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Immune competence of the *Ciona intestinalis* pharynx: Complement system-mediated activity

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ABSTRACT

In the tunicate *Ciona intestinalis*, the ciliated pharynx, which connects the external environment to a highly developed and compartmentalized gastrointestinal system, represents the natural portal of entry for a vast and diverse, potentially pathogenic microbial community. To address the role of the pharynx in immune surveillance in *Ciona*, we asked whether C3, the key component of the complement system, was expressed in this organ and whether the encoded protein was functionally active. We found by real-time PCR that C3, constitutively expressed in the pharynx, is up-regulated by LPS injection. Using two specific anti-CiC3 and anti-CiC3a polyclonal antibodies in immunohistochemical staining of pharynx sections, we found that the gene product was localized to hemocytes of the pharyngeal bars (identified as granular amoebocytes) and in stigmata ciliated cells. Use of the same antibodies in Western blot analysis indicated that CiC3 and its activation products CiC3b and CiC3a are present in pharynx homogenates. Our observation that the amount of the bioactive fragment CiC3a increased in the pharynx of LPS-treated animals provides the first molecular and functional evidence for complement-mediated immunological activity in the tunicate pharynx.

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1. Introduction

In ascidians, which are filter-feeding animals, the pharynx occupies an extensive part of the body and corresponds to the branchial sac of lower vertebrates. The development and morphology of this organ in the adult have been studied in detail in many different species of colonial as well as solitary ascidians [1–3]. In adults, the pharynx consists of two epithelial monolayers perforated by rows of anteroposteriorly elongated elliptical, ciliated structures, the stigmata [4], 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 blood flows. The ciliated cells of the stigmata generate water currents, which are crucial for the two main activities of this organ, respiration and food collection [2]. Absorption of dissolved organic molecules [5,6], apocrine secretion,

and phagocytosis of food particles [7] have been also suggested as additional functions of this organ.

In the ascidian pharynx, mature and immature blood cell types are abundant in both the longitudinal and transversal bars [8], whose epithelia host clusters of stationary cells, called lymph nodules. These lymph nodules consist of dividing hemoblast-like cells surrounded by non-dividing mature hemocytes [9]. Experiments carried out with [³H]thymidine have created a dynamic picture of blood cell generation in the pharynx nodules, providing compelling evidence of a hematopoietic role for this organ [10]. The high rate of this proliferative activity has been further demonstrated through stimulation of pharyngeal explants by interleukins and mitogenic lectins [11,12].

Recently, evidence in the ascidian *Ciona intestinalis* has indicated that the pharynx is involved in the inflammatory reaction induced by lipopolysaccharide (LPS) injection into the body wall. Immune-related genes encoding TNF α , CAP, and the a and b galectins, are expressed in pharynx hemocytes, and are up-regulated by the inflammatory agent LPS [13–16]. In fact, *Ciona*-specific hemocytes have been demonstrated to play important roles in the immune response. Granular amoebocytes (GAs) and univacuolar refractile granulocytes participate in the encapsulation processes [17,18], and are involved in phenoloxidase production [19]. Furthermore, the univacuolar granulocytes have been shown to

Abbreviation: GA, granular amoebocyte.

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synthesize anti-microbial peptides [20,21], and GAs are involved in the phagocytosis [22,23], and the production of the immune receptor variable region-containing chitin-binding proteins (VCBP) [23].

GAs are also involved in the production of C3, the key molecule of the complement system. Elements of this system, which represents one of the major effector mechanisms of vertebrate innate immunity, have been identified in many invertebrate species belonging to different branches of the metazoan phylogenetic tree [24]. *Ciona* has been shown to have an almost complete set of complement gene families [25–27], 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 *Ciona*, providing compelling evidence of the presence of a complement system-related inflammatory pathway in deuterostome invertebrates [28]. This finding has been extended through the identification of the C3a-specific receptor, CiC3aR, on GAs, giving a complete representation of the mechanisms and partners involved in the pro-inflammatory activity mediated by the complement system [29].

To determine whether the pharynx might be involved in the immune response in *Ciona*, we asked whether C3 transcripts were present in this tissue and, if so, whether the encoded protein was functionally active. We have found by real-time PCR that C3 expression in the pharynx is up-regulated by LPS injection. Furthermore, Western blot analysis indicated that C3 activation generates the C3a anaphylatoxin fragment. Our results provide experimental evidence that the *Ciona* pharynx, the organism's most anterior outpost toward the external environment, has a role in the immune surveillance.

