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REGULAR ARTICLE

Inflamed adult pharynx tissues and swimming larva of *Ciona intestinalis* share *Ci*TNFα-producing cells

Nicolò Parrinello · Aiti Vizzini · Giuseppina Salerno · Maria Antonietta Sanfratello · Matteo Cammarata · Vincenzo Arizza · Mirella Vazzana · Daniela Parrinello

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Abstract In situ hybridisation and immunohistochemistry analyses have shown that the *Ciona intestinalis* tumour necrosis factor alpha gene (*Ci*TNFα), which has been previously cloned and sequenced, is expressed either during the inflammatory pharynx response to lipopolysaccharide (LPS) or during the swimming larval phase of development. Granulocytes with large granules and compartment/morula cells are *Ci*TNFα-producing cells in both inflamed pharynx and larvae. Pharynx vessel endothelium also takes part in the inflammatory response. Haemocyte nodules in the vessel lumen or associated with the endothelium suggest the involvement of *Ci*TNFα in recruiting lymphocyte-like cells and promoting the differentiation of inflammatory haemocytes. Specific antibodies against a *Ci*TNFα peptide have identified a 43-kDa cell-bound form of the protein. Observations of pharynx histological sections (at 4 and 8 h post-LPS inoculation) from naive and medium-inoculated ascidians have confirmed the *Ci*TNFα-positive tissue response. Larval histological sections and whole-mount preparations have revealed that *Ci*TNFα is expressed by trunk mesenchyme, preoral lobe and tunic cells, indicating *Ci*TNFα-expressing cell immigration events and an ontogenetic role.

Keywords Tumour necrosis factor · Pharynx · Inflammation · Haemocytes · Larval development · Innate immunity · Evolution · Ascicia, *Ciona intestinalis* (Tunicata)

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N. Parrinello (✉) · A. Vizzini · G. Salerno · M. A. Sanfratello · M. Cammarata · V. Arizza · M. Vazzana · D. Parrinello
Department of Animal Biology, Laboratory of Marine Immunobiology, University of Palermo,
Via Archirafi 18,
90123 Palermo, Italy
e-mail: nicpar@unipa.it

Introduction

The *Ciona intestinalis* inflammatory response involves both a cellular and humoral component. Following lipopolysaccharide (LPS) challenge or inoculation of a foreign agent into the ascidian body wall, several inflammatory events are initiated, including haemocyte recruitment into the inflamed tissue (Parrinello 1981; Parrinello et al. 1984; Parrinello and Patricolo 1984), tissue damage (Parrinello et al. 1984, 1990), encapsulation (Parrinello and Patricolo 1984; De Leo et al. 1997), enhancement of serum haemolymph lectins with human interleukin-1α epitopes (Parrinello et al. 2007), proPO system activity (Cammarata et al. 2008) and upregulation of FACIT-type IX-like collagen (Vizzini et al. 2008) and *Ciona intestinalis* tumour necrosis factor-α (*Ci*TNFα; Parrinello et al. 2008).

The vertebrate TNFα cytokine is a type II transmembrane protein with an extracellular homotrimeric C-terminal domain that shares several conserved features with components of the TNF superfamily (Magor and Magor 2001; Bodmer et al. 2002). Following LPS stimulation, the membrane form is cleaved and mature TNFα is secreted by a variety of immune cells. TNFα participates in a number of inflammatory responses, including the recruitment and activation of inflammatory cells, and plays a pivotal role in cell proliferation, differentiation, cytotoxicity and apoptosis and in the remodelling of the extracellular matrix and the regulation of cell adhesion molecule and integrin expression (Akira et al. 1990; Idriss and Naismith 2000; Goetz et al. 2004; Ordás et al. 2007; Wride and Sanders 1995). Members of the TNF superfamily have also been found to be critically involved in mouse and chicken development (Wride and Sanders 1995), which forms the basis of the "ontogenic inflammation" model (Kohchi et al. 1991, 1994).

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Because recent phylogenetic evidence suggests that tunicates are the sister group of vertebrates (Bourlat et al. 2006; Delsuc et al. 2006; Vienne and Pontarotti 2006), the debate concerning chordate phylogenetic relationships has enhanced the interest in considering ascidians (tunicates) as a unique experimental model for evolutionary studies. Progress in *C. intestinalis* genome sequencing has led to the examination of cytokine-like gene expression and function in *C. intestinalis* immune responses. Parrinello et al. (2008) have reported the sequence of *Ci*TNFα, the *C. intestinalis* TNFα-like molecule, as a component of the TNFα family. *Ci*TNFα cDNA was obtained by reverse transcription of mRNA extracted from the pharynx of ascidians at 4 h post-injection (p.i.) with LPS. The putative mature protein contained 185 residues (20.9 kDa theoretical size) and the deduced amino acid sequence showed 33% similarity and 15% identity to TNFα of the fish, *D. rerio*, and 37% similarity and 19% identity to human TNFα. A preliminary in situ hybridisation (ISH) assay (Parrinello et al. 2008) identified *Ci*TNFα-positive granulocytes with large granules in the pharynx vessels, granular amoebocytes in the tunic matrix and amoebocytes and haemocytes with large granules in the circulating haemolymph. Although Parrinello et al. (2008) discuss the relationship between *Ci*TNFα expression and the inflammatory response, no attempts have previously been made to examine cells and tissues containing both transcript and protein by performing a time-course of gene expression. In addition, although monoclonal anti-human TNFα antibody cross-reactions have been used to identify human TNFα epitopes in a 43-kDa protein found in haemocyte lysates, the actual *Ci*TNFα protein has not been identified by using specific antibodies.

