system is activated during the tunic inflammatory reaction of Ciona intestinalis. Cell Tissue Res. 333, 481–492.

Cheng, W., Liu, C.H., Tsai, C.H., Chen, J.C., 2005. Molecular cloning and characterisation of a pattern recognition molecule, lipopolysaccharide- and beta-1,3-glucan binding protein (LGBP) from the white shrimp Litopenaeus vannamei. Fish Shellfish Immunol. 18, 297–310.

Dehal, P., Satou, Y., Campbell, R.K., Chapman, J., Degnan, B., De Tomaso, A., Davidson, B., Di Gregorio, A., Gelpke, M., Goodstein, D.M., Harafuji, N., Hastings, K.E., Ho, I., Hotta, K., Huang, W., Kawashima, T., Lemaire, P., Martinez, D., Meinertzhagen, I.A., Necula, S., Nonaka, M., Putnam, N., Rash, S., Saiga, H., Satake, M., Terry, A., Yamada, L., Wang, H.G., Awazu, S., Azumi, K., Boore, J., Branno, M., Chin-Bow, S., DeSantis, R., Doyle, S., Francino, P., Keys, D.N., Haga, S., Hayashi, H., Hino, K., Imai, K.S., Inaba, K., Kano, S., Kobayashi, K., Kobayashi, M., Lee, B.I., Makabe, K.W., Manohar, C., Matassi, G., Medina, M., Mochizuki, Y., Mount, S., Morishita, T., Miura, S., Nakayama, A., Nishizaka, S., Nomoto, H., Ohta, F., Oishi, K., Rigoutsos, I., Sano, M., Sasaki, A., Sasakura, Y., Shoguchi, E., Shin-i, T., Spagnuolo, A., Stainier, D., Suzuki, M.M., Tassy, O., Takatori, N., Tokuoka, M., Yagi, K., Yoshizaki, F., Wada, S., Zhang, C., Hyatt, P.D., Larimer, F., Detter, C., Doggett, N., Glavina, T., Hawkins, T., Richardson, P., Lucas, S., Kohara, Y., Levine, M., Satoh, N., Rokhsar, D.S., 2002. The draft genome of Ciona intestinalis: insights into chordate and vertebrate origins. Sci. (New York, N. Y. 298, 2157–2167.

Delsuc, F., Brinkmann, H., Chourrout, D., Philippe, H., 2006. Tunicates and not cephalochordates are the closest living relatives of vertebrates. Nature 439, 965–968.