2. Materials and methods

2.1. Preparation of pharynx samples

Samples of *C. intestinalis* were collected in the Gulf of Napoli and maintained in circulating seawater until used. To induce an inflammatory reaction, 0.4 ml of 5 mg/ml LPS (*Escherichia coli*, serotype O55:B5, Sigma) in PBS were injected into the tunic between the two siphon. Control individuals were injected with the same volume of PBS. Animals were maintained in tanks containing aerated seawater and sacrificed at different time intervals between 1 and 48 h, and large fragments (about 1 cm²) of the pharynx from the injured areas were collected. Each sample to be processed for either RNA or protein extraction was a pool of pharynx fragments from three control or treated individuals.

2.2. RNA isolation from the pharynx and cDNA synthesis

Total RNA was extracted from *C. intestinalis* pharynx samples using the SV Total RNA Isolation System Kit (Promega). Samples were weighed and immediately homogenized with an Ultra-Turrax T25 (Janke and Kunkel, Ika-Labortechnik) at 0 °C with 3 cycles of 30 s. According to the manufacturer's instructions, 175 µl of SV RNA Lysis Buffer were added to 60 mg of tissue. Oligo(dT)-primed single-stranded cDNA was synthesized from 2 µg of pharynx RNA using the Superscript First-Strand Synthesis System Kit for RT-PCR (Invitrogen).

2.3. Real-time PCR

Real-time PCR experiments were carried out in a Chromo4™ Real-time PCR Detector (BioRad) with FastStart SYBR Green Master (Roche) chemistry.

Specific primers for CiC3-1 (which, for simplicity, we will hereafter refer to as CiC3) (sense primer, 5'-AGCGAAA-GATCCATTGGTAC-3'; antisense primer, 5'-GCAATTCCTGGTCCACAGT-3') were designed according to a CiC3-1 nucleotide sequence (GenBank ID: AJ320542) [26]. Cytoskeletal actin (GenBank ID: AJ297725) was used as a reference gene. Actin-specific primers were: sense primer, 5'-ATGTGCAAGGCCGGTTT-3'; antisense primer, 5'-GACACGGAGTTCGTGT-3'. Both CiC3 and actin primers produced single-band amplicons of the expected size. The amplicons were verified by DNA sequencing.

Reactions were performed in triplicate in 96 multi-well plates. The final PCR reaction volume was 25 µl, containing 2 µl of cDNA diluted 1:10, 12.5 µl of FastStart SYBR Green PCR Master Mix (Roche), and 10.5 µl of a mix containing 0.7 pmol/µl of each specific primer. The PCR program included a denaturation step (95 °C for 10 min) followed by 40 cycles of amplification (95 °C for 15 s and 60 °C for 1 min), and a final extension step (72 °C for 5 min). The specificity of the amplification reactions was verified by melting curve analysis. PCR amplification efficiencies, calculated for primer pairs of the reference and target genes, were both 2. All data were normalized against cytoskeletal actin using the 2^{-ΔΔCT} method [30], where 2 is the multiplier for each amplification cycle, ΔCT the difference between the target gene and the reference gene, and ΔΔCT the difference between ΔCT at a specific time and ΔCT at T₂₄, which was used as a reference for the experiment. Real-time PCR results are reported as a percentage of the maximum expression value of CiC3.

2.4. Anti-CiC3, anti-CiC3a, and anti-CiC3aR antibody production

Three specific antibodies, anti-CiC3, anti-CiC3a, and anti-CiC3aR, were prepared for use in Western blot and immunohistochemical analysis. The anti-CiC3 was directed against two synthetic peptides, KREQALIKLSVFNYGD (CiC3^{840–855}) and ELKDIYETF-PIDLKNSR (CiC3^{941–957}), in the α'-chain of CiC3b. Anti-CiC3a and anti-CiC3aR were against the peptides RLNSGTRRRQRVQGR and HELPTEPTLQANAEPPT, respectively, corresponding to the predicted immunogenic epitopes CiC3a^{63–76} and CiC3aR^{65–81}, localized at the CiC3-1a carboxy-terminus [28] and in the CiC3aR first extracellular loop, respectively [29]. The synthetic peptides were coupled to OVA and used to immunize rabbits (PRIMM, Milan, Italy). Specific antibodies were purified by affinity chromatography using the corresponding synthetic peptides coupled to cyanogen bromide-activated Sepharose 4B (GE Healthcare, Life Sciences).

