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REVIEW

Gene expression and regulation of molecules involved in pharynx inflammatory response induced by LPS in *Ciona intestinalis*

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Abstract

In the ascidian *Ciona intestinalis*, the pharynx (hemopoietic organ) connects the external environment to the gastrointestinal system for two main activities, respiration and food collection, potentially exposing the ascidian to high concentrations of pathogenic microorganisms. Recently, evidence in *C. intestinalis* has indicated that the pharynx is involved in the inflammatory reaction induced by lipopolysaccharide (LPS) injection into the body wall. Immune-related genes such as cytokines, galectins, pro-PO, CAP are expressed in pharynx hemocytes and are up-regulated by the inflammatory agent LPS. Studies of the expression pattern of the immune gene clearly show that in *C. intestinalis*, as in vertebrates, immune gene expression can be regulated through Alternative Polyadenylation (APA) Mechanism and GAIT element al 3'UTR.

Key Words: ascidian; inflammation; pharynx; LPS; *Ciona intestinalis*

Introduction

Inflammation is a rapid protective response to microbial infection, tissue injury, and insults (Nathan, 2002; Medzhitov, 2008). Infection by microbial invaders is often implicated as the major promoter of inflammatory responses, though injury or trauma (in the absence of parasitic infection) and exposure to foreign particles/irritants/pollutants are also potent activators of inflammation (Medzhitov, 2008), suggesting that this response likely evolved as a general adaptation to solve tissue damage (Matzinger, 2002). A common explanation for why infection and trauma might evoke similar inflammatory responses is that both pathogens and wounding cause damage to cells and tissue, which implies that similar responses to trauma and infection could have advantageous results (Bianchi, 2007). On an evolutionary level, inflammation is a highly conserved phenomenon and appears to be an important first line of defense for both invertebrates and vertebrates. Many of the processes associated with the inflammatory cascade, such as chemotaxis and phagocytosis, are readily employed by unicellular organisms and were later coopted as defensive components to maintain the integrity of more complex multicellular organisms (Rowley, 1996).

When host cells capable of innate immune responses encounter a pathogenic microbes or another foreign or host irritant, the inflammatory response initiates within minutes. The host cells first recognize the stimulus through a wide variety of sensing mechanisms involving transmembrane receptors. These interactions transmit signals to the nucleus, resulting in the activation and regulation of numerous genes via both transcriptional and post-transcriptional mechanisms (Akira *et al.*, 2006; Medzhitov, 2007; Ishii *et al.*, 2008; Beutler, 2009). Some inducible gene products, such as antimicrobial peptides and complement factors, directly target infectious microorganisms. Other inducible gene products, including proinflammatory cytokines and chemokines, activate endothelial cells and recruit immune system cells to the site of infection.

The innate immune system is the major contributor to acute inflammation (Akira *et al.*, 2006; Beutler *et al.*, 2006). Innate immunity is a conserved, complex and multi-pronged response to overcoming infection that is present in all metazoans. The ascidian *C. intestinalis* is a powerful model for studying innate immunity because it occupies a key phylogenetic position in chordate evolution and is considered the sister group of vertebrates (Zeng and Swalla, 2005;

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Delsuc *et al.*, 2006; Tsagkogeorga *et al.*, 2009). Ascidians possess an exclusively innate immune system, including inflammatory, humoral and cellular responses. In vertebrates, the development of the adaptive immune system is linked to the acquisition of the enzyme machinery encoded by Recombination Activating Genes (RAG) that provide for the rearrangement of immunoglobulin (Ig) and T Cell Receptor (TCR) genes. Analysis of *C. intestinalis* genome sequences has not revealed any of the pivotal genes and molecules for adaptive immunity, such as Major Histocompatibility Complex (MHC) genes, TCRs, or dimeric Igs (Dehal *et al*., 2002; Azumi *et al.*, 2003; Shida *et al.*, 2003;).

In *C. intestinalis*, challenge with Pathogen-Associated Molecular Patterns (PAMPs), such as Gram-negative lipopolysaccharide (LPS), induces inflammatory-like reactions in the pharynx (immunocompetent organ). These responses can induce several immunological phenomena, including the expression of characteristic innate immune genes and a repertoire of innate effectors.

This review provides an overview of the molecular and cellular events that are involved in pharynx inflammatory response induced by LPS in *C. intestinalis.*

Inflammatory response in the pharynx
Effector molecules

Following an LPS challenge or the inoculation of a foreign agent into the ascidian body wall, several inflammatory events are initiated, including hemocyte recruitment into the inflamed tissue (Parrinello, 1981; Parrinello *et al.*, 1984a, b; Parrinello and Patricolo, 1984). The well-studied organ involved in the inflammatory reaction induced by LPS injection into the body wall is the pharynx. In ascidians, which are filter-feeding animals, the pharynx occupies an extensive part of the body. It consists of two epithelial monolayers perforated by rows of antero-posteriorly elongated elliptical ciliated structures, the stigmata (Martinucci *et al.*, 1988), which are aligned dorsoventrally. Each row of stigmata is enclosed in a mesh of vessels (forming the so called transversal and longitudinal bars) that originate from the two epithelial leaflets, where the hemolymph, containing abundant mature and immature hemocytes, flows (De Leo *et al.*, 1987). The ciliated cells of the stigmata generate water currents, which are crucial for the two main activities of this organ, respiration and food collection that expose ascidians to high concentrations of microorganisms.

