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IL-17 signaling components in bivalves: Comparative sequence analysis and involvement in the immune responses

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ABSTRACT

The recent discovery of soluble immune-regulatory molecules in invertebrates takes advantage of the rapid growth of next generation sequencing datasets. Following protein domain searches in the transcriptomes of 31 bivalve *spp.* and in few available mollusk genomes, we retrieved 59 domains uniquely identifying interleukin 17 (IL-17) and 96 SEFIR domains typical of IL-17 receptors and CIKS/ACT1 proteins acting downstream in the IL-17 signaling pathway. Compared to the *Chordata* IL-17 family members, we confirm a separate clustering of the bivalve domain sequences and a consistent conservation pattern of amino acid residues. Analysis performed at transcript and genome level allowed us to propose an updated view of the components outlining the IL-17 signaling pathway in *Mytilus galloprovincialis* and *Crassostrea gigas* (in both species, homology modeling reduced the variety of IL-17 domains to only two 3D structures). Digital expression analysis indicated more heterogeneous expression levels for the mussel and oyster IL-17 ligands than for IL-17 receptors and CIKS/CIKSL proteins. Besides, new qPCR analyses confirmed such gene expression trends in hemocytes and gills of mussels challenged with heat-killed bacteria. These results uphold the involvement of an ancient IL-17 signaling pathway in the bivalve immune responses and, likewise in humans, suggest the possibility of distinctive modulatory roles of individual IL-17s/IL-17 receptors. Overall, the common evidence of pro-inflammatory cytokines and inter-related intracellular signaling pathways in bivalves definitely adds complexity to the invertebrate immunity.

1. Introduction

Innate mechanisms based on non-self recognition, signal transduction and finely regulated gene expression provide invertebrate organisms with an effective protection against possible pathogens such as bacteria and viruses. Invertebrates are not able to mount long-lasting immune responses but rapidly respond to aggressors with multifaceted hemocytes and a large variety of effector molecules such as antimicrobial peptides, heterogeneous lectins, lysozymes, proteases and protease inhibitors (Buchmann, 2014). Reports on the existence of soluble immune-regulatory molecules in invertebrate animals are relatively recent and the possibility of a cytokine-like signaling network definitely adds complexity to the innate immune systems (Malagoli, 2010; Raftos and Nair, 2004). Spätzle in insects, astakine in crustaceans and invertebrate homologs of MIF, TNF and IL-17 might regulate the behavior of target cells in terms of growth and motility, thus orchestrating hematopoiesis, inflammation and immunity likewise their mammalian counterparts (Annunziato et al., 2014; Saenz et al., 2008).

According to their cellular origin and pleiotropic actions, the vertebrate cytokines are classified as interleukins, chemokines and interferons (Turner et al., 2014). Interleukin-17 (IL-17) was first recognized as cytolitic T-cell factor with pro-inflammatory activity (Rouvier et al., 1993) and it is the unique member of an interleukin class with no homology to any other known cytokine family (Moseley et al., 2003; Xu and Cao, 2010). Soluble IL-17 ligands and related membrane receptors (IL-17Rs) exist in humans as gene families and their effects on the target cells depend on differences in the expression levels of individual family members, receptor usage, and on a highly cross-regulated signaling system (Li P. et al., 2014; Sabat et al., 2013; Wang et al., 2014). The IL-17 signaling pathway starts with the binding of IL-17 homo- or hetero-dimers to specific membrane-bound IL-17R complexes. The signal transduction proceeds in the cytosol through the tumor-necrosis factor receptor-associated factor 6 (TRAF6, a key adaptor also in the TLR- and

Abbreviations: AA, amino acid; cds, protein coding sequence; hpi, hours post-injection; IL-17, interleukin 17 gene or transcript; *IL-17R*, receptor gene or transcript for interleukin 17; *CIKS/ACT1*/TRAFIP2, gene or transcript for a TRAF-interacting protein involved in regulating responses to vertebrate cytokines; SEFIR, SEF/IL-17R domain present in IL-17R and CIKS/ACT1/TRAFIP2 proteins; M, million; *Mg*, *Mytilus galloprovincialis*; qPCR, quantitative real-time PCR; RPKM, reads per kilobase per million mapped reads; RQ, relative quantification.

