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Ciona intestinalis interleukin 17-like genes expression is upregulated by LPS challenge

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ABSTRACT

In humans, IL-17 is a proinflammatory cytokine that plays a key role in the clearance of extracellular bacteria promoting cell infiltration and production of several cytokines and chemokines. Here, we report on three *Ciona intestinalis* IL-17 homologues (*Ci*IL17-1, *Ci*IL17-2, *Ci*IL17-3). The gene organization, phylogenetic tree and modeling supported the close relationship with the mammalian IL-17A and IL-17F suggesting that the *C. intestinalis* IL-17 genes share a common ancestor in the chordate lineages. Real time PCR analysis showed a prompt expression induced by LPS inoculation suggesting that they are involved in the first phase of inflammatory response. *In situ* hybridization assays disclosed that the genes transcription was upregulated in the pharynx, the main organ of the ascidian immune system, and expressed by hemocytes (granulocytes and univacuolar refractile granulocyte) inside the pharynx vessels.

1. Introduction

The innate invertebrate immune response involves both cellular and humoral components, and cytokine-like factors can be engaged (Beschin et al., 2004; De Zoysa et al., 2009; Parrinello et al., 2008; Roberts et al., 2008; Wu et al., 2013; Zhang et al., 2008). These factors show pleiotropicity, functional redundancy and receptor promiscuity affecting cell motility, chemotaxis, phagocytosis, cytotoxicity and wound repair (Ottaviani et al., 2004). In vertebrates, cytokines are a class of proteins that includes interleukins (ILs), interferons (IFNs), colony-stimulating factors (CSFs), and tumor necrosis factors (TNFs). They are produced and secreted by cells in response to various stimuli including LPS, and mediate their effects by binding to specific receptors on the surface of target cells (Ihle, 1995).

In mammals, the interleukin 17 (IL-17), at first identified as cytolytic T-lymphocyte (CTL)-associated antigen 8 (Rouvier et al., 1993), is a T-cell derived cytokine characterized by the IL-17 cysteine knot fold formed by two sets of paired β-strands stabilized by three disulfide interactions (Pappu et al., 2010). This key structure is shared with components of transforming growth factor (TGFβ), bone morphogenic protein, and nerve growth factor superfamilies (Gerhardt et al., 2009). In contrast to the other cysteine knot proteins, the IL17 F C-terminal region contains five spatially conserved cysteine residues, four of them form a cysteine bridge fold (Pappu et al., 2010). In humans, six IL-17 family members (IL-17A-F) and five IL-17 receptors (IL-17RA-E) have been identified (Kolls and Linden, 2004). The IL-17 A,E and F share the highest amino acid sequence identity (50%), whereas IL-17E is the most divergent displaying 16% identity to IL-17A. The sequences of IL-17 B,C,E differ from those of IL-17A,F at the N-terminal region, with longer extensions for the former three proteins, whereas IL-17B, C, and E may be included into a distinct subclass (Gerhardt et al., 2009). In humans and mice (human chromosome 6, mouse chromosome 1, respectively), the IL-17F gene is located close to the IL-17A gene, whereas genes for the other members are located on different chromosomes (Iwakura et al., 2011). The IL-17A and F are produced by a class of effectors αβ T cells (Th17 and CD8+ cells), NK cells and neutrophils (Ciric et al., 2009; Michel et al., 2008; Weaver et al., 2007). They induce the expression of numerous inflammatory mediators including IL-8, CXC chemokines, granulocyte-colony stimulating factor and prostaglandin E2 (Ferretti et al., 2003; Jones and Chan, 2002; Ruddy et al., 2004). IL-17 displays proinflammatory properties, like those of tumour necrosis factor α (TNFα), in its capacity to induce other inflammatory effectors (Benderdour et al., 2002; Gaffen, 2004), and synergizes with other cytokines at the center of the inflammatory network (Gaffen, 2004) to activate the nuclear factor κB (Hata et al., 2002).

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In the Japanese pufferfish, *Takifugu rubripes*, seven IL-17 genes have been identified (Korenaga et al., 2010). They are expressed in the head, kidney and gill, and are upregulated by lipopolysaccharide stimulation. In the rainbow trout, *Oncorhynchus mykiss*, two IL-17C-like genes are modulated by LPS and IL-1β (Wang et al., 2010), and IL-17A/F2 molecule plays a role in promoting inflammatory and host innate immune responses directed against different pathogens (Monte et al., 2013). In addition, IL-17D homologs have been found in the Atlantic salmon, *Salmo salar* (Kumari et al., 2009), and in the lamprey, *Lethenteron japonicum* (Tsutsui et al., 2007). The lamprey skin cell IL-17 gene transcription can be upregulated by LPS (Tsutsui et al., 2007).

In invertebrates, IL-17 genes have been identified in the sequenced genomes of *Strongylocentrotus purpuratus* and *Caenorhabditis elegans*, and in the Pacific oyster, *Crassostrea gigas*, hemocyte cDNA library, two clones encode a protein similar to vertebrate IL-17s. The putative oyster IL-17 (*CgIL-17*) is homolog to rainbow trout IL-17D and human IL-17D. In both, invertebrates and fish IL-17s contained conserved cysteine residues that characterize all mammalian IL-17 forms (Roberts et al., 2008). In addition in the pearl oyster, *Pictada fucata*, an IL-17 homolog has been identified and characterized, while gene expression analysis indicates that it is involved in defence response to bacteria (Wu et al., 2013).