The inflammatory response induced by LPS challenge in the pharynx has been extensively studied, including the expression of characteristic innate immune genes and of a repertoire of innate effectors in pharynx hemocytes. Granular cells and univacuolar refractile granulocytes are active in galectin and phenoloxidase (PO) production (Vizzini *et al.*, 2012, 2015). The genome-wide analysis of *C. intestinalis* has provided a comprehensive picture of immune-related genes and annotated galectin. The sequences of two *C. intestinalis* genes encoding galectins, characterized by two carbohydrate recognition domains (CRD) homologous but not identical, connected by a short linker peptide (bi-CRD) (*Ci*Lgals-a and *Ci*Lgals-b), have been deduced from ESTs and the genome (Houzelstein *et al.*, 2004). *Ci*Lgals-a exhibits F4-CRD-liker-F3-CRD organization which is typical of all vertebrate bi-CRD galectin genes, and *Ci*Lgals-b shows an F4-CRD-linker-F4-CRD structure which is unknown in vertebrate genes. *In situ* hybridization assays and Real time PCR show that both galectins appear to be inducible and promptly expressed after LPS inoculation in pharynx hemocytes (Vizzini *et al.*, 2012) (Fig. 1).

In *C. intestinalis*, *in vivo* challenge of LPS resulted in the upregulation of a mannose-binding lectin-like collectin (*Ci*MBL) (Bonura *et al.,* 2009) (Fig. 1). Collectins are a family of calcium-dependent lectins that are characterized by their collagen-like domains and exert several functions, including complement activation, microbe agglutination, opsonization, and the modulation of inflammatory response. In pharynx hemocytes *Ci*MBL transcription results strongly upregulated by LPS inoculation (Fig. 1) and expressed by granular amebocytes and hemocytes with large granules (Bonura *et al.,* 2009). *C. intestinalis* has been shown to have an almost complete set of complement gene families (Marino *et al*., 2002; Azumi *et al*., 2003; Nonaka and Satake, 2010), suggesting the existence of complement cascade pathways in this ascidian. Indeed, a specific chemotactic activity exerted on hemocytes by C3a, the anaphylatoxin produced by C3 activation, has been demonstrated in *C. intestinalis*, providing compelling evidence of the presence of a complement system-related inflammatory pathway in deuterostome invertebrates (Pinto *et al*., 2003). This finding has been extended through the identification of the C3a-specific receptor, CiC3aR, on hemocytes (granular amebocytes) (Melillo *et al*., 2006). C3, which is constitutively expressed in the pharynx, is up-regulated by LPS injection. Using two specific anti-CiC3 and anti-CiC3a polyclonal antibodies, it was found that the gene product was localized to the hemocytes of the pharynx vessels (identified as granular amebocytes) and in stigmata ciliated cells (Giacomelli *et al*., 2012).

Hemocytes inside the pharynx vessels are also engaged in the PO expression genes CinPo-1 and Cin-PO2 (Vizzini *et al.*, 2015). A basal expression of both genes was found in naïve ascidians, indicating an active role of the enzymes as a constitutive defense activity as befits innate-type defense factors. In the pharynx, expression gene analysis showed an increase of transcripts of these genes following LPS inoculation (Fig. 1). Immune gene expression analysis of galectins, proPO, and cytokines with a time course of 48 h after LPS challenge, showed a peculiar upregulation at 1 - 4 h post injection, followed by a second wave of activation at the stage of 12 - 48 h (Fig. 1). Interestingly, a similar pattern of activation has been observed for *Ci*-type-IX-collagen (Vizzini *et al.*, 2008), presumably involved as an inducer of inflammation as well as playing a role in the formation of granulation tissue, matrix, and tissue remodeling.

These data are indicative of an early phase of inflammatory response activation and a late phase which precedes the resolution of inflammation.