2.5. Immunohistochemical staining

Dissected pharynx samples were fixed in Bouin's fluid (saturated picric acid:formaldehyde:acetic acid 15:5:1) for 24 h. To completely remove the fixative, the specimens were rinsed with 75% ethanol until the brownish color disappeared. After dehydration, the samples were embedded in paraffin. The immunohistochemical staining was conducted on 7.0-µm sections mounted on Superfrost Plus slides (Thermo Scientific), using the Vectastain Elite ABC Kit for peroxidase (Vector Laboratories) and the Fast DAB set (Sigma–Aldrich). Sections were incubated with anti-CiC3 or anti-CiC3aR affinity-purified antibody (15 µg/ml) overnight at 4 °C. Controls were run in parallel by using the corresponding pre-immune rabbit IgG at the same concentration. Staining was observed under a Zeiss Axiophot microscope.

2.6. Western blot analysis

Fragments of pharynx, collected as indicated above, were homogenized in lysis buffer (50 mM Tris, 150 mM NaCl, and 1% NP-

40, pH 6.8, containing the Mini Complete protease inhibitor cocktail, Roche) by using an Ultra-Turrax T25 (Janke & Kunkel, Ika-Labortechnik). The homogenate was centrifuged at 4 °C for 30 min at 10,000 rpm in an Eppendorf 5810R centrifuge, and the recovered supernatant was divided into aliquots and stored at –20 °C until use. Protein determination assays were performed using the QuantiPro BCA Assay Kit (Sigma–Aldrich).

Aliquots of pharynx samples containing 80 µg of protein were resuspended in sample buffer (50 mM Tris, 2% SDS, 10% β-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue, pH 6.8, containing the Mini Complete protease inhibitor mixture), boiled for 4 min, subjected to 7.5% and 15% SDS/PAGE [31], transferred to a Hybond-ECL nitrocellulose membrane [32] (GE Healthcare, Life Sciences), and probed with the anti-CiC3 and anti-CiC3a antibodies; detection was performed with HRP-conjugated secondary antibody and ECL-Plus detection reagent (GE Healthcare, Life Sciences).

3. Results

3.1. CiC3 is constitutively expressed in the pharynx

In order to verify the presence of CiC3 transcripts in the pharynx of adult animals, we performed a preliminary analysis by PCR of oligo(dT) first-strand cDNA synthesized from pharynx total RNA. PCR amplification of *Ciona* pharynx cDNA was conducted with two specific primers designed from the CiC3 nucleotide sequence deposited in GenBank. This analysis resulted in a single DNA band of the expected size (174 bp) (Fig. 1A). Control PCR was carried out on a cDNA template synthesized without reverse transcriptase (Fig. 1B). Sequencing of the PCR product confirmed that the 174 bp band corresponded to the CiC3 nucleotide sequence.

Expression of CiC3 in the pharynx was then further characterized by conventional immunohistochemical staining with the anti-CiC3 antibody and DAB substrate (Fig. 2A–D). Reactivity with the anti-CiC3 antibody in pharynx sections was found mainly in the stigmata and transversal bars of this organ (Fig. 2A). Observation of the pharynx stigmata at higher magnification indicated that the staining was restricted to the ciliated cells (Fig. 2B). A more detailed analysis of the transversal bars revealed a distinct staining pattern in some of the blood cells populating the bars (Fig. 2C). The immunostained blood cells, which were often organized in strongly reactive clusters, may be GAs. This possibility was conclusively confirmed by examination at higher magnification of antibody-positive isolated hemocytes that, according to their morphology, could be classified as GAs (see Fig. 2C inset). We observed inter-individual variations in the staining patterns in terms of both intensity and distribution. This variability hampered the use of immunostaining in a rigorous quantitative expression analysis.

To further investigate the possibility that the pharynx has a role in the complement-mediated inflammatory response, we also performed immunostaining with an antibody specific for the *Ciona* C3a receptor that had been raised against the CiC3aR^{65–81} synthetic peptide, which reproduces a sequence of the first extracellular loop [29]. This antibody produced a staining pattern superimposable on that obtained with the anti-CiC3 antibody (Fig. 2E).