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IL-1R-signaling cascades) up to the activation of canonical transcription factors, such as NF-κB, and the expression of cytokines, chemokines and antimicrobial peptides among other target genes (Hartupee et al., 2007; Steinman, 2007). The crucial element of the IL-17 signaling is the SEF/IL-17R (SEFIR) domain which displays similarity to the Toll/IL1 (TIR) domain and mediates the interaction between the cytoplasmic tail of IL-17Rs and CIKS (alias Act1 or TRAFIP2), a proximate adaptor protein with TRAF-binding motifs (Chang et al., 2006; Novatchkova et al., 2003).

Humans possess six IL-17s (named IL-17A/F), five receptors (named IL-17RA/E) and one CIKS protein (Aggarwal and Gurney, 2002; Gaffen, 2009). IL-17s are produced by activated T lymphocytes and other cell types relevant to the host immunity such as the mucosal epithelial cells (Saenz et al., 2008). On the whole, the IL-17 family members are potent pro-inflammatory cytokines involved in host defense, autoimmunity and cancer (Annunziato et al., 2014; Wang et al., 2014). IL-17 is now recognized as central regulator of inflammatory responses in the human brain (Liu et al., 2014).

IL-17 and the related signaling pathway have been considered as exclusive vertebrate features until 2006, when thirty IL-17 and two IL-17R gene models were reported in the genome of the sea urchin *Strongylocentrotus purpuratus* (Hibino et al., 2006). Sequences similar to IL-17, IL-17R and CIKS have been found since then in several non-vertebrate organisms (Valenzuela-Muñoz and Gallardo-Escárate, 2014; Vizzini et al., 2015; Wu et al., 2011). In mollusks, IL-17 was first identified in *Crassostrea gigas* in 2008 (Roberts et al., 2008) and subsequently in *Pinctada fucata* (Wu et al., 2013) and *Hydnum rufescens* (Valenzuela-Muñoz and Gallardo-Escárate, 2014). Five IL-17 genes were reported in the *C. gigas* genome (Li J. et al., 2014) with more recent analyses indicative of eight IL-17s and five IL-17Rs (Zhang et al., 2015).

In this work, we update and describe the transcriptional landscapes of IL-17 and related signaling components of *Bivalvia*, focusing in particular on mussel and oyster *spp*. Aiming to validate the *M. galloprovincialis* sequence findings, we also report expression data of six selected IL-17s, three IL-17Rs and the CIKSL adaptor, as measured in hemocytes and gill of mussels injected with a mixture of heat-killed bacteria.

2. Materials and methods

2.1. Identification of protein domains for interleukins, IL-17s, IL-17Rs and CISK in Mytilus and other mollusks

To obtain a *Mytilus galloprovincialis* (*Mg*) reference transcriptome, we used a collection of 18,788 ESTs obtained by Sanger sequencing from mixed tissues (Venier et al., 2009) and 642.5 million (M) reads obtained by paired-end Illumina sequencing from digestive gland (97 Mreads, SRA ID: PRJNA88481 (Gerdol et al., 2014), gills (189.4 Mreads, unpublished) and from mantle, muscle, gill and immuno-stimulated hemocytes (108 Mreads, SRA ID: SRP033481). A global *de-novo* assembly was performed with Trinity (Grabherr et al., 2011) and CLC Genomic Workbench v.7 (Qiagen, Germany). The draft assembly of the mussel genome was downloaded from NCBI (GenBank ID: APJB000000000.1) (Nguyen et al., 2014).

Available RNA sequencing datasets were *de-novo* assembled separately for 31 bivalve species with the CLC Genomics Workbench using the same parameters as earlier (Table 1). In the case of *Crassostrea gigas*, we processed together other 704 Mreads (Illumina Hiseq2000, 2 × 50 bp paired end reads, SRA IDs: SRR334212-20, ENA ID: E-MTAB-2552 or unpublished) to check and expand the already available gene predictions. The genome drafts of *C. gigas*, *P. fucata*, *Lottia gigantea* and *Aplysia californica* were also downloaded from the public repositories with their gene predictions (details in Table 1).