Du Pasquier, L., Zucchetti, I., De Santis, R., 2004. Immunoglobulin superfamily

receptors in protochordates: before RAG time. Immunol. Rev. 198, 233–248.
Du, X.J., Zhao, X.F., Wang, J.X., 2007. Molecular cloning and characterization of a lipopolysaccharide and beta-1,3-glucan binding protein from fleshy prawn (Fenneropenaeus chinensis). Mol. Immunol. 44, 1085–1094.
Ermak, T.H., 1976. The hematogenic tissue of tunicates. In: The Phylogeny of Thymus and Bone-marrow-bursa Cells, pp. 45–56.
Giacomelli, S., Melillo, D., Lambris, J.D., Pinto, M.R., 2012. Immune competence of the Ciona intestinalis pharynx: complement system-mediated activity. Fish Shellfish Immunol. 33, 946–952.
Ishida, Y., Tsuchiya, W., Fujii, T., Fujimoto, Z., Miyazawa, M., Ishibashi, J., Matsuyama, S., Ishikawa, Y., Yamazaki, T., 2014. Niemann-Pick type C2 protein mediating chemical communication in the worker ant. Proc. Natl. Acad. Sci. U. S. A. 111, 3847–3852.
Iwanaga, S., Lee, B.L., 2005. Recent advances in the innate immunity of invertebrate animals. J. Biochem. Mol. Biol. 38, 128–150.
Khalturin, K., Panzer, Z., Cooper, M.D., Bosch, T.C., 2004. Recognition strategies in the innate immune system of ancestral chordates. Mol. Immunol. 41, 1077–1087.
Lee, S.Y., Wang, R., Soderhall, K., 2000. A lipopolysaccharide- and beta-1,3-glucan-binding protein from hemocytes of the freshwater crayfish Pacifastacus leniusculus. Purification, characterization, and cDNA cloning. J. Biol. Chem. 275, 1337–1343.
Liao, J.X., Yin, Z.X., Huang, X.D., Weng, S.P., Yu, X.Q., He, J.G., 2011. Cloning and characterization of a shrimp ML superfamily protein. Fish Shellfish Immunol. 30, 713–719.
Martinucci, G.B., Dallai, R., Burighel, P., Lane, N.J., 1988. Different functions of tight junctions in the ascidian branchial basket. Tissue & Cell 20, 119–132.
Park, B.S., Song, D.H., Kim, H.M., Choi, B.S., Lee, H., Lee, J.O., 2009. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. Nature 458, 1191–1195.
Parrinello, N., Arizza, V., Cammarata, M., Giaramita, F.T., Pergolizzi, M., Vazzana, M., Vizzini, A., Parrinello, D., 2007. Inducible lectins with galectin properties and human IL1alpha epitopes opsonize yeast during the inflammatory response of the ascidian Ciona intestinalis. Cell Tissue Res. 329, 379–390.
Parrinello, N., Vizzini, A., Arizza, V., Salerno, G., Parrinello, D., Cammarata, M., Giaramita, F.T., Vazzana, M., 2008. Enhanced expression of a cloned and sequenced Ciona intestinalis TNFalpha-like (CiTNF alpha) gene during the LPS-induced inflammatory response. Cell Tissue Res. 334, 305–317.
Parrinello, N., Vizzini, A., Salerno, G., Sanfratello, M.A., Cammarata, M., Arizza, V., Vazzana, M., Parrinello, D., 2010. Inflamed adult pharynx tissues and swimming larva of Ciona intestinalis share CiTNFalpha-producing cells. Cell Tissue Res. 341 (2), 299–311.
Pinto, M.R., Chinnici, C.M., Kimura, Y., Melillo, D., Marino, R., Spruce, L.A., De Santis, R., Parrinello, N., Lambris, J.D., 2003. CiC3-1a-mediated chemotaxis in the deuterostome invertebrate Ciona intestinalis (Urochordata). J. Immunol. 171, 5521–5528.
Roux, M.M., Pain, A., Klimpel, K.R., Dhar, A.K., 2002. The lipopolysaccharide and beta-1,3-glucan binding protein gene is upregulated in white spot virus-infected shrimp (Penaeus stylirostris). J. Virol. 76, 7140–7149.
Sasaki, N., Ogasawara, M., Sekiguchi, T., Kusumoto, S., Satake, H., 2009. Toll-like receptors of the ascidian Ciona intestinalis: prototypes with hybrid functionalities of vertebrate toll-like receptors. J. Biol. Chem. 284, 27336–27343.
Shi, X.Z., Zhong, X., Yu, X.Q., 2012. Drosophila melanogaster NPC2 proteins bind bacterial cell wall components and may function in immune signal pathways. Insect Biochem. Mol. Biol. 42, 545–556.
Swalla, B.J., Cameron, C.B., Corley, L.S., Garey, J.R., 2000. Urochordates are monophyletic within the deuterostomes. Syst. Biol. 49, 52–64.
Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.
Tsagkogeorga, G., Turon, X., Hopcroft, R.R., Tilak, M.K., Feldstein, T., Shenkar, N., Loya, Y., Huchon, D., Douzery, E.J., Delsuc, F., 2009. An updated 18S rRNA phylogeny of tunicates based on mixture and secondary structure models. BMC Evol. Biol. 9, 187.
Vanier, M.T., 2015. Complex lipid trafficking in Niemann-Pick disease type C. J. Inherit. Metab. Dis. 38, 187–199.
Vizzini, A., Arizza, V., Cervello, M., Cammarata, M., Gambino, R., Parrinello, N., 2002. Cloning and expression of a type IX-like collagen in tissues of the ascidian Ciona intestinalis. Biochim. Biophys. Acta 1577, 38–44.
Vizzini, A., Bonura, A., Parrinello, D., Sanfratello, M.A., Longo, V., Colombo, P., 2013a. LPS challenge regulates gene expression and tissue localization of a Ciona intestinalis gene through an alternative polyadenylation mechanism. PLoS One 8, e63235.
Vizzini, A., Di Falco, F., Parrinello, D., Sanfratello, M.A., Mazzarella, C., Parrinello, N., Cammarata, M., 2015a. Ciona intestinalis interleukin 17-like genes expression is upregulated by LPS challenge. Dev. Comp. Immunol. 48, 129–137.
Vizzini, A., Parrinello, D., Sanfratello, M.A., Mangano, V., Parrinello, N., Cammarata, M., 2013b. Ciona intestinalis peroxinectin is a novel component of the peroxidase-cyclooxygenase gene superfamily upregulated by LPS. Dev. Comp. Immunol. 41, 59–67.
Vizzini, A., Parrinello, D., Sanfratello, M.A., Salerno, G., Cammarata, M., Parrinello, N., 2012. Inducible galectins are expressed in the inflamed pharynx of the ascidian Ciona intestinalis. Fish Shellfish Immunol. 32, 101–109.
Vizzini, A., Parrinello, D., Sanfratello, M.A., Trapani, M.R., Mangano, V., Parrinello, N., Cammarata, M., 2015b. Upregulated transcription of phenoloxidase genes in the pharynx and endostyle of Ciona intestinalis in response to LPS. J. Invertebr. Pathol. 126C, 6–11.
Vizzini, A., Pergolizzi, M., Vazzana, M., Salerno, G., Di Sano, C., Macaluso, P., Arizza, V., Parrinello, D., Cammarata, M., Parrinello, N., 2008. FACIT collagen (1alpha-chain) is expressed by hemocytes and epidermis during the inflammatory response of the ascidian Ciona intestinalis. Dev. Comp. Immunol. 32, 682–692.