A TNF-like molecule has also been identified in *Ciona savignyi* (*Cs*TNF; Zhang et al. 2008). Quantitative polymerase chain reaction (PCR) analysis of *Cs*TNF-like mRNA has revealed constitutive expression in various tissues and a low transcription level in the pharynx. The expression decreases in haemocytes at 2–3 h after LPS challenge. However, *Cs*TNF expression in stimulated pharynx has not been examined and the delayed and weak response in other tissues has been attributed to infiltrating haemocytes. At present, an MTT (tetrazole) assay showing the cytotoxic activity of the *Cs*TNF recombinant protein on the L929 cell line (Zhang et al. 2008) is the only insight into ascidian TNF-like cytokine activity. Based on this study, *Ci*TNFα expression during the pharynx inflammatory response and during a crucial developmental phase (swimming larva) of the ascidian life cycle might be useful in determining the functional significance of *Ci*TNFα. The swimming tadpole-like larva exhibits a remarkable chordate body plan, with metamorphosis into a sessile filter-feeding adult representing an extensive reorganisation of the body plan, which includes inflammatory processes (Chambon et al. 2002; Tarallo and Sordino 2004; Baghdiguian et al. 2007). Davidson and Swalla (2002) have reported the expression of innate immune-related genes in the solitary ascidian, *Boltenia villosa*, during the early development stages and have demonstrated the expression of the same genes at larval and post-larval stages. The activation of innate immune genes during larval phases and metamorphosis (Roberts et al. 2007) might represent the programmed maturation of the adult immune system and may be necessary for signalling and the resorption and re-structuring of larval tissues.

We report, for the first time, that inflamed pharynx and naive swimming larvae share *Ci*TNFα-producing "compartment/morula" cells. Moreover, trunk mesenchymal cells express *Ci*TNFα, migrate to the preoral lobe and reach the tunic matrix across an epithelial layer in a fashion similar to that of cell migration during inflammation.

Materials and methods

Ascidians

Adult ascidians were gathered from Termini Imerese marinas (Sicily, Italy), maintained in tanks with aerated seawater at 15°C and fed every second day with a marine invertebrate diet (Coraliquid, Sera Heinsberg, Germany).

To obtain larvae, oocytes and sperm were collected by dissecting gonoducts and cross-fertilisation was performed in plastic Petri dishes. Embryos were cultured at 18°C in filtered seawater containing 100 U/ml penicillin and 100 μg/ml streptomycin sulphate. Under these conditions, larvae hatched approximately 18–20 h after fertilisation. Mid-late swimming larvae were examined 3–4 h after hatching when competence for metamorphosis was reached (Chiba et al. 2004; Nakayama et al. 2005).

For histological studies, 10 ascidians inoculated with LPS were examined at 4 and 8 h p.i. by ISH and the same number of LPS-inoculated ascidians were examined by immunohistochemistry (IH). As a control, 5 naive and 5 sham-injected ascidians were examined.

Approximately 60 larvae and 50 specimens were examined by histology and in whole-mount preparations, respectively.

LPS inoculation

LPS (*Escherichia coli* 055:B5, LPS; Sigma-Aldrich, Germany) was prepared in sterile marine solution (MS: 12 mM $CaCl_2.6H_2O$, 11 mM KCl, 26 mM $MgCl_2.6H_2O$, 43 mM TRIS HCl, 0.4 M NaCl, pH 8.0). As described previously (Vizzini et al. 2008; Parrinello et al. 2008), LPS (100 μg in 100 μl MS per animal) was inoculated into

the body wall under the tunic at the median body wall region. Ascidians either untreated or injected with MS (100 µl, sham-injected ascidians) were used as controls.

Haemolymph collection and haemocyte suspension preparation

The haemolymph was withdrawn from the heart with a sterile syringe containing ice-cold anticoagulant (11 mM KCl, 43 mM TRIS HCl, 0.4 M NaCl, 10 mM EDTA, pH 7.4) isosmotic (1:2 ratio) with *C. intestinalis* haemolymph (1090 mOsm kg^{-1}) and immediately centrifuged at 800*g* (10 min, 4°C). The haemocytes were washed and resuspended in MS. Dead haemocytes were assessed by trypan blue staining (0.05% final concentration in MS); cell death (counted by using an improved Neubauer chamber) was found to be lower than 2.0%.

Haemocyte lysate supernatant and pharynx tissue supernatant preparations

Haemocytes were suspended in RIPA buffer (50 mM TRIS-HCl, pH 7.4, 150 mM NaCl, 0.1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) with a protease inhibitor cocktail (pepstatin A, E-64, bestatin, leupeptin, aprotinin and AEBSF). After incubation on ice for 20 min, the haemocyte suspension was vigorously shaken and centrifuged at 14,000*g* for 20 min at 4°C and the separated haemocyte lysate supernatant was maintained at −80°C.

Pharynx fragments (200 mg) were excised from the body wall injection site and homogenised on ice in lysis buffer (10 mM sodium deoxycholate, pH 8; 1 ml/g) by using an Ultra-Turrax homogeniser. The supernatant, separated by centrifugation of the homogenate at 13,000*g* for 30 min at 4°C, was mixed (1:200) with the protease inhibitor cocktail and stored at −80°C.