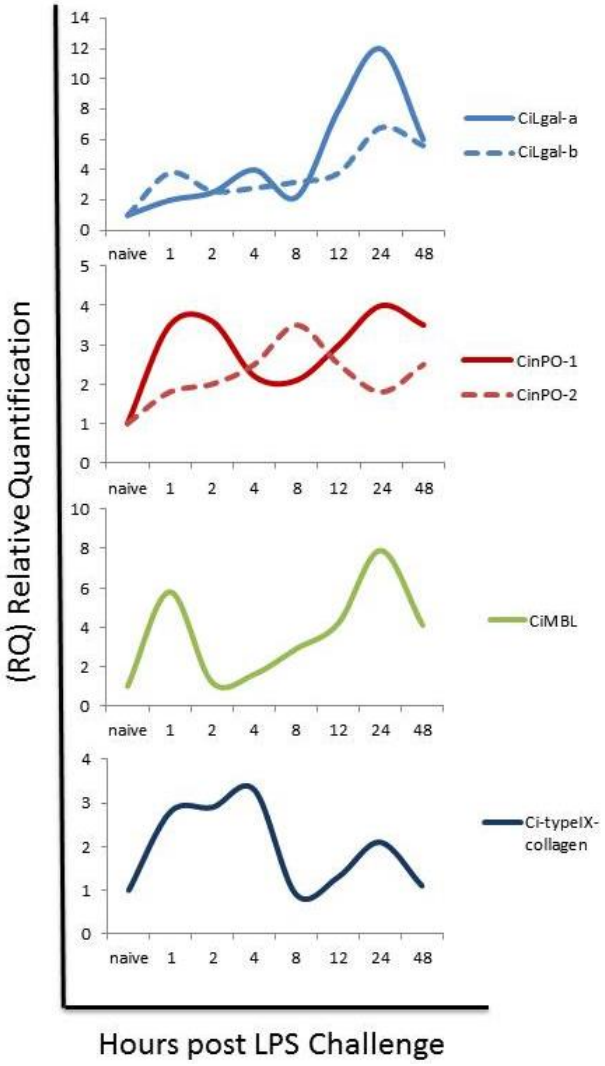

Fig. 1 *Ci*Lgal-a,-b, CinPO-1,-2, *Ci*MBL, *Ci*-typeIX-collagen gene expression in *C. intestinalis* pharynx after inoculation of lipopolysaccharide (LPS) into the body wall.

Receptors and signal transduction

The initial sensing of cell infection is mediated by innate Pattern Recognition Receptors (PRRs), which include Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs) and C-type lectin receptors (Beutler, 2009; Foster and Medzhitov, 2009; Takeuchi and Akira, 2010). The intracellular signaling cascades triggered by these PRRs lead to the transcriptional expression of inflammatory mediators that coordinate the elimination of pathogens and infected cells. In *C. intestinalis*, screenings of the genome sequence have identified homologues to human TLRs (hTLRs) (Azumi *et al.*, 2003; Sasaki *et al.*, 2009), designated *Ci*-TLR1 and *Ci*-TRL2 and composed of a Toll/interleukin-1 receptor (TIR) domain, a transmembrane, and a leucine-rich repeat (LRR) domain. Ci-TLR1 and Ci-TRL2 result most homologue to hTLR 7 and hTLR 8. The TIR domains of Ci-TLR1 and Ci-TRL2 were most similar to hTLR 4 and hTLR 6 respectively. They show hybrid functionality since both bind the same ligands of hTLR 2, 3, 5. The *Ci*-TLR1 and *Ci*-TRL2 genes are expressed intensively in the stomach, intestine, and hemocytes (Sasaki *et al.*, 2009). *Ci*-TLRs, like vertebrate TLRs, directly recognize PAMPs and trigger the transactivation of nuclear factor kB (NF-κB) by orthologs of TLR-signaling factors such as Myeloid differentiation primary response protein 88 (MyD88), Interleukin 1 receptor-associated kinase (IRAK), Tumor necrosis factor receptor-associated factors (TRAF), inhibitor of kappa B protein (IκB), and NF-κB, as well as induce the expression of Tumor necrosis factor α (*Ci* TNFα) (Fig. 2) (Azumi *et al.*, 2003; Sasaki *et al.*, 2009).

In mammals, LPS is recognized by a complex of TLR4 and Myeloid Differentiation protein-2 (MD2) (Miyake *et al.*, 2000; Dunne and O'Neill, 2005; Takeda and Akira, 2005). In humans, MD2 is a component of the MD-2-related Lipid-recognition (ML) superfamily which contains a large set of genes encoding proteins such as MD-1, MD-2, Niemann-Pick