No staining was observed when control immunocytochemical reactions were performed using the affinity-purified IgGs from the corresponding pre-immune rabbit sera (Fig. 2D and F).

3.2. CiC3 expression in the pharynx is up-regulated by LPS treatment

To determine whether inflammatory agents influence the expression of CiC3 in the pharynx, we analyzed total RNA samples

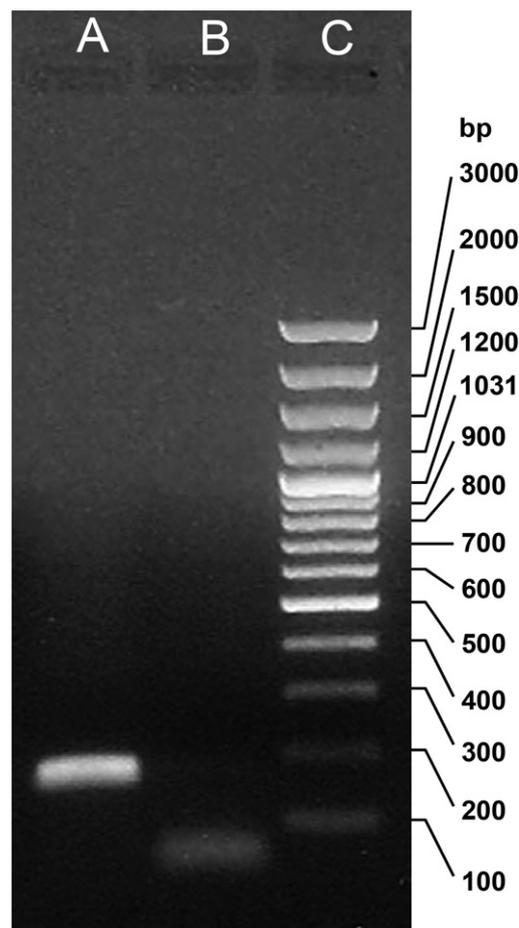


Fig. 1. PCR amplification of CiC3. CiC3 expression was detected by PCR in pharynx cDNA. (A) Amplification with specific primers produced a single band of 174 bp; (B) control (transcription without RT); (C) DNA ladder with molecular weights on the right.

from the pharynx of adult LPS-injected individuals by real-time PCR. After the injection, animals were sacrificed at different time intervals, from 1 to 48 h; tissue fragments from non-treated animals were processed as controls for the constitutive expression of the CiC3 gene.

The expression level of CiC3 mRNA in the *Ciona* pharynx was then determined at 0, 1, 3, 6, 24, 30, and 48 h post-LPS stimulation. While no variations in CiC3 transcript were detected at 1 or 3 h post-injection, a slight mRNA increase and an ~4-fold increase were observed at 6 and 24 h post-injection, respectively; gene expression was down-regulated to basal levels at 30 and 48 h after the injection (Fig. 3). In the control wells, no significant variations in CiC3 transcription levels were recorded between 0 and 48 h. Also, the expression of cytoplasmic actin, selected as reporter gene, was constant throughout the experiment (data not shown).

3.3. Generation of CiC3a in response to LPS treatment

To assess the presence of the CiC3 gene product and its activation fragments (CiC3b and the anaphylatoxin CiC3a) in the pharynx, we used Western blotting to examine the reactivity of two specific polyclonal antibodies, anti-CiC3 and anti-CiC3a, with pharynx protein extracts. We generated these anti-CiC3 and anti-CiC3a antibodies against synthetic peptides reproducing distinct regions of the CiC3 deduced amino acid sequence, localized in the α'-chain of CiC3b and at the carboxy-terminus of CiC3a, respectively.