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; Zeng and Swalla, 2005). They are chordate invertebrates provided by an innate immune system, including inflammatory humoral and cellular responses. The *Ciona intestinalis* humoral and cellular inflammatory responses have been previously reported (Parrinello and Rindone, 1981; Parrinello et al., 1984a, 1984b), and, due to the knowledge of the genome (Dehal et al., 2002), this ascidian has become a model to study the evolution of immune related genes (Iwanaga and Lee, 2005; Pinto et al., 2003; Zucchetti et al., 2008). In the pharynx, that is retained the main organ of the ascidian immune system (Giacomelli et al., 2012), the LPS inoculation upregulates the transcription of hemocyte genes coding for type IX collagen-like (Vizzini et al., 2002, 2008), CAP-like (Bonura et al., 2010), MBL-like (Bonura et al., 2009), Galectins (Vizzini et al., 2012) and TNFa-like (Parrinello et al., 2008, 2010).

In the present paper, we report on the identification, characterization and expression of *C. intestinalis* IL-17 genes (*Ci*IL17-1, *Ci*IL17-2, *Ci*IL17-3). Real time PCR analysis revealed that their transcription is upregulated by LPS inoculation, while *in situ* hybridization assay revealed that they are expressed by hemocytes (granulocytes, URG) in the inflamed pharynx tissues.

2. Materials and Methods

2.1. Tunicates and LPS inoculation

Ascidians were collected from Sciacca Harbour (Sicily, Italy), maintained in tanks with aerated sea water at 15 °C, and fed every second day with a marine invertebrate diet coraliquid (Sera Heinsberg, Germany). The ascidian tunic surface was cleaned and sterilized with ethyl alcohol.

Lipopolysaccharide (*Escherichia coli* 055:B5, LPS, Sigma-Aldrich, Germany) solution was prepared in sterile sea water (12 mM $CaCl_2$, 11 mM KCl, 26 mM $MgCl_2$, 43 mM Tris–HCl, 0.4 M NaCl, pH 8.0). LPS solution (100 μg LPS in 100 μl sea water per animal) was inoculated into the tunic matrix close to the pharynx wall at the median body region. Untreated Ascidians were used as controls.

Table 1
Primers used for cloning and expression.

Primer name	Sequence 5′-3′	Application
IL17-1	5′-GTCGGAACAAAACGGGGGCATGTTGAT- 3′	RACE 5′
	5′-CACGTTCCCGGCGCTTTGTTTCTTT- 3′	NESTED5′
IL17-1	5′-GAATCCCTATGGTGCTTCCACGAGCA-3′	RACE3′
	5′-CGTTGGTTGTACTTGTGTTGTGCCTCGT-3′	NESTED3′
IL17-2	5′-TGCGCAACTAAAGGCACCGACTTCC-3′	RACE 5′
	5′-AAGCAATGCACCCGCTACACAAGCA-3′	NESTED5′
IL17-2	5′-TGCTTGTGTAGCGGGTGCATTGCTT-3′	RACE3′
	5′-TGTTGGGAAGTCGGTGCCTTTAGTTGC-3′	NESTED3′
IL17-3	5′-CGGACATTGAAGGCTCCGCTTTGTT-3′	RACE 5′
	5′-TCGCCGTCTTTTTCCTCGTATTGCAG-3′	NESTED5′
IL17-3	5′-GTCCGACGTTTAACCCAGCCAGCAC-3′	RACE3′
	5′-CCAAGACCGTAATAGGCGACCAAGCA-3′	NESTED3′
IL17-1 F	5′- GCCGGGAACGTGACAGAA- 3′	Real-time PCR
IL17-1 R	5′-GGCATGTTGATTGCGACCTT- 3′	Real-time PCR
IL17-2 F	5′-GTGTAGCGGGTGCATTGCT-3′	Real-time PCR
IL17-2 R	5′-GGCACCGACTTCCCAACA-3′	Real-time PCR
IL17-3 F	5′-CAAAGCGGAGCCTTCAATGT-3′	Real-time PCR
IL17-3 R	5′-GCTTCTTTGCTCGACACTTGTG-3′	Real-time PCR
Actin F	5′-TGATGTTGCCGCACTCGTA-3′	Real-time PCR
Actin R	5′- TCGACAATGGATCCGGT-3	Real-time PCR

2.2. Total RNA extraction

Ascidian tissue fragments (200 mg) explanted at various times (from 1 to 72 h) were immediately soaked in RNAlater Tissue collection (Ambion, Austin, TX), and stored at –80 °C. Total RNA extraction was performed by using an RNAqueous™-Midi Kit purification system (Ambion, Austin, TX).

2.3. Cloning and sequences analysis

A search at NBCI (http://www.ncbi.nlm.nih.gov) identified three sequences: IL17-1 (acc no. NP_001123347.1), IL17-2 (NP_001123346.1), IL17-3 (NP_001123348.1). The sequence of the *Ci*IL17-1, *Ci*IL17-2 and *Ci*IL17-3 cDNA was obtained by using the GeneRacerTM kit (Invitrogen, USA). 5′ and 3′ RACE was conducted using primers listed in Table 1. Three overlapping RACE products were cloned into the pCR™IIvector (TA cloning Kit, Invitrogen) and sequenced. They contained the complete coding region.