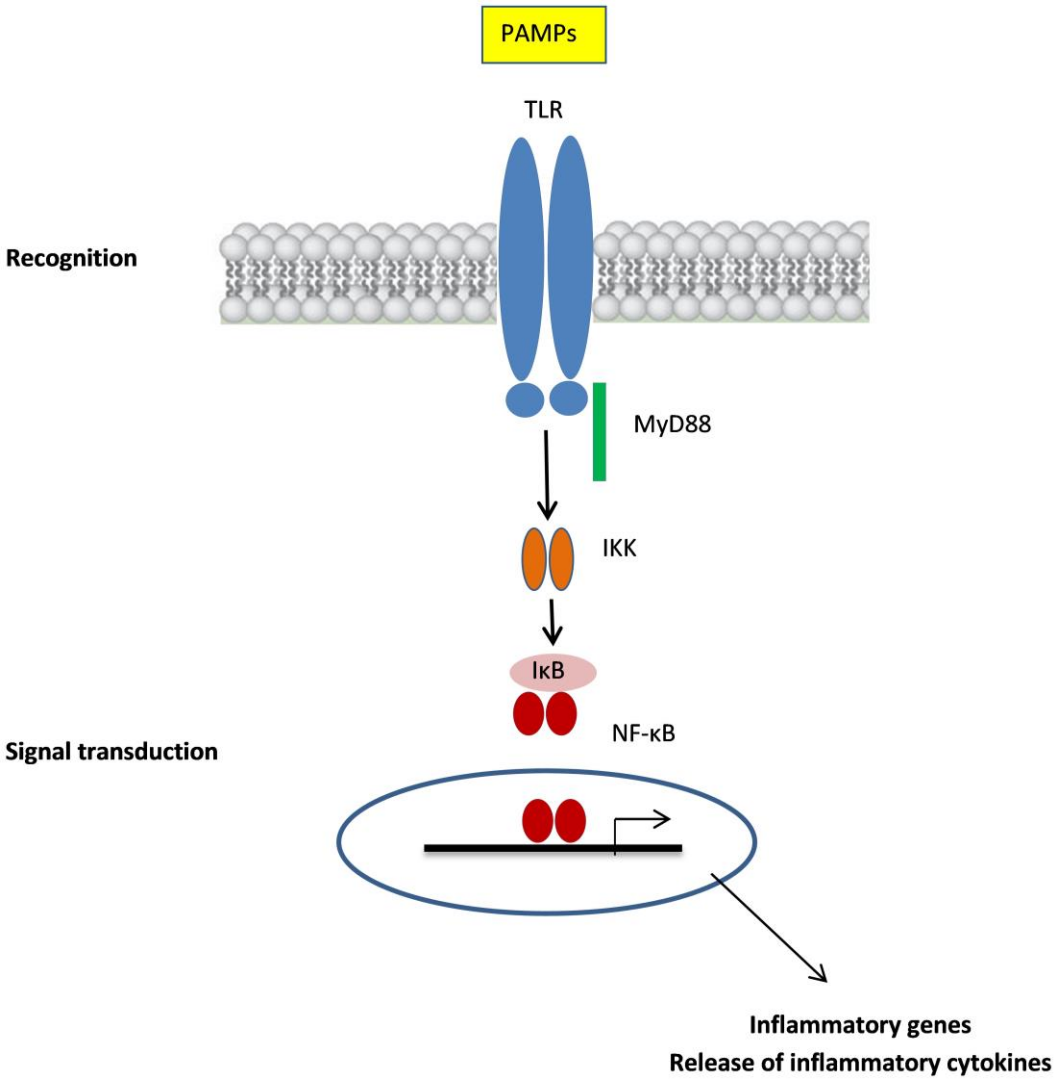

Fig. 2 Inflammatory cascade: pathogens induce inflammation. TLRs activate an MyD88-dependent signal transduction pathway. NF-κB is released from IκB and translocates to the nucleus where transcription is upregulated through binding to target inflammatory genes.

type C2 (NPC2) protein. NCP2 proteins play important roles in lipid metabolism and innate immune response. In *C. intestinalis*, the use of a subtractive hybridization strategy to identify differentially expressed LPS sequences allowed the identification of a *Ci*-NPC2 mRNA (Vizzini *et al.*, 2015a) that becomes upregulated after LPS challenge and is preferentially expressed in hemocytes (compartment cells and signet ring cells) inside the vessel lumen of the pharynx. Sequence analysis showed a significant homology of *Ci*-NCP2 with several components of the ML superfamily, supporting a conserved evolution of the NCP2 protein and suggesting an evolutionary model based on gene duplication and sequence diversification of ML family components (Vizzini *et al.,* 2015a). This finding supported the hypothesis that the recognition of LPS by TLR4 through MD2 binding may have been acquired during the evolution of vertebrates and that *C. intestinalis* may respond to LPS through a complex with other alternative LPS sensors (*i.e.*, the *Ci*-NCP2 protein) before the differentiation of the MD-2 protein in vertebrates (Vizzini *et al.,* 2015a).

Inflammation mediators

Inflammation consists of a tightly regulated cascade of immunological processes that are orchestrated by soluble immune signaling molecules called cytokines. The net effect of an inflammatory response is determined by the balance between pro-inflammatory and anti-inflammatory cytokines. In *C. intestinalis*, the recognition of LPS (PAMPs) activates a signaling pathway that culminates in the activation of NF-kB and the transcription and translation of genes that lead to the expression of inflammatory cytokines such as Transforming growth factor β (TGF-β), Interleukin 17 (IL-17), and TNF-α (Azumi *et al.,* 2003; Sasaki *et al.,* 2009).