Production of specific antibodies against a *Ci*TNFα peptide

Specific antibodies were raised against a peptide that was selected from the mature *Ci*TNFα sequence and that demonstrated adequate immunogenic properties (Sigma-GenoSys, The Woodlands, Tex., USA). Peptide synthesis and antiserum production were conducted by Sigma-GenoSys. Briefly, the peptide ($_{251}$*C*-FIAHHRKRRG SESKT$_{265}$) from the *Ci*TNFα sequence was synthesised, conjugated to 150 mg keyhole limpet haemocyanin and used to immunise rabbits according to standard and proprietary protocols. The final serum antibody titre (1:10,000) was determined by enzyme-linked immunosorbent assay (ELISA) with the peptide antigen. An ELISA performed on the rabbit pre-immune serum excluded non-specific reactivity.

Specific antibodies were isolated by affinity chromatography. Briefly, the peptide (5 mg) was coupled to 20 ml CNBr-activated Sepharose 4B (GE Healthcare) according to the manufacturer's instructions. Aliquots of 25 ml filtered (0.45 µm filter) rabbit serum were then passed through a Sepharose-peptide column (1×5 cm). After extensive washes with phosphate-buffered saline (PBS, pH 7.4), and once the baseline absorbance (280 nm) was reached, antibodies adsorbed to the column were eluted with 0.1 M glycine-HCl, pH 2.8. The specific antibody fractions were collected into tubes containing 100 µl 1 M TRIS, pH 8, pooled and dialysed against PBS. The antibody titre of the pooled samples (1.5 mg/ml protein content) was assayed by ELISA.

Indirect peptide ELISA and competitive ELISA

The peptide ELISA was performed as described by Plagemann (2005). In brief, wells of Nunc Maxisorp ELISA plates (Nunc, Denmark) were coated overnight with the peptide (10 µg/well) dissolved in carbonate buffer, pH 9.6. The peptide-coated wells were rinsed with PBS containing 0.1% (v/v) Tween 20 (PBS-T1) and incubated with blocking buffer consisting of 0.1% w/v bovine serum albumin (BSA) in PBS-T1, at room temperature, for 1 h. The wells were then incubated (1 h at room temperature) with either anti-*Ci*TNFα antiserum diluted (1:1000–1:50,000) in blocking solution, pre-immune rabbit serum in blocking solution (1:50–1:200) or the purified antibody fraction diluted (1:1000–1:50,000) in blocking solution. After being rinsed with PBS-T1, the wells were treated (60 min at room temperature) with peroxidase-conjugated anti-rabbit IgG diluted 1:1000 in blocking solution, rinsed four times with PBS-T1 and incubated (15–30 min) with o-phenylenediamine (2 mg in 0.1 M sodium citrate, pH 4.0) substrate (100 µl/well). The final protein in each sample was quantified by recording absorbance at 492 nm with an automatic plate reader.

To check for antibody specificity (competitive ELISA), a mixture of 100 µl/well of specific antibody preparation (1:3000 or 1:5000 in PBS-T1) and *Ci*TNFα peptide (10 µg/well) was incubated in the peptide-coated wells and the procedure was performed as described above.

Western blot analysis

SDS-polyacrylamide gel electrophoresis (12% acrylamide) of the sample (2 µg protein) was carried out according to the method of Laemmli (1970). The gel was soaked in transfer buffer (20 mM TRIS, 150 mM glycine, pH 8.8) for 10 min and the proteins were transferred (1 h at 210 mA) onto a nitrocellulose sheet in transfer buffer. The filter was soaked for 2 h in blocking solution (PBS containing 2%

BSA and 0.05% Tween 20), incubated with anti-*Ci*TNFα antibodies (1:5000 in blocking buffer) for 1 h, washed with blocking buffer and incubated for 1 h with an anti-rabbit IgG-alkaline phosphatase conjugate (1:20,000 in blocking buffer). After being washed with PBS, the nitrocellulose sheet was treated with the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) liquid substrate system (3 ml).

To check for specificity, an antibody preparation (0.1 µl with 2.5 µg/µl protein) was treated overnight at 4°C with 125 µl *Ci*TNFα synthetic peptide (10 µg/ml, synthesised by Sigma-Aldrich) and centrifuged at 27,000*g* for 45 min at 4°C. Untreated and treated (absorbed) IgGs were diluted (1:2500) and used in Western blot assays.

To estimate the molecular weight of the proteins, the immunostained proteins and low-molecular-weight protein standards (low range 6.5–66.0 kDa, SigmaMarker) were stained with Coomassie Brilliant Blue. The molecular sizes were recorded by using AlphaEaseFC v. 4 image analysis software (Alpha Innotech). Four distinct Western blot patterns were analysed.

Histological methods

The ascidian tunic surface was cleaned and sterilised with ethyl alcohol. Body wall fragments containing pharynx alone or both tunic and pharynx were excised from the ascidians at the injection site. Tissues were fixed in Bouin's fluid (saturated picric acid:formaldehyde:acetic acid, 15:5:1) for 24 h, paraffin-embedded and serially cut into 6 µm sections (Leica RM2035 microtome, Solms, Germany). Histological sections were examined under a Leica DMRE microscope. Body wall tissues and cells were identified as reported previously (Vizzini et al. 2008; Parrinello et al. 2008).