2.4. Phylogenetic analysis and comparative modeling

Similarity searches were performed using the FASTA algorithm (http://www.ebi.ac.uk/Tools/fasta/). Sequences were subjected to multiple alignments and a phylogenetic tree was made by the neighbor-joining method (NJ) after 1000 bootstrap iterations by using CLC workbench 6.4. The accession numbers are as follows: AAH66251.1 (*Homo sapiens* IL-17A), NP_443104.1 (*H. sapiens* IL-17F), CAG33473.1 (*H. sapiens* IL-17B), AAH36243.1 (*H. sapiens* IL-17D), AAH69152.1 (*H. sapiens* IL-17C), AAN39038.1 (*H. sapiens* IL-17E), AAI15082.1 (*Danio rerio* IL- 17a/f3), AAH75405.1 (*Xenopus tropicalis* IL-17B), AAH02271.1 (*Mus musculus* IL-17B), AAI62897.1 (*D. rerio* IL-17D), AAI45854.1 (*M. musculus* IL-17C), AAI19310.1 (*M. musculus* IL-17A), AAK59816.1 (*M. musculus* IL-17E), NP_665836.2 (*M. musculus* IL-17D), NP_001107719.1 (*X. tropicalis* IL-17D), XP_005653873.1 (*Sus scrofa* IL-17D), ACG70182.1 (*Salmo salar* IL-17 isoform D), NP_001018634.1 (*D. rerio* IL-17a/f2), NP_001018623.1 (*D. rerio* IL-17a/f1), NP_001018624.1 (*D. rerio* IL-17D), NP_001117871.1 (*Oncorhynchus mykiss* L-17D), NP_001171958.1 (*O. mykiss* IL-17C1), CAW30795.1 (*O. mykiss* IL-17C2), CCG00933.1 (*O. mykiss* IL-17A), XP_004915036.1 (*X. tropicalis* IL-17F), AAQ88439.1 (*M. musculus* IL-17F), XP_003641993 (*Gallus gallus* IL-17C), EMP36259.1 (*Chelonia mydas* IL-17C), XP_003124134 (*S. scrofa* IL-17B), XP_003730558.1 (*Strongylocentrotus purpuratus* IL-17),

AGZ03660.1 (*Haliotis rufescens* IL-17), ETN80850.1 (*Necator americanus* IL-17), ABO93467.1 (*Crassostea gigas* IL-17), AGC24392 (*Pinctata. fucata* IL-17).

The signal peptide was predicted using the SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP-4.0). The protein structural models were developed with SWISS-MODEL and the Swiss-PdbViewer (Arnold et al., 2006; Guex and Peitsch, 1997; Schwede et al., 2003) by using human IL-17F (PDB 1jpy) as template.

2.5. Real-time PCR analysis

Tissue differential expression of the three cDNAs were studied by real-time PCR using the Sybr–Green method and specific sets of primers listed in Table 1. Real-time PCR analysis was performed using the Applied Biosystems 7500 real-time PCR System. Tissue differential expression was performed in a 25 µl PCR reaction containing 2 µl cDNA converted from 250 ng of total RNA, 300 nM forward and reverse primers and 12.5 µl of Power Sybr–Green PCRMasterMix (Applied Biosystems).

The 50 cycles of the two-step PCR program consisted of initial polymerase activation for 3 min at 95 °C followed by denaturing step at 95 °C for 15 s, and then the 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 with 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 analyzed as described in the Applied Biosystems Use Bulletin N.2 (P/N 4303859). To check how ΔCt varied with template dilution, dilutions (1.0, 0.5, 0.2, 0.1, 0.05, 0.02, 0.01) of cDNAs were amplified by real-time PCR using target and actin gene-specific primers, and the ΔCt, i.e. CT(target)–CT(reference gene), was calculated, in three replicates for each cDNA dilution. Data were fit using least-squares linear regression analysis. The amplification efficiencies of the target and reference gene were approximately equal.

The amount of *Ci*IL17-1, *Ci*IL17-2 and *Ci*IL17-3 transcripts from different tissues was normalized to actin in order to compensate for variations in input RNA amounts. Relative *Ci*IL17-1, *Ci*IL17-2 and *Ci*IL17-3 expression was determined by dividing the normalized value of the target gene in each tissue by the normalized value obtained from the untreated tissue.

2.6. Pharynx preparation and histology

The tunic surface was cleaned and sterilized with ethyl alcohol and body wall fragments (200 mg), containing both tunic and pharynx tissue, were excised from the injection site at various times after the injection (1–48 h).

For *in situ* hybridization studies, body wall 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).

2.7. In situ *hybridization assay (ISH)*

To examine tissue excised from the inflamed body wall, ISH was carried out with digoxigenin-11-UTP-labeled riboprobes (1 µg/ml final concentration). The *Ci*IL17-1 probe was generated by PCR amplifying a cDNA fragment of 165 bp covering the 3′ region from nucleotide 472 to nucleotide 721.

The DNA fragments were cloned in the pCR4-TOPO vector (Invitrogen, USA). The *Ci*IL17-2 probe was generated by PCR amplifying a cDNA fragment of 138 bp covering the 3′ region from nucleotide 348 to nucleotide 486. The *Ci*IL17-3 probe was generated by PCR amplifying a cDNA fragment of 138 bp covering the 3′ region from nucleotide 348 to nucleotide 486. The digoxigenin-11-UTP-labeled 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

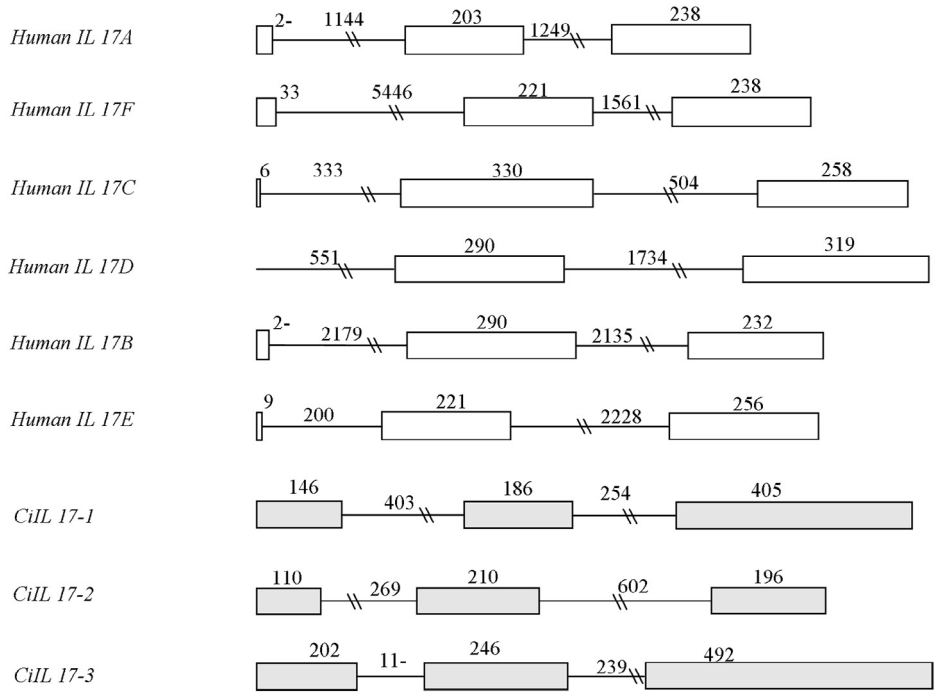

Fig. 1. Comparison of the intron/exon sizes between human IL-17 family genes (introns are reduced) and the *C. intestinalis* IL-17 genes (in gray). The thin line represents the introns and the open boxes indicate the exons of the respective genes.