Protein coding sequences (cds) were predicted in each species-specific assembly using Transdecoder (Haas et al., 2013). Selected HMM profiles (Interleukin-17: PF06083, SEFIR: PF08357, other interleukin domains: PF00715, PF00727, PF02025, PF02059, PF03039, PF00340, PF00489, PF00726, PF01415, PF02372, PF03487, PF09238, PF09240, PF10420, PF00048, PF14565, PF15036, PF15095, PF02394, PF05566, PF07400, PF06529, PF15037, PF15177, PF15209, PF15225, PF12233) were preliminary retrieved from the PFAM database release 27 (Finn et al., 2014) and used for HMMER scanning analysis (Eddy, 2011). The resulting positive hits were then screened for the presence of putative signal peptide and trans-membrane domains by SignalP-3.0 (Petersen et al., 2011) and TMHMM v.2.0 (Krogh et al., 2001), respectively.

The protein sequences found positive for one reference domain were subsequently aligned using MUSCLE (Edgar, 2004) and trees were generated with a neighbor joining clustering method and Jukes–Cantor substitution model with 1000 bootstrap replicates using MEGA6 (Tamura et al., 2013). To compute more precise phylogenetic distances, multiple alignments were performed on conserved domain positions previously identified with Gblocks (Castresana, 2000). A position was considered 'conserved' if it was common to 51% of the locally aligned sequences.

The *Mg* transcript sequences outlining a mussel IL-17 signaling pathway were used as blast query against the genomic mussel contigs in order to recover the corresponding gene structures. Only hits with an E-value lower than 10^{-20} were extracted and *de-novo* assembled to identify overlapping regions. The resulting contigs and singletons were manually combined to obtain the gene sequence; then, correct gene assembly and prediction of alternative splicing events were ascertained by RNA-seq read mapping with appropriate analysis parameters.

The three-dimensional structure of the identified IL-17 domains and proteins was predicted by homology using Phyre2 (Kelley and Sternberg, 2009).

2.2. Digital gene expression analysis of IL-17 signaling components identified in mussels and oysters

Using CLC Genomics Workbench, we analyzed different Illumina RNAseq samples from *Mg* (SRR442031/6 and SRP033481) and from *C. gigas*: different oyster tissues (SRR334212/20), oysters treated with *Vibrio anguillarum*, *V. tubiashii*, *V. aestuarianus*, *V. alginolyticus* and *Micrococcus lysodeikticus* (SRR796582-98), oysters at different developmental stages (SRR334222-59, Zhang et al., 2015) and OsHV-1-positive spat (E-MTAB-2552, Rosani et al., 2014). Details are reported in Supplementary Table S1. The *Mg* and *C. gigas* reads were mapped on the gene sequences denoting *IL-17* and proximate signaling components, separately for each species. Sequence length and similarity values were set to 0.75 and 0.95, respectively, whereas mismatch/insertion/deletion penalties were set to 2/3/3. The total number of reads of each dataset was counted and used to calculate digital expression values as RPKM (reads per kilobase per million mapped reads) (Mortazavi et al., 2008), then datasets were normalized by the total mapped reads and related to the expression levels of Elongation Factor 1α (EF1α).

2.3. Mussel immunostimulation and tissue sampling

Native mussels of commercial size (*Mg*, 5.7 ± 0.4 cm shell length) were collected from one outlet of the Venice lagoon (Italy) in May 2014, acclimatized at 23 ± 1 °C in artificial sea water (32‰ salinity, 22 °C) and fed with certified food (Plancto® Aqua Medic, Bissendorf, Germany). Mussels were placed in two plastic tanks (50 mussels/tank, 1 l sea water/mussel) and were injected into the posterior adductor muscle either with 0.1 ml of NaCl-enriched PBS (PBS–NaCl) or with 0.1 ml of a mixture of heat-killed Gram positive and Gram negative bacteria (10^8 CFU/ml). The bacterial cocktail was prepared from equal amounts of *Micrococcus lysodeiticus*, *Vibrio*