ISH protocol

The ISH method was as previously described (Vizzini et al. 2008; Parrinello et al. 2008). In brief, histological sections were treated with digoxigenin-11-UTP-labelled riboprobes (1 µg/ml final concentration; Roche Diagnostics). The riboprobe contained the sequence corresponding to positions 600–1080 of the *Ci*TNFα-like cDNA. After digestion with proteinase K (10 µg/ml) in PBS-T1 for 5 min, sections were washed with PBS-T1 and treated with prehybridisation buffer containing 50% formamide, 5×SSC (1×SSC: 0.15 M NaCl/0.015 M sodium citrate, pH 7), 50 µg/ml heparin, 500 µg/ml yeast tRNA and 0.1% Tween 20 at 37°C overnight. After a 1 h incubation with an alkaline-phosphatase-conjugated anti-digoxygenin antibody (Roche Diagnostics) diluted 1:500, the sections were washed in PBS-T1 and incubated in BCIP/NBT (Sigma-Aldrich, Germany). Colour development was stopped after incubation for 30 min at room temperature

To examine cells from the haemolymph, haemocytes (5×10^5 in 100 µl) were placed on a Super Frost microscope slide, allowed to settle for 30 min at 18°C and fixed with 100 µl isotonic solution (0.01 M TRIS-HCl, 0.5 M NaCl) containing 4% paraformaldehyde (pH 7.4, ISO) for 30 min at 18°C. After being washed with PBS-T1, the settled haemocytes were incubated with the specific *Ci*TNFα riboprobe at 42°C overnight and treated as described above.

Protocols for IH and immunocytochemistry

Histological sections washed in PBS-T1 were incubated with 3% BSA in PBS-T1 for 2 h at room temperature and then with primary antibody (1:500 in PBS-T1) overnight at 4°C. An anti-rabbit IgG alkaline-phosphatase-conjugated antibody was used as the secondary antibody (1:10,000; 90 min at room temperature). The sections were rinsed with PBS-T and stained with the BCIP/NBT liquid substrate system.

Fixed haemocytes on a microscopic slide (see above) were treated (3 h at room temperature in a moist chamber) with 5% BSA in PBS-T1 and incubated (overnight at 4°C) with anti-*Ci*TNFα purified antibodies diluted 1:100 in blocking buffer. After being washed, the cell preparation was treated (2 h at room temperature) with alkaline-phosphatase-conjugated anti-rabbit IgG (1:10,000 in blocking buffer), washed 3 times for 10 min at room temperature with PBS-T1 and incubated for 1 h with 1 mM levamisole in PBS-T1. The cell preparation was treated with the BCIP/NBT liquid substrate system and haemocyte types were identified according to Arizza and Parrinello (2009).

Whole-mount ISH and IH protocols

Larvae fixed with 4% paraformaldehyde in PBS for 30 min were transferred into methanol for 5 min at −20°C and rehydrated by successive incubation with 70%, 50% and 30% ethanol.

For ISH, the fixed larvae were treated with 10 µg/ml proteinase K in PBS (15 min, 37°C) and digestion was stopped with PBS-T (twice for 5 min). After fixation with 4% paraformaldehyde in PBS for 1 h, the specimens were treated (1 h at 42°C) with a prehybridisation buffer containing 50% formamide, 5×SSC, 50 µg/ml heparin, 50 µg/ml tRNA, 5×Denhardt's solution and 0.1% Tween 20. The prehybridisation buffer was replaced with hybridisation buffer containing anti-sense or sense transcript labelled with 1 µg/ml digoxygenin and incubated overnight at 42°C. After hybridisation, the specimens were washed sequentially in 50% formamide, 4×SSC, 0.1% Tween 20 (10 min, 42°C); 50% formamide, 2×SSC, 0.1% Tween 20

(10 min, 42°C); and buffer A (0.5 M NaCl, 10 mmol/l TRIS, pH 8.0, 5 mmol/l EDTA, 0.1% Tween 20; 10 min, 37°C). Digestion with RNaseA (20 µg/ml RNaseA in buffer A) was carried out for 30 min at 37°C, followed by sequential washes with 50% formamide, 2×SSC, 0.1% Tween 20 (10 min, 42°C); 50% formamide in 1×SSC, 0.1% Tween 20 (10 min, 42°C); and PBS-T (twice for 5 min). After a blocking step in 5% horse serum in PBS-T1 (30 min), RNA hybrids were detected after treatment (1 h) with a 1:100 dilution of an alkaline-phosphatase-conjugated anti-digoxygenin antibody (Roche Diagnostic). Larvae, washed in PBS-T1 and incubated (1 h) with 1 mM levamisole in PBS-T1, were incubated with the BCIP/NBT liquid substrate system. Colour development was stopped in 30%, 50% and 70% ethanol and 80% glycerol.

For IH, the pre-treated larvae were washed with PBS containing 0.5% Triton X-100 (PBS-T2) permeabilised in PBS-T2 (1 h) and then in acetone (10 min, −20°C). After being washed (twice in PBS-T2), the larvae were incubated in PBS-T2 containing 10% BSA (blocking solution; 1 h at room temperature) and treated (1 h at 37°C and overnight at 4°C) with blocking solution containing anti-*Ci*TNFα antibodies (1:50). After further washes with PBS-T2, the samples were incubated overnight at 4°C with an anti-rabbit IgG alkaline phosphatase conjugate (1:10,000 in PBS-T2), washed in PBS-T2 and incubated for 1 h with 1 mM levamisole in PBS-T2. Finally, the samples were incubated in BCIP/NBT. Colour development was stopped in 30%, 50% and 70% ethanol and 80% glycerol.