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 1 h 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). Color development was stopped after 30 min at room temperature.

2.8. Statistical methods

The analysis of variance was run adopting a multifactorial experimental design dealing with three main effects. Multiple comparisons were performed with factorial analysis of variance (ANOVA), and different groups were compared by using *post hoc* Tukey's test. Standard deviation was calculated on four experiments and differences considered significant at $P < 0.001$.

3. Results

3.1. Sequence analysis of CiIL17-1, CiIL17-2 and CiIL17-3

A search in Ensembl identified three IL17-like genes localized on the chromosome (Chr)1: *Ci*IL17-1 (ENSCING00000006967), *Ci*IL17-2 (ENSCING00000024333), *Ci*IL17-3 (ENSCING00000005269). In particular they were localized as follows: Chr. 1: 9,918,822-9,920,215; Chr. 1: 10,012,187-10,013,573, and Chr. 1: 4,830,906-4,832,198. Each gene encodes for a unique transcript.

The cDNA sequence analysis showed that the *Ci*IL17-1 cDNA presents a 5′-UTR of 72 bp, an ORF of 516 bp enconding 171 aa and a 3′-UTR of 87 bp, the *Ci*IL17-2 cDNA presents a 5′-UTR of 51 bp, an ORF of 516 bp enconding 171 aa and a 3′-UTR of 56, and the *Ci*IL17-3 cDNA presents a 5′-UTR of 62 bp, an ORF of 612 bp enconding 204 aa and a 3′-UTR of 67bp (Fig. S1). The *Ci*IL17-1 amino acid sequence contains a predicted 19 aa signal peptide and a 152 aa mature polypeptide with a predicted molecular size of 19 kDa. The *Ci*IL17-2