TGF-β belongs to a family of regulatory cytokines that have pleiotropic functions in a broad range of cell lineages involved in numerous physiological and pathological processes, such as embryogenesis, carcinogenesis, and immune response (Blobe *et al.*, 2000; Li *et al.*, 2006; Wharton and Derynck, 2009). The TGF-β signaling process appears to be widely conserved in the animal kingdom since components of the pathway are characteristic of protostomes and deuterostomes (Huminiecki *et al.*, 2009). In comparative genome analysis of the TGF-β pathway genes in 33 species and in bilateria, at least one type II receptor and multiple type I receptors were detected, and the ancestral bilaterian repertoire can be inferred as consisting of two type II receptors and three type I receptors (Huminiecki *et al.,* 2009). In ascidians, the ancestral bilaterian TGF receptor repertoire is expanded to three type II receptors: this is the first example of bilaterian TGF-β receptor duplication, mapping to chordates which is propagated through vertebrates (Huminiecki *et al.*, 2009). The signaling output of TGF-β elicits diverse cellular responses that are primarily mediated through the actions of SMAD transcription factors (Massague, 1998; Shi and Massague, 2003; Massague and Gomis, 2006). In ascidians, at least two R-SMADs (one TGF-β and one bone morphogenetic protein), one Co-SMAD, and one I-SMAD have been described (Huminiecki *et al.*, 2009).

*Ci*TGF-β is structurally related to members of the superfamily, is synthesized as a large pre-pro-protein composed of a hydrophobic signal peptide, a N terminal pro-domain, and a C-terminal active peptide, and also exhibits a cleavage site at a consensus site (RRRK) for generating a C-terminal domain. As with all members of the superfamily, the pro-domain shows a low degree of conservation for correct processing and secretion of the mature dimeric complex, and C-terminal active peptide exhibits high cysteine residues at invariant positions (Munger *et al.*, 1999). These are engaged in intramolecular disulphide bonds, resulting in the adoption of a tridimensional structure with a cysteine knot motif and shared secondary structures with two α helix and seven β sheets. In addition, an RGD motif is present with a potential binding of integrins that can activate the TGF-β1 present in LAP-b conformation. In mammals, upon binding, it induces adhesion-mediated cell forces that are translated into biochemical signals which can lead to the liberation activation of TGF β from its latent complex (Munger *et al.*, 1999). *Ci*TGF-β become transcriptionally upregulated during the inflammatory process induced by LPS inoculation, suggesting that it is involved in the first phase (1-4 hrs) and significant in the secondary phase (48 h) of the inflammatory response in which cell differentiation occurs (Vizzini *et al.,* 2016) (Fig. 3). This double peak of *Ci*TGF-β mRNA production could be related to the potential function of TGF-β as a pro-inflammatory cytokine and anti-inflammatory molecule that, at the end of the reaction, helps to restore homeostasis. In situ hybridization assays show that the *Ci*TGF-β gene is expressed in tightly packed hemocyte clusters within the vessel lumen by inflammatory hemocytes (granulocytes and univacuolar refractile granulocytes) in the pharynx vessels (Vizzini *et al.,* 2016). In mammals, TGF-β has a role in T cell differentiation during immune response, in particular for T helper 17 cells (Th17), and in IL-17 production (Lohr *et al.*, 2006). In humans, IL-17 is a proinflammatory cytokine that plays a key role in the clearance of extracellular bacteria promoting cell infiltration and the production of several cytokines and chemokines. IL-17 displays the 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 NF-kB (Hata *et al.*, 2002).

In *C. intestinalis*, IL-17 genes (*Ci*IL17-1,*Ci*IL17-2, *Ci*IL17-3) show their involvement in the inflammatory response toward LPS via pharynx tissue (Vizzini *et al.*, 2015b) (Fig. 3). *C. intestinalis* IL-17 genes share a prompt expression induced by LPS inoculation, suggesting that they are involved in the first phase of inflammatory response, and a significant expression was also found in hemocytes (granulocytes and univacuolar refractile granulocyte) inside the pharynx vessels at 24 h post-inoculation (Vizzini *et al.,* 2015b). Sequence and structural analysis of *Ci*IL-17s revealed that these genes shared similar features with vertebrate orthologs (human IL-17A/F) which are involved in inflammation and host defense, including the production of proinflammatory cytokines such as TNFα, chemokines and antimicrobial peptides (Iwakura *et al.*, 2011).