Chemicals and estimation of protein content

Unless otherwise reported, chemicals and secondary antibodies were from Sigma-Aldrich (Germany). Protein content was measured according to the method of Bradford (1976) with BSA as a standard.

Results

Antibody specificity

The ELISA data showed that the antiserum generated against the selected *Ci*TNFα peptide reacted with the immobilised peptide up to a 1:10,000 antibody dilution, whereas the antibodies purified through peptide-bound affinity chromatography reacted up to a 1:25,000 dilution. An ELISA was also conducted in the presence of solubilised peptide (10 µg/ml per well) and this competitive inhibition abolished the reaction, supporting the specificity of the antibody (data not shown).

To determine whether the anti-*Ci*TNFα IgG reacted with the *Ci*TNFα protein, an immunoblotting assay was performed. As shown in Fig. 1, the antibodies reacted with a 43-kDa protein both from the haemocyte lysate (Fig. 1, lane b) and from pharynx homogenate supernatants (Fig. 1, lane c). No additional bands were observed in the lanes. When the immunoblot was conducted with an antibody preparation absorbed with the peptide, the antibody reaction was inhibited (Fig. 1, lane d).

Pharynx haemocytes and vessel endothelium express *Ci*TNFα protein

Examination of histological sections of the pharynx of ascidians examined at 4 and 8 h p.i. and of sections from untreated and sham ascidians revealed that few cells expressed *Ci*TNF mRNA (Fig. 2a) or *Ci*TNF protein (Fig. 2b) in naive ascidian pharynx vessels, whereas some positive cells were observed in haemocytes in the vessel lumen of sham ascidians examined 4 h after MS inoculation (Fig. 2c, d). In addition, no positive expression of *Ci*TNF was observed in the endothelium of naive ascidians. Although a haemocyte differential count was not performed, we clearly observed that, 4 h after the LPS inoculation, a large part of the vessels was densely populated with haemocytes expressing the transcript and

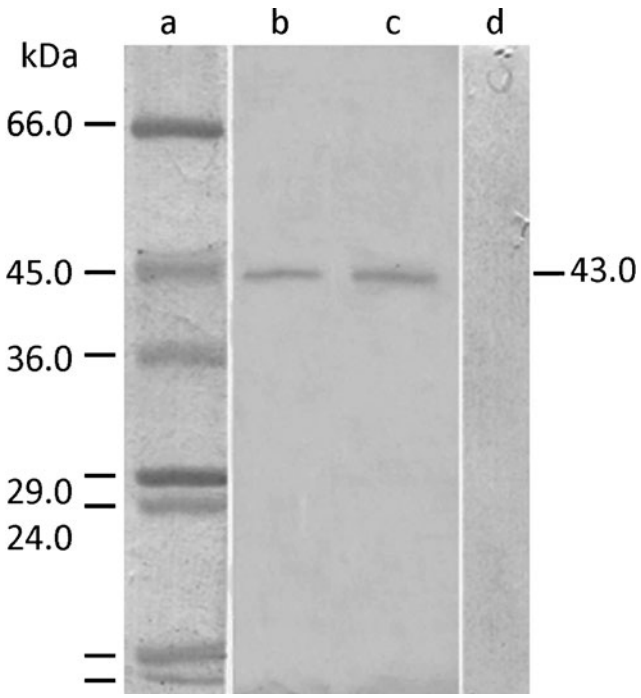

Fig. 1 Western blot analysis with an anti-*Ci*TNFα purified antibody identified *Ci*TNFα protein in ascidians at 4 h post-LPS inoculation (*lane a* standard proteins with sizes indicated in kDa *left*, *lane b* haemocyte lysate supernatant, *lane c* pharynx homogenate supernatant, *lane d* samples examined as in *lanes b*, *c* but after treatment with the antibody preparation absorbed with a *Ci*TNFα peptide (antigen) preparation