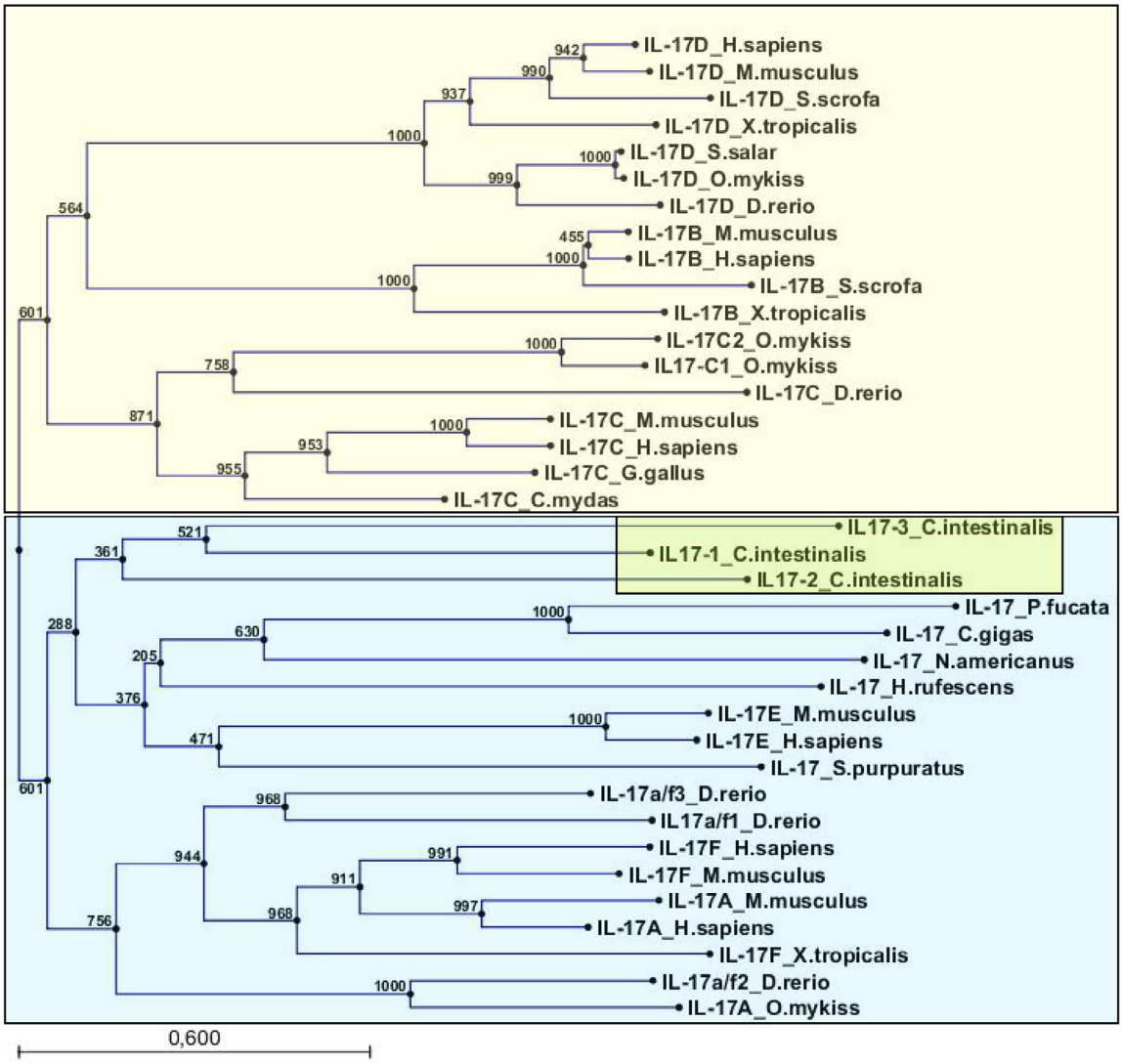

Fig. 2. Phylogenetic tree of IL-17 family of vertebrates (*H. sapiens, M. musculus, S. scrofa, X. tropicalis, D. rerio, O. mykiss, S. salar, G. gallus, C. mydas*) and invertebrates (*C. intestinalis, S. purpuratus, P. fucata, C. gigas, N. americanus, H. rufescens*). The tree was constructed by the neighbor-joining 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 residue substitutions for site.

amino acid sequence contains a 16 aa predicted signal peptide and a 155 aa mature polypeptide with a predicted molecular size of 19 kDa. The *Ci*IL17-3 amino acid sequence presents a 21 aa predicted signal peptide and a 165 aa mature polypeptide with a predicted molecular size of 23.41 kDa (Fig. S1). The Pfam software supported that the three mature polypeptide sequences belong to the IL-17 family with a conserved C-terminal CXC signature motif and with four additional conserved cysteine residues that could be related to a cysteine fold superfamily. The three *Ci*IL17-like deduced amino acid sequences, examined in GeneBank through FASTA analysis, showed significant homologies with components of the IL17 family. In particular, the highest similarity (S) and identity (I) percentages were found as follows: 64.3% S and 39.1% I between *Ci*IL17-1 and *Chelonia midas* IL-17C; 56.9% S and 30.7% I between *Ci*IL17-2 and *Homo sapiens* IL-17D; 54.2% S and 30% I between *Ci*IL17-3 with *Homo sapiens* IL-17C.

The genomic structure of the *Ciona intestinalis* IL-17 family genes was found to be similar to those of the human IL-17 family genes, with three exons and two introns, but *Ci* IL-17 family genes were found to be more compact than the human IL-17 family genes with short introns (Fig. 1). In addition, the exon 1 of *Ci*IL-17s is longer respect to vertebrate exon 1, and the last exon of the *Ci*IL17-1 and 3 genes is longer than the correspondent vertebrate exons.

3.2. Sequence phylogenetic analysis

The amino acid sequences of *Ci*IL17-1, *Ci*IL17-2, *Ci*IL17-3, of the vertebrate IL-17A,B,C,D,E,F (*H. sapiens, M. musculus, X. tropicalis, D. rerio, O. mykiss, G. Gallus, C. mydas*) and the invertebrate IL-17like (*P. fucata, C. gigas, N. americanus, H. rufescens*) were aligned and the derived phylogenetic tree (Fig. 2) shows the following two main clusters: the first one includes vertebrate IL-17D,B and C (*H. sapiens, M.*