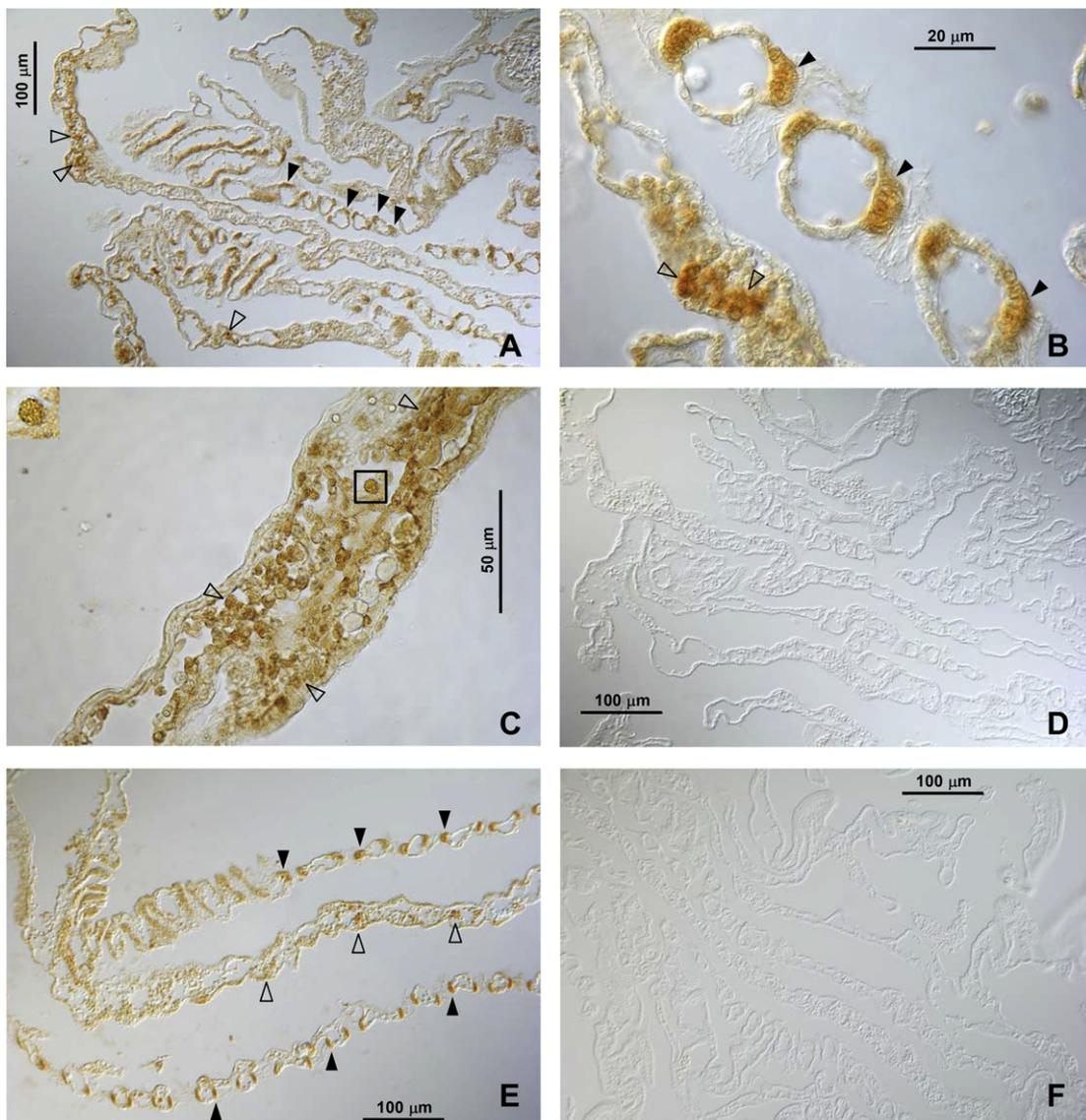


Fig. 2. Immunolocalization of CiC3 and CiC3aR in the pharynx. The immunohistochemical localization of CiC3 was performed with the anti-CiC3 polyclonal antibody. (A) CiC3 was detected in the stigmata (black arrowheads) and in hemocytes of the transversal (white arrowhead) and longitudinal bars. Higher magnification analysis indicates that staining (B) in the stigmata is confined to the ciliated cells, and (C) in the bars, to hemocytes identified as granular amoebocytes (see inset). Immunostaining with the anti-CiC3aR antibody (E) produced a pattern superimposable to that obtained with the anti-CiC3 antibody. Control reactions for anti-CiC3 (D) and anti-CiC3aR (F) were performed with the corresponding affinity-purified pre-immune rabbit IgG.