In *C. intestinalis*, tumor necrosis factor α gene (*Ci*TNFα) is expressed either during the inflammatory pharynx response to LPS or during the swimming larval phase of development. Granulocytes and compartment/morula cells are *Ci*TNFα-producing cells in both the inflamed pharynx and larvae (Parrinello *et al.,* 2010). The pharynx vessel endothelium also takes part in the inflammatory response. The presence of hemocyte nodules in the vessel lumen or associated with the endothelium suggests the involvement of *Ci*TNFα in recruiting lymphocyte-like cells and promoting the differentiation of inflammatory hemocytes. In larvae, *Ci*TNFα is expressed by trunk mesenchyme, preoral lobe, and tunic cells, indicating *Ci*TNFα-expressing cell immigration events and an ontogenetic role (Parrinello *et al*., 2008, 2010).

In *C. intestinalis*, as in vertebrates, the complex interplay between multiple cytokines, cells, and the extracellular matrix is central to the initiation, progression, and resolution of inflammation.

Transcriptional and post-transcriptional regulation of gene expression in inflammation

Inflammation is a multicomponent response, and a crucial point of its control is at the level of gene transcription (mRNA splicing, mRNA polyadenylation, mRNA stability, protein translation) which plays an instrumental role in controlling both the magnitude and duration of the inflammatory response. Transcriptional and post-transcriptional mechanisms that modify mRNA expression, stability, and/or translation provide for the rapid and

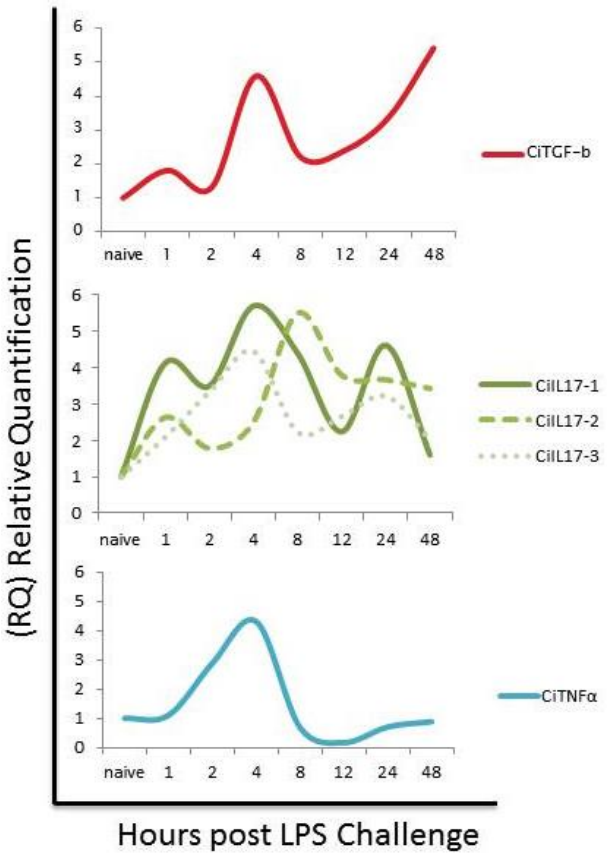

Fig. 3 *Ci*TGFβ, *Ci*IL-17s, *Ci*TNFα gene expression in *C. intestinalis* pharynx after inoculation of lipopolysaccharide (LPS) into the body wall.

flexible control of this process and are particularly important in coordinating the initiation and resolution of inflammation (Anderson, 2010).

The use of Alternative Polyadenylation (APA) sites is a regulatory level by which cells can generate different protein isoforms with different functions or with mRNAs differing in the length of their 3' untranslated regions (3'UTR) (Batra *et al.*, 2015). APA mechanisms can be divided into two major types. The simplest and most frequent are named Untranslated Region-Alternative Polyadenylation (UTR-APA) in which the alternative poly(A) sites are located in the 3'UTR of the mRNAs, with the majority of them residing closer to the stop codon (proximal) compared to canonical poly(A) sites (distal). This kind of alternative mechanism results in the shortening of the 3'UTR without changing coding capacity. The second type, named Coding Region-Alternative Polyadenylation (CR-APA), is a mechanism by which the alternative poly(A) sites reside in the upstream regions of genes. In particular, the less frequent intronic APA involves the recognition of a cryptic intronic poly(A) signal that involves premature polyadenylation within the coding region affecting the sequence of the protein (Elkon *et al.*, 2013). Through the alteration of the 3'UTRs, APA potentially regulates the stability, cellular localization, and translation efficacy of target RNAs as 3'UTRs serve as binding regions for factors that control these regulatory layers (*i.e.,* microRNAs and RNA binding proteins). Furthermore, 3'UTRs of the mRNA of pro-inflammatory proteins can contain regulatory elements, such as Interferon-γ-activated inhibitor of translation (GAIT), that direct their degradation and/or translational repression (Fox, 2015).