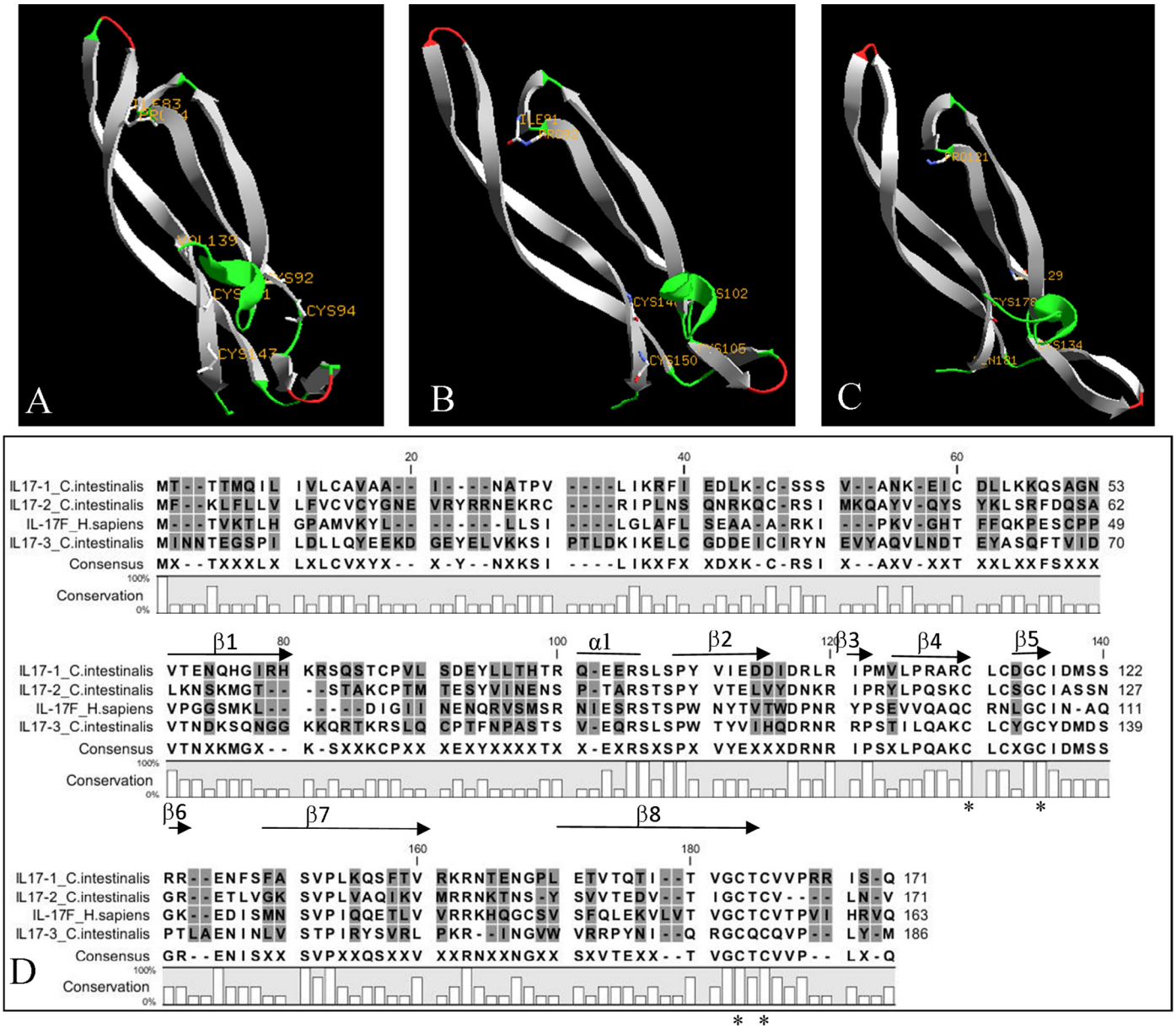

Fig. 3. Homology model of the homo-dimeric *Ci*IL17-1 (A), *Ci*IL17-2 (B), *Ci*IL17-3 (C) superimposed on human IL-17F(PDB 1JPY). The conserved intra-molecular disulphide bridges are shown in yellow. (D) Alignment and secondary structure elements of human IL-17F and *Ci*IL17-1, *Ci*IL17-2, *Ci*IL17-3. The conserved cysteines that may form intra-molecular disulphide linkages are indicated with black asterisks. Arrows represent secondary structure elements: a – helices, b – strands. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Statistical evaluation of *Ci*IL17-1, *Ci*IL17-2 and *Ci*IL17-3 transcripts differential expression of disclosed by real time PCR. Effect of LPS treatment (LPS/MS) and the time course profiles (TIME). The table summarizes the main results of the factorial ANOVA analysis. Separate sums-of-squares F-tests and *P* value are computed for each effect.

Effect	Interleukin	Degree of freedom	MS	F	*P* value
LPS/MS	IL17/1	1	82.77	137.302	>0.0001
LPS/MS	IL17/2	1	67.46	1987.21	>0.0001
LPS/MS	IL17/3	1	80.55	1560.28	>0.0001
TIME	IL17/1	6	2.88	4.78	>0.0001
TIME	IL17/2	6	2.61	77.05	>0.0001
TIME	IL17/3	6	1.05	20.41	>0.0001

Mean of squares (MS), F-test value (F).

musculus, S. scrofa, X. tropicalis, S. salar, O. mykiss, D. rerio, M. musculus, C. mydas), the second includes the invertebrate IL-17 (*C. intestinalis, P. fucata, C. gigas, N. americanus, H. rufescens*) and vertebrate IL-17A, F and IL-17a/f1,2,3 (*H. sapiens, M. musculus, S. scrofa, X. tropicalis, S. salar, O. mykiss, D. rerio, M. musculus*).

3.3. Structural analysis

A structural homology analysis was carried out using the SWISS-MODEL program, and a modeling process was performed on the basis of the known crystal structure of human IL-17F (1jpyB; 2.85 A). Figure 3A shows the *Ci*IL17-1,2,3 molecular models resulting from sequence superimpositions. The *Ci*IL17-1 model resulted from the superimposition of the 62-146 residue sequences that share 33.3% identity. The *Ci*IL17-2 model resulted from the superimposition of the 71–154 residue sequences that share 34.4% identity. The *Ci*IL17-3 model resulted from the superimposition of the 96–184 residue sequences that share 31.183% identity. Although the target and template sequences are too diverse in term of identity to infer a conservation of the oligomeric state, Fig. 3B shows that the domains of the IL-17 of all three genes showing four cysteine residues involved in the formation of disulphide bonds of the theoric dimer, are highly conserved like functional and structural motifs.

3.4. Differential expression of CiIL17-1, CiIL17-2 and CiIL17-3 transcripts disclosed by real time PCR

Quantitative mRNA expression of *Ci*IL17-1, *Ci*IL17-2 and *Ci*IL17-3 in naive, sham (inoculated with marine solution) and LPS inoculated ascidians, was evaluated by real time PCR analysis. Four naive, sham and LPS-treated ascidians in three distinct experiments were examined at different post-inoculation time points (1, 4, 8, 12, 24, 48 h p.i.).

As shown in Table 2 the factorial ANOVA analysis of the time course profiles ($P > 0.001$) and LPS treatment ($P > 0.001$) have disclosed that the RNA level raised significant effects for each of the three genes.

The LPS-treated ascidians were compared to specimens inoculated with marine solution, and the latter compared to naive ascidians. In the LPS-treated ascidians, *Ci*IL17-1, *Ci*IL17-2 and *Ci*IL17-3 disclosed a significantly higher RNA level, in particular, the *Ci*IL17-1 expression was enhanced at 1 h and reached the highest expression at 4 h p.i., then decreased at 48 h p.i. (Fig. 4A), the *Ci*IL17-2 expression was enhanced at 1 h and reached the highest expression at 8 h p.i., then decreased at 48 h p.i. (Fig. 4B), the *Ci*IL17-3 expression was enhanced at 2–4 h and then decreased at 48 h p.i. (Fig. 4C). The inoculation procedure (sham ascidians) slightly modulated ($P > 0.05$) the expression levels in comparison to the naive specimens (Fig. 4A,B,C).