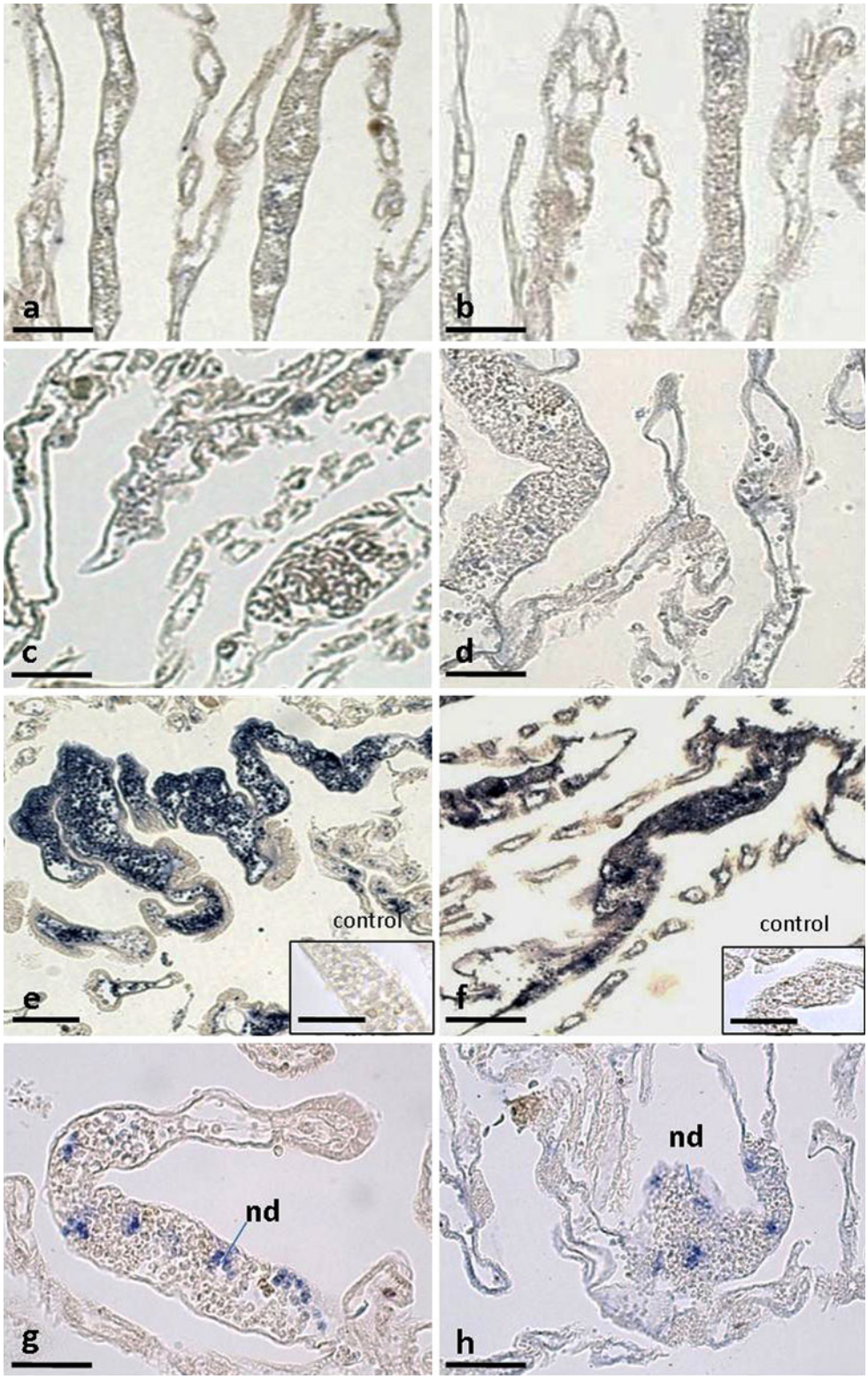

Fig. 2 Histological sections of pharynx before and after (4 h) LPS inoculation showing vessels containing *Ci*TNFα-producing cells. **a**, **c**, **e**, **g** In situ hybridisation (ISH) with a *Ci*TNFα riboprobe. **b**, **d**, **f**, **h** immunohistochemistry (IH) with an anti-*Ci*TNFα antibody. **a**, **b** Naive ascidians. **c**, **d** Sham-injected ascidians inoculated with marine solution. **e**, **f** Pharynx vessels at 4 h post-LPS inoculation showing densely populated *Ci*TNFα-producing haemocytes and nodules marked by the riboprobe (**e**) or the antibody (**f**). **g**, **h** Pharynx vessels at 8 h p.i. with LPS showing small numbers of haemocyte nodules and positive cells (**g** ISH, **h** IH) scattered in the vessel lumen (*nd* nodule). *Insets* Controls: treatment with a sense strand or omission of the primary antibody. *Bars* 100 μm

protein (Fig. 2e, f), whereas the sections examined 8 h after the LPS injection had smaller numbers of *Ci*TNF-positive haemocytes scattered among mostly non-positive haemocytes inside the vessels (Fig. 2g, h).

As shown in Fig. 3a, b, at 4 h post-LPS inoculation, *Ci*TNF-positive (ISH, IH) haemocytes formed numerous large nodules inside the vessel lumen. Positive staining was also observed in the endothelium, indicating variable

expression of *Ci*TNF within this tissue (Fig. 3c, d). Antibody staining revealed that the cytokine was present mainly in the basal membrane lining the endothelium (Fig. 3d, e).

Higher magnification showed various types of haemocytes inside the vessels. Granulocytes (7–9 μm in diameter) with several large granules or cells resembling compartment/morula cells were mainly observed (Fig. 3a inset, b inset). All cells expressed both *Ci*TNF mRNA and protein, with localisation mainly in the nucleus and cytoplasm, respectively. The protein was not observed within the large granules or compartments but, instead, was localised to their outside edge.

Haemocyte nodules were also observed near the endothelium and were composed mainly of small cells with a large nucleus, similar to lymphocyte-like cells (LLCs; Fig. 3f, g). These cells contained nuclear *Ci*TNF transcripts (Fig. 3f inset) and the antibody identified the protein at the periphery of the cytoplasm (Fig 3g inset). In some sections, LLCs close to the nodules could be identified.