LPS challenge in *C. intestinalis* induces a CR-APA mechanism that generates an alternative transcript in two genes, named *Ci*8 (Vizzini *et al.*, 2013) and CAP (Vizzini *et al.*, 2016) (Fig. 4). In particular, LPS was able to weakly modulate the expression of the *Ci*8long transcript and to induce the activation of a LPS-induced APA mechanism responsible for the generation of a shorter mRNA (*Ci*8short). In fact, in silico analysis identified a non-canonical poly(A) site within the first intron of the annotated gene. Sequence analysis showed that the *Ci*8long-deduced amino acid sequence displays a protein domain homologous to components of the Receptor Transporting Protein (RTP) family (Mainland and Matsunami, 2012). The RTP family consists of four members (RTP1-4): RTP1 and RTP2 are expressed in olfactory neurons and vomeronasal neurons, RTP3 is expressed in the liver, lungs and testes, and RTP4 is expressed in a wide variety of tissues, including lymph nodes, peripheral blood

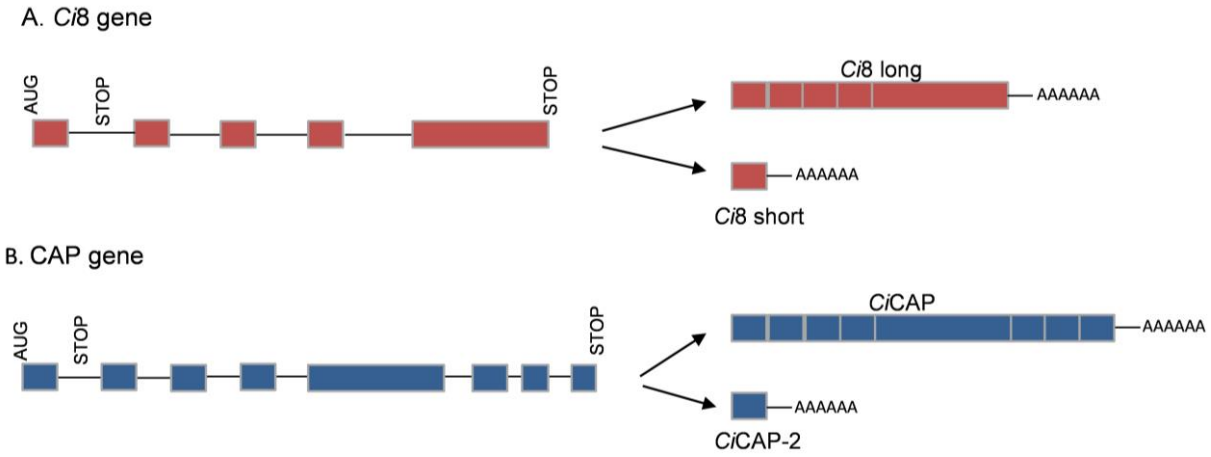

Fig. 4 LPS challenge in the ascidian *C. intestinalis* induces a Coding Region Alternative Polyadenylation (CR-APA) event in the annotated *Ci*8 and CAP gene. Intron-exon organization of the *Ci*8 (A) and CAP (B) gene. The thin lines represent the introns, and the open boxes indicate the exons. An intronic polyadenylation signal within the first intron leads to the transcription of variants named, respectively, *Ci*8short and CiCAP-2 mRNA.

leucocytes, spleen, and thymus (Mainland and Matsunami, 2012). The action mechanism of this family of proteins is poorly understood, and the existence of several closely related family members with disparate phenotypes suggests a wide role for these proteins. The short isoform does not contain the RTP domain and does not display any other homologs in the data banks different from the *C. intestinalis* annotated transcript (Vizzini *et al.,* 2013).

Furthermore, in silico prediction demonstrated that the *Ci*8long derived protein contains two transmembrane regions which are not present in the *Ci*8short protein, suggesting that the short isoform may represent an LPS-induced secreted form of the constitutively expressed gene. The *Ci*8short expression profile showed a peak of activation within 1 h post inoculation, followed by a second wave of activation at 12 h. The tissue localization of the *Ci*8short and *Ci*8long transcripts showed that LPS inoculation also induced a differential tissue localization of the two mRNAs, probably related to the CR-APA mechanism. The *Ci*8long transcript was expressed in some hemocytes of pharynx vessels, whereas the *Ci*8short mRNA appears to be strongly upregulated in compartment/morula and signet ring cells as well as in vessel endothelial cells and epithelium (Vizzini *et al.*, 2013).