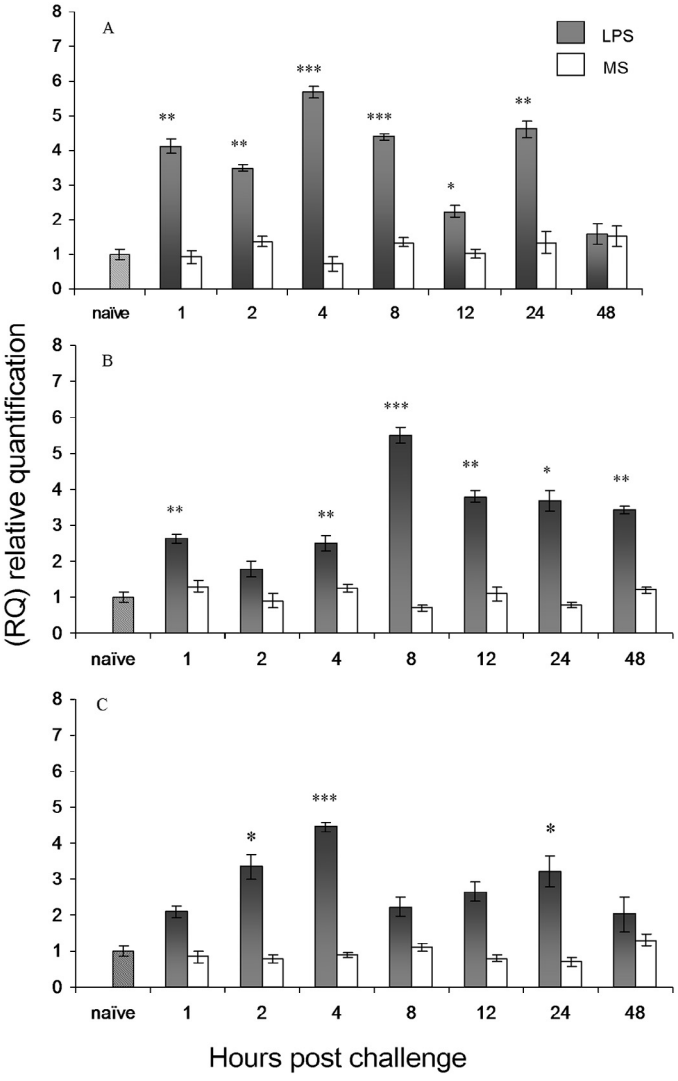

Fig. 4. Real-time PCR analysis. Time-course of *Ci*IL17-1 (A), *Ci*IL17-2 (B), and *Ci*IL17-3 (C), gene expression in *C. intestinalis* pharynx after inoculation into the body wall of 100 µg bacterial lipopolysaccharide (LPS) in 100 µl marine solution (MS) (in gray), compared with the gene expression in ascidians injected with 100 µl MS (in white). Values, plotted as mean ± SD, were inferred from four ascidians examined in three distinct experiments; each assay was performed in triplicate. Asterisks indicate significant differences, at each time point, between LPS and SW inoculation (post hoc Tukey *t*-test). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ($n = 4$).

3.5. In situ *hybridization assay of pharynx*

Observations of pharynx histological sections from naive ascidians (Fig. 5B,G,I) and LPS treated specimens (Fig. 5C,D,E,F,H,L) revealed that *Ci*IL17-1, *Ci*IL17-2 and *Ci*IL17-3 transcripts are preferentially expressed by granulocytes and URG cells located in tightly packed cell groups within the vessel lumen (Fig. 5E,F). The hemocyte population marked by *Ci*IL17-1, *Ci*IL17-2 and *Ci*IL17-3 riboprobes appeared to be increased following LPS stimulation. The density of hemocytes that express *Ci*IL17-1 transcript increased already at 1 h p.i. (Fig. 5C) with peaked at 4 h p.i. (Fig. 5D), whereas the density of hemocytes that expressed *Ci*IL17-2 and *Ci*IL17-3 transcript increased at 8 h and 4 h p.i., (Fig. 5H, L respectively). Histological sections treated only with the sense strand as controls showed no positive staining (Fig. 5A).