Higher magnification of ISH showed that amoebocytes and compartment/morula cells populated the inflamed tunic matrix (Fig. 3h insets). To check for circulating haemocytes that produced *Ci*TNFα, the haemolymph was withdrawn from ascidians at 4 h post-LPS inoculation. ISH and IH showed that a portion (not counted) of LLCs (Fig. 4a, d), granulocytes with large granules and compartment/morula cells (Fig. 4b, c, f), in the haemolymph were positive. Amoebocytes also stained positive for the protein (Fig. 4e). The wide nucleus of the LLCs was densely stained with the riboprobe, whereas the protein was observed at the periphery of the cytoplasm (Fig. 4a, d). Compartment/morula cells exhibited *Ci*TNFα transcripts in the nucleus (Fig. 4c) and, in the cytoplasm, the protein was distributed in a layer above the granules/compartments (Fig. 4f). The content of the granules/compartments showed little to no positive staining (Fig. 4b, c, f). Histological sections treated only with the sense strand or secondary antibody as controls showed no positive staining (Fig. 4, right, top and bottom).

*Ci*TNFα is expressed in swimming larvae

In histological sections of mid-late swimming larvae, ISH and IH detected a goblet-like group of positive cells localised in the posterior region of the trunk in which mesenchymal cells were situated (Fig. 5a, d). Mesenchymal cells were stained with the antibody (Fig. 5e); the protein appeared to be distributed close to the epidermis of the posterior trunk.

In histological sections of some specimens, a few large cells (compartment/morula cells) stained positively with the riboprobe and were either localised close to the epidermis

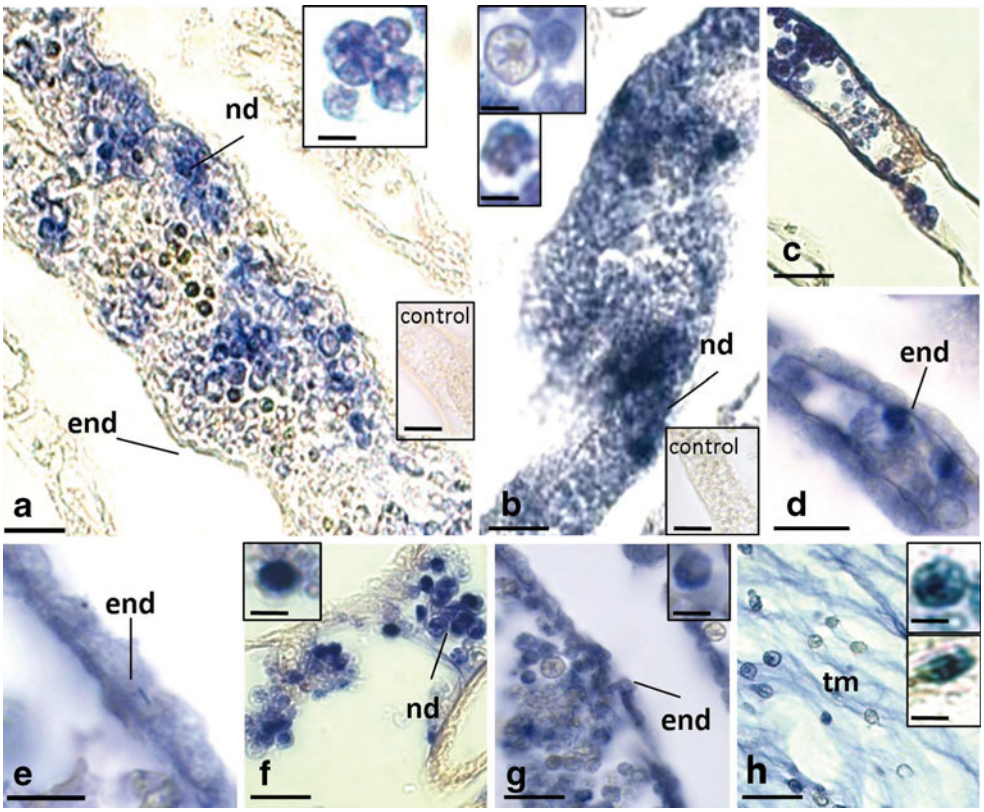

Fig. 3 Magnification of pharynx histological sections prepared from ascidians at 4 h post-LPS inoculation. **a**, **b** Haemocyte nodules (*nd*) in the vessels, with *Ci*TNFα riboprobe signal (**a**) and anti-*Ci*TNFα antibody reaction (**b**). *Insets top* Compartment/morula cells. *Insets (control)* Controls with sense strand or omission of the primary antibody. **c** Endothelium (*end*) stained with riboprobe. **d** Antibody reaction in the endothelial cells. **e** Endothelium treated with antibody immunostaining the basal membrane of the tissue. **f** Nodules associated with the endothelium containing the *Ci*TNFα riboprobe. *Inset* Lymphocyte-like cell. **g** Endothelium-associated nodule after antibody treatment. *Inset* Lymphocyte-like cell. **h** Tunic cells stained with the riboprobe (*tm* tunic matrix). *Inset* Compartment/morula cell (*top*) and amoebocyte (*bottom*). *Bars* 20 μm (**a**–**h**), 5 μm (*insets*), 50 μm (*controls*)