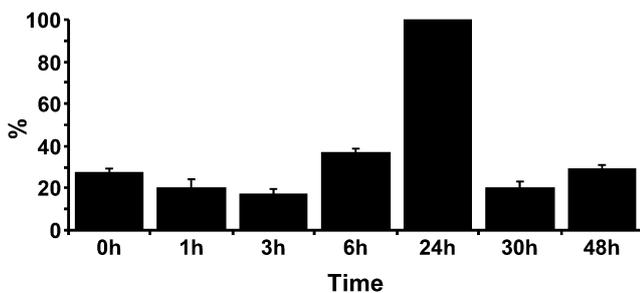


Fig. 3. Expression level of C3 at 0, 1, 3, 6, 24, 30, and 48 h after LPS challenge. Quantitative PCR reactions were carried out in triplicate, and cytoskeletal actin was used as the reference gene. The C3 expression level is reported as a percentage of the maximum expression value reached at 24 h after LPS injection. Results are expressed as the mean \pm the standard deviation of the biological replicates ($n = 3$).

The anti-CiC3 antibody recognized two bands on 7.5% SDS-PAGE with apparent molecular masses of 140 and 114 kDa (Fig. 4A). The lower-mobility band, always more abundant in non-treated animals, corresponded to the CiC3 α -chain; the difference between the observed (140 kDa) and calculated (123 kDa) molecular mass on SDS-PAGE can be attributed to glycosylation or other post-transcriptional modifications. The faster band revealed by the anti-CiC3 antibody, with a molecular mass of 114 kDa, corresponded to the CiC3b α' -chain produced by CiC3 activation.

Consistent with this result, the anti-CiC3a antibody, which recognizes an epitope localized in the CiC3a fragment, identified only a single band of 140 kDa (Fig. 4B), corresponding to the lower-mobility band revealed by the anti-CiC3 antibody and representing the CiC3 α -chain. As expected, the anti-CiC3a antibody recognized a band of ~ 9 kDa (Fig. 4C), corresponding to the anaphylatoxin CiC3a, the small fragment produced during CiC3 activation. A schematic representation of CiC3 activation is provided in Fig. 5.

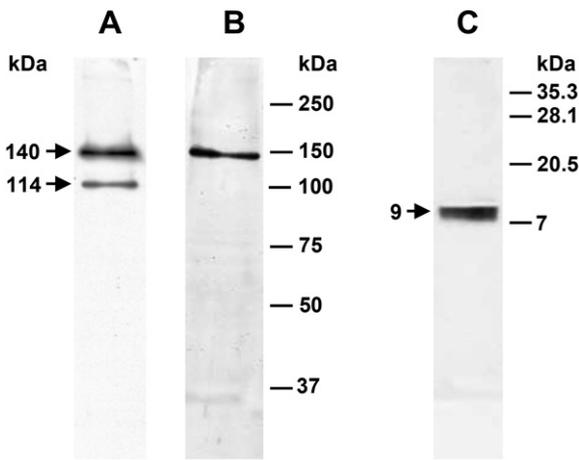


Fig. 4. Reactivity of anti-CiC3 and anti-CiC3a antibodies with *Ciona intestinalis* pharynx proteins. Proteins extracted from *Ciona* pharynx (80 µg/lane) were subjected to electrophoresis on a 7.5% SDS polyacrylamide gel under reducing conditions, blotted onto nitrocellulose membranes, and then probed with anti-CiC3 (A), or anti-CiC3a (B) affinity-purified antibody, followed by HRP-conjugated anti-rabbit Ig and ECL-plus. In (C), the same amount of pharynx proteins was run on a 15% gel and probed with anti-CiC3a antibody. On the left (A–C), the molecular mass of C3-reactive bands is indicated, and on the right (B,C), the mobility of the molecular weight markers is shown.

In another series of Western blot experiments, we analyzed the possible effects of the LPS treatment on pharynx CiC3 activation by comparing the expression of CiC3 and its activation fragments in the pharynx of untreated animals to that in LPS-injected individuals at 24 h after LPS treatment. As revealed by real-time PCR analysis, *CiC3* gene transcripts peaked at this time interval. As expected, the anti-CiC3 antibody detected the 140- and 114-kDa bands in the control samples, with a strong prevalence of the 140-kDa band corresponding to the CiC3 α -chain (Fig. 6A). The anti-CiC3 antibody detected the same two bands in the protein extracts from the pharynx of LPS-treated animals, but with a reversed quantitative ratio, indicating a predominance of the 114-kDa band, corresponding to the CiC3b α' -chain (Fig. 6B). Consistent with these results, the Western blot analysis carried out on the same samples with the anti-CiC3a antibody detected a considerable increase in