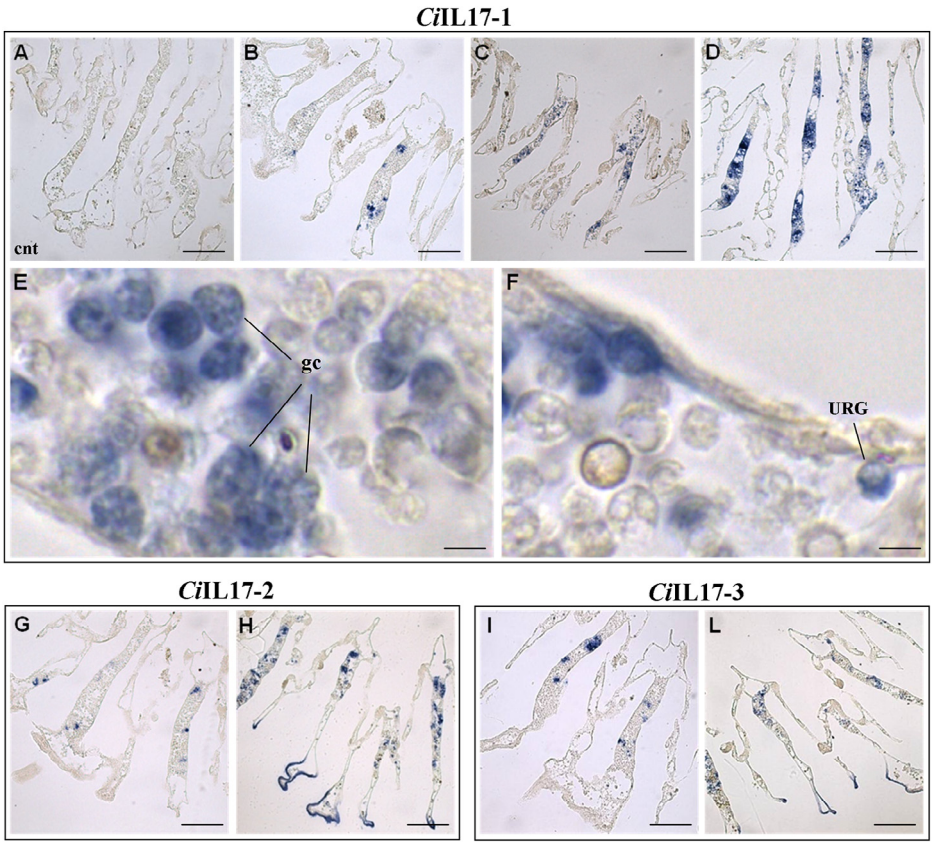

Fig. 5. Histological sections of *C. intestinalis* pharynx vessels. *In situ* hybridization with the *Ci*IL17-1, *Ci*IL17-2, *Ci*IL17-3 riboprobes. *Ci*IL17-1 riboprobe: sham ascidian (B), ascidian at 1 h (C) and 4 h. (D–F) After LPS inoculation. *Ci*IL17-2 riboprobe: sham ascidian (G), ascidian at 8 h. (H) After LPS inoculation. *Ci*IL17-3 riboprobe: sham ascidian (I), ascidian at 4 h. (L) After LPS inoculation. Control with the sense strand (A). Bars size: A–D, G–L bar 100 μm, E–F bar 5 μm. URG (unique retractile granulocyte), gc: granular cells.

4. Discussion

Immunity involves an intracellular signaling pathway that leads to the transcription of soluble mediators such as cytokines (Chen et al., 2007), small proteins that regulate and mediate, hematopoiesis, inflammation and immunity (Savan and Sakai, 2006). In mammals, interleukin 17 is a member of the proinflammatory cytokine family, it bridges the innate and adaptive arms of the immune system, and is mainly produced by a subset of T helper (Th) cells named Th17 (Moisan et al., 2007). Two forms, IL-17A and F, play an important role in host defence via the induction of proinflammatory gene expression, promoting cell infiltration and production of several proinflammatory cytokines and chemokines (Hartupee et al., 2007; Mesquita et al., 2009). These two genes are present in tandem on the same chromosome. Likewise, teleost IL-17 A and F homolog genes (IL17-A/F) are located on the same chromosome, and present the same genomic organization (three exons and two intron) (Gunimaladevi et al., 2006).

In this study, we identified three *C. intestinalis* IL-17 genes (*Ci*IL17-1,*Ci*IL17-2, *Ci*IL17-3) located on the chromosome 1, and showed their involvement in the inflammatory response toward LPS by the pharynx tissue which is retained the main immune organ of ascidians (Giacomelli et al., 2012). Phylogenetic analysis supported a conserved evolution of the IL-17 genes, and *Ci*IL17-1, *Ci*IL17-2 and *Ci*IL17-3 sequences grouped with human IL-17A and F which are involved in inflammation and host defence, including the production of proinflammatory cytokines such as TNFα, chemokines and antimicrobial peptides (Iwakura et al., 2011).

The similarity of the *Ci*IL17s gene organization and sequences suggests an evolutionary model based on gene duplication and sequence diversification. The close position in a same chromosome, the sequences and exon/intron similarity between *Ci*IL17-1 and *Ci*IL17-3, indicate a recent event of gene duplication, while the IL17-2 gene has probably been a product from a first IL17 gene duplication.

Sequence and structural analysis of *Ci*IL-17s revealed that these genes, shared similar features with vertebrate orthologs. They show the same gene organization of the human IL-17A/F, formed with two introns and three exons, differing in the length of the introns which are longer in human IL-17A/F. Sequence alignments showed four cysteins strictly conserved in regions that in human IL-17A and F are correspondent to functionally and structurally essential motifs (Witowski et al., 2004). The three-dimensional structure model, made on the basis of the crystal structure of human IL-17F, displays the cysteines localized in β-sheets, and supported the preservation of the disulphide linkages position in all the IL-17 homologs (Hymowitz et al., 2001). The model was generated respectively by superimposition of the *Ci*IL17-1 residues (62–146) that share 33.3% sequence identity, the *Ci*IL17-2 residues (71–154) that share 34.4% sequence identity, the *Ci*IL17-3 residues (96–184) that share 31.183% sequence identity. These are the sequences of the C-terminal region that is critical for the receptor binding (Hymowitz et al., 2001), and this finding is supported by the presence in the *C. intestinalis* genome of a gene homologous to IL-17 receptor (gene ID: ENSCING00000023310).

The *Ci*IL-17 genes appeared to be involved in the pharynx inflammatory response. Factorial ANOVA analysis between the time

course profiles disclosed that the *Ci*IL17 genes transcription following the LPS inoculation was significantly higher than that due to the SW injection.

The real time PCR profile shows that they are upregulated at 1–8 h p.i., and a significant expression was also found at 24 h p.i. Previous findings on inflammatory genes expression, including *Ci*TNFα (Parrinello et al., 2008, 2010) and galectins (Vizzini et al., 2012), revealed a prompt (4–8 h) upregulation of immune-related genes transcription by pharynx hemocytes, and the present paper suggest that *Ci*IL17 genes, such as vertebrate ortologs, are involved in the inflammatory response (Parrinello et al., 2007, 2008, 2010; Vizzini et al., 2012). *In situ* hybridization assay showed that *Ci*IL17 genes are expressed by inflammatory hemocytes (granulocytes and URGs) in the pharynx vessels, and, according to previous findings (Bonura et al., 2009, 2010; Cammarata and Parrinello, 2009; Cammarata et al., 2008; Parrinello et al., 2008, 2010; Vizzini et al., 2008, 2012), the LPS inoculation enhanced the population density of hemocytes expressing immune-related genes including *Ci*IL-17s. Finally these findings suggest that *Ci*IL-17 genes may be key genes in cellular defence reaction.

Uncited reference

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Appendix: Supplementary Material

Supplementary data to this article can be found online at doi:10.1016/j.dci.2014.09.014.

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