In *C. intestinalis*, LPS induces an CR-APA event in the CAP gene that generates a shorter mRNA *Ci*CAP-2 gene through an CR-APA site contained within the first intronic sequence and derived from the sequence of the first exon plus a stretch of nucleotides contained within the first intron with coding capacity (Vizzini *et al.,* 2016). CAP proteins are a wide group of proteins that belong to the cysteine-rich secretory protein, antigen 5 and pathogenesis-related 1 superfamily which, it has been proposed, play key roles in the infection process and the modulation of immune responses in host animals (Gibbs *et al.*, 2008). The *Ci*CAP-2 mRNA potentially codifies for a deduced 51 amino acid long protein containing a predicted signal peptide of 22 amino acids and a 29 amino acid mature peptide that does not possess CAP elements identified in the *Ci*CAP protein (Bonura *et al.*, 2010; Vizzini *et al.*, 2016). Expression studies performed in pharynx tissue showed that the *Ci*CAP-2 mRNA is upregulated a few hours after LPS injection (1 - 4 h), while the LPS injection procedure slightly modulated the expression levels of *Ci*CAP. This data is in agreement with the colocalisation of the two mRNAs within the hemocytes flowing through the pharynx vessels and epithelium of stigmata, and the net increase of cells expressing the *Ci*CAP-2 mRNA with respect to the cells expressing *Ci*CAP after the LPS challenge (Vizzini *et al.,* 2016).

Furthermore, an in silico analysis identified the presence of a GAIT element in the 3'UTR of *Ci*CAP-2 mRNA which was not identified in the 3'UTR of *Ci*CAP (Vizzini *et al.,* 2016). This GAIT element is a cis-acting RNA element first identified in the 3'UTR of the ceruloplasmin (Cp) mRNA in humans. In a more general way, GAIT elements have been found in several immune-related mRNAs, showing an important role in gene-specific translation control in innate immunity (Vyas *et al.*, 2009). These elements form a stem-loop structure that is involved in the selective translational silencing of mRNA transcripts. The GAIT complex identified in human myeloid cells is hetero-tetrameric, consisting of glutamyl-prolyl-tRNA synthetase (EPRS), NS1-associated protein 1 (NSAP1), ribosomal protein L13a, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fox, 2015). A search of the *C. intestinalis* genome showed annotated ortholog genes for EPRS, ribosomal protein L13a, and GAPDH except for NSAP1. In this respect, ascidians seem to have a GAIT structure similar to the murine heterotrimeric GAIT complex, lacking NSAP1 (Arif *et al.*, 2012; Vizzini *et al.*, 2016).

Genes encoding cytokines are regulated at transcriptional and post-trascriptional level, mRNA stability and at translational level (Sariban *et al.*, 1988). TNF-α is a key cytokine regulator of the immune process and of tissue homeostasis. A computational analysis of *C. intestinalis* was performed to identify cis-regulatory elements in the 3'UTR of *Ci*TNFα, and a GAIT element, AU rich elements (AREs), Musashi binding element (MBE), and a TNF element were identified (Vizzini *et al.*, 2017).

In humans, the biosynthesis of TNFα is tightly regulated at the transcriptional (Beisang and Bohjanen, 2012) and post-transcriptional levels (Jensen *et al.*, 2001; Karpova *et al.*, 2001, Hao and Baltimore, 2013). AREs are present in the 3'UTRs of many cytokine and inflammatory genes (Lu *et al.*, 2006; Sandberg *et al.*, 2008; Wang *et al.*, 2008). The production of TNF-α is mainly controlled post-transcriptionally by cis-elements located in the 3'-UTR which regulate mRNA stability and translation efficiency. In addition to a class II AU-rich element (ARE2), another RNA binding motif has been characterized about 150 nt downstream. This element contains a consensus core of AUAUUUA, which is highly conserved in evolution and has been suggested to synergize with the ARE2 element in the repression of TNF-α mRNA translation and in translation regulation (Carpenter *et al.*, 2014). Finally, in the 3'UTR of *Ci*TNF-α an MBE was identified that is involved in mRNA translational control during cell cycle progression and cancers (Arumugam *et al.*, 2012; Fox *et al.*, 2015).

Concluding remarks

In *C. intestinalis*, inflammation is a complex reaction of host defense mechanisms (innate immunity), initiated by the presence of trauma, pathogenic microbes, or foreign materials, aiming at the neutralization of the insult and restoring normal tissue structure and function. This reaction proceeds via an overlapping pattern of events including coagulation, inflammation, epithelialization, formation of granulation tissue, matrix and tissue remodeling. This review describes the mediators and effectors involved and regulated at the transcriptional and post-transcriptional levels in mechanisms of the innate immune response in *C. intestinalis*. In silico and in vitro studies of the expression pattern of the immune genes clearly show that ascidians are able to respond to the inflammatory injury induced by LPS through the prompt transcriptional activation and regulation by CR-APA event and/or additional regulatory elements in the 3'UTR, implying tight translational control during inflammatory response. These findings suggest that in ascidians, transcriptional and post-transcriptional regulation can rapidly activate and repress immune genes, controlling the initiation phase of immune response and the resolution of the inflammation through a mechanism which has been conserved during evolution.

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