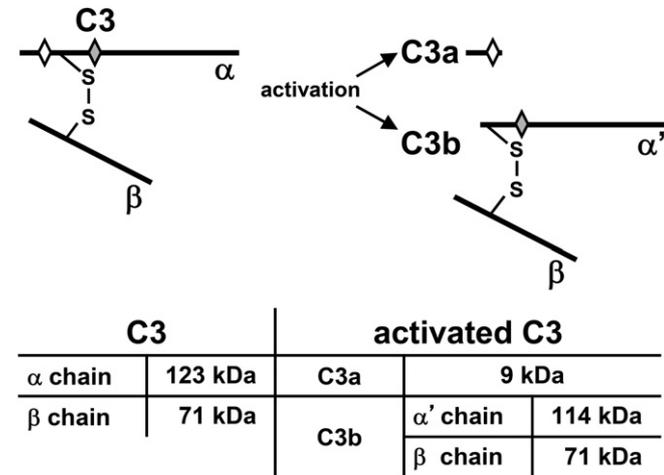


Fig. 5. Schematic representation of the CiC3 activation process. The calculated molecular sizes of the reduced chains of the generated fragments are indicated in the table associated with the scheme. The empty and gray diamonds indicate the positions of the antigenic peptides used to raise the anti-CiC3a and anti-CiC3 antibodies, respectively.

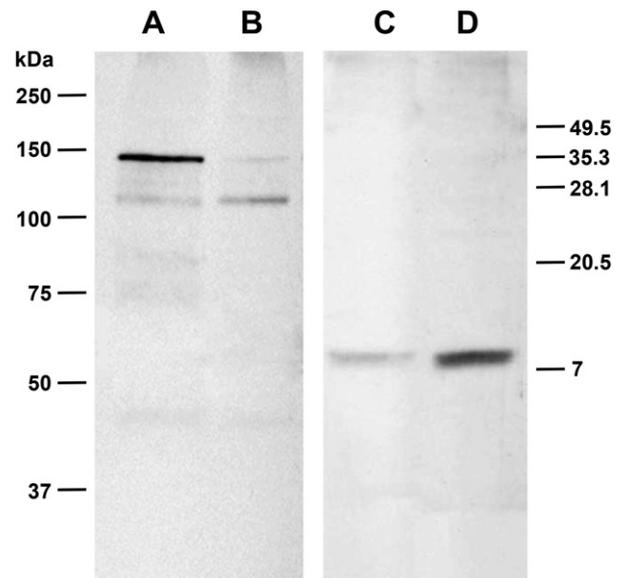


Fig. 6. Western blot analysis of pharynx protein extracts from naïve and LPS-challenged animals. Proteins extracted from pharynx samples (80 µg/lane) of (A) naïve and (B) LPS-treated animals were subjected to 7.5% SDS-PAGE under reducing conditions, Western blotted, and probed with anti-CiC3 antibody. The reactivity of the anti-CiC3a antibody against the same (C) naïve and (D) LPS-treated pharynx samples was tested on a 15% gel blotted on nitrocellulose membrane. On the left and the right are the molecular weight standards used for the 7.5% and 15% gels, respectively.

the 9-kDa band in the LPS-treated animals, corresponding to the CiC3a anaphylatoxin fragment generated by CiC3 cleavage (Fig. 6C and D).

4. Discussion

In previous papers, we have demonstrated that CiC3 is constitutively expressed in both hyaline and GAs of the tunic and circulating hemolymph [28,29]. In the present study, we examined the expression of this molecule in the pharynx. For this purpose, we preliminarily carried out a PCR analysis that demonstrated that CiC3 is constitutively expressed in the pharynx. To further characterize C3 expression in the pharynx, immunohistochemical staining was carried out on sections of this organ using anti-CiC3 polyclonal antibodies. This experimental approach revealed staining in two different locations: ciliated cells of the stigmata and hemocytes of the blood flowing through the net of transversal and longitudinal bars.

These results were confirmed and extended by Western blot analysis indicating the presence of both C3 and its activation products, namely C3a and C3b, in the pharynx, providing the first compelling evidence that C3, constitutively present in the *Ciona* pharynx, is also functionally active.

These findings were further strengthened by the results of the pharyngeal response to LPS injected into the tunic. C3 expression levels were recorded by real-time PCR at different time intervals after LPS challenge. The CiC3 transcript concentration reached a peak (about four times higher than basal level) at 24 h after the LPS injection. At the same time, Western blot experiments demonstrated that the ratio between the band intensities of the C3 α -chain and C3b α' -chain was inverted, with a concomitant significant increase of the C3a band in the LPS-injected animals. A previous analysis of the effects of LPS injection into the tunic tissue revealed a relevant increase in the number of GAs actively engaged in C3 production in the injured area during the same time period

[28]. The present results indicate that, apart from its local effect, LPS injection into the tunic also triggers a systemic reaction, as evidenced by the up-regulation of the *CiC3* gene and activation of the encoded protein in the pharynx tissue. However, at present it is unclear whether the cleavage of *CiC3* is the result of a complement convertase or a proteolytic enzyme acting via the extrinsic complement activation pathway [33,34].

Our results are also consistent with evidence from several papers dealing with the effects of LPS injection into the tunic on the expression of immune-related genes in *Ciona* pharynx. Indeed, real-time PCR analysis recorded an enhanced expression of genes encoding the TNF- α -like cytokine [14], a component of the CAP superfamily proteins [13], and the inducible a and b galectins in the pharynx [15].

In mammals, the response to LPS is mediated by the Toll-like receptor 4 (TLR4) in association with the adapter protein MD-2 and results in the activation of the transcription factor NF- κ B. In *Ciona*, only two TLRs, *Ci*-TLR1 and *Ci*-TLR2, have been identified and characterized. *In vitro* challenge of *Ciona* TLR-transfected HEK293 cells with LPS did not influence the expression of either TLR [35]. This result, together with the absence of an MD-2 gene model in *Ciona* genome [25], suggests that a TLR-independent pathway mediates the response to LPS. Our finding that, in *Ciona*, LPS enhances *CiC3* expression and its subsequent cleavage, points to the complement system as a possible key pathway of the immune response to LPS. The interesting finding from our immunohistochemical experiments that C3 is constitutively present in the stigmata ciliated cells points to a possible role of these cells in the early recognition of PAMPs. In fact, the ciliated cells of the stigmata come in contact with a very large variety of microbes because of their physiological role in generating water currents, which drive the ingestion of food particles, and they could be involved in the sampling of potentially pathogenic organisms. This is, to our knowledge, the first report attributing an immunological function to *Ciona* stigmata ciliated cells.

In the blood of ascidians, the C3-producing hemocytes are essentially GAs [28,29,36], which, because of their documented phagocytic activity [22,23] and ability to synthesize immune mediators [13,16,23,28,29,36] could be considered the organism's principal immunocytes. Thus, the presence of C3-labeled GAs in the pharynx bars of *Ciona*, together with the up-regulation of C3 synthesis by LPS injection and resulting increase of C3a production, indicate that the pharynx tissue is clearly capable of mounting an immune response.

We have also found in the present study that the staining patterns of pharynx sections with anti-*CiC3aR* and anti-*CiC3* antibodies are superimposable and that GAs are able to synthesize C3aR as well as C3, the precursor of C3a. In this context, it must be recalled that the bar epithelia of the pharynx have been described as specific sites of hematopoietic proliferation, which occurs in cells assembled in lymph nodules embedded in the epithelia [10]. In humans, normal hematopoietic stem and progenitor cells express C3aR and respond to C3a. The C3aR–C3a axis has been suggested to be involved in promoting the homing of both cell types to the bone marrow, via cross-talk with other factors [37].

Recently, studies carried out in *Xenopus* and zebrafish have revealed an unexpected role for complement proteins in early vertebrate development. C3a and its receptor C3aR are co-expressed in neural crest cells and apparently control mutual cell attraction during directional collective cell migration [38]. The C3a–C3aR interaction could operate in the *Ciona* pharynx to modulate the homing of GAs. In particular, we hypothesize that *CiC3* gene up-regulation in the pharynx after LPS injection causes an increase of secreted C3, whose activation, in turn, generates a C3a concentration gradient in an environment crowded of

proliferating and maturing GAs, which constitutively express C3aR. We assume that the interaction between soluble C3a and its specific receptor C3aR on the GAs cell surface contributes, together with unknown factors, to the migration of these cells toward injured areas, with a mechanism resembling the coordinated collective movement used by the neural crest cells of lower vertebrates in their homing process.

Taken together, our results provide compelling evidence of a key role for the pharynx in immune defense in the ascidian *C. intestinalis*. In this context, the complement system seems to be a major player with several different functions, as suggested its C3 expression pattern and activation dynamic.

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