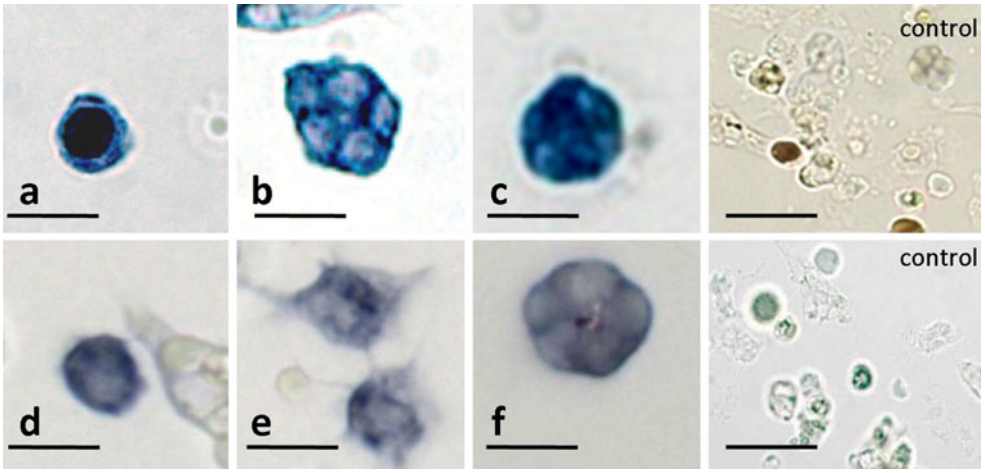

Fig. 4 *Ci*TNFα-producing haemocytes from circulating haemolymph of ascidians at 4 h post-LPS inoculation. Lymphocyte-like cell marked by the riboprobe (**a**) or by the anti-*Ci*TNFα antibody (**d**). Compartment/morula cells were stained by the riboprobe (**b**, **c**) and the antibody (**f**). Amoebocytes after antibody reaction (**e**). Controls: haemocytes treated only with sense strand or secondary antibodies (*control*). *Bars* 10 μm (**a**–**f**), 25 μm (*controls*)

of the middle-anterior part of the trunk (Fig. 5b) or in the anterior part of the pre-oral lobe (Fig. 5f). A large compartment/morula cell was also observed at the most anterior part of the trunk close to papillae (Fig. 6a, c).

*Ci*TNFα-containing cells lining the sensory vesicle were observed mainly at the posterior side in which a diffuse antibody reaction was visible (Fig. 5g). However, the ISH method did not identify transcript-containing cells in the same area.

To examine the tunic of the swimming larvae, ISH and IH of whole-mount preparations were performed. The treated specimens displayed numerous positive (ISH and IH) compartment/morula cells in the tunic of the trunk and tail (Fig. 6d-f, insets). Many of the cells positively stained with the riboprobe were in close contact with the epidermis; this contact was suggestive of cell migration (Fig. 6d, e).

Discussion

In accordance with previous results (Parrinello et al. 2008), our present work further supports the involvement of *Ci*TNFα in the pharynx inflammatory response of *C. intestinalis* by demonstrating the *Ci*TNFα-positive staining of tissues and cells with ISH and IH methods. In particular, we have raised rabbit antibodies against a *Ci*TNFα peptide selected for its optimal antigenicity, isolated them from the antiserum through a peptide-bound Sepharose affinity chromatography column and checked them for their specificity. The design of the peptide ($_{251}$*C*-FIAHHRKRRGSESKT$_{265}$) is based on the deduced sequence of the *Ci*TNFα mature protein (Parrinello et al. 2008). The anti-*Ci*TNFα antibody identifies a 43-kDa protein in the inflamed pharynx and haemocyte lysate supernatants at 4 h post-LPS inoculation, suggesting that a cell-bound form is expressed in inflammatory haemocytes and inflamed tissues. A similar result has been reported by treating *C. intestinalis* haemocyte lysates with an anti-human TNFα monoclonal antibody (Parrinello et al. 2008) and likewise, in vertebrates, a cell-bound form of TNFα is probably oligomeric in its structure. In our studies, competitive ELISA and antibody absorption with the synthetic peptide support these previous studies, confirming that residues of the *Ci*TNFα sequence contribute to the epitope.

LPS inoculation stimulates the pharynx and the response profile shown by ISH and IH corresponds to the real-time PCR analysis of tissue extracts (Parrinello et al. 2008). Upregulation of *Ci*TNFα has been found at 4 h p.i. when *Ci*TNFα-producing haemocytes (ISH and IH) densely populate the lumen of the pharynx vessels, whereas at 8 h p.i., *Ci*TNFα-producing haemocytes are a minor component of the vessel haemocyte population. In addition, at 4 h p.i, numerous nodules are formed in the vessels by *Ci*TNFα-producing haemocytes, giving a distinct inflammatory signature to the vessels. The LPS-induced inflammatory response is supported by the lack of pharynx inflammation observed in sham-injected ascidians inoculated with MS.

Although the classification of ascidian haemocytes is controversial (Arizza and Parrinello 2009; De Leo 1992), and although ISH and IH are not the most suitable methods for identifying cells, granulocyte-type cells have been identified as *Ci*TNFα-producing haemocytes. Granulocytes with irregularly shaped large granules, compartment cells with a few regularly shaped large granules (giving the cells a compartmentalised appearance) and morula cells with a few regularly shaped large granules (giving the cells a morular appearance) are all *Ci*TNFα-producing cells. Because only slight morphological differences distinguish compartment and morula cells, we refer to them as "compartment/morula cells". As each of these granulocyte-type cells are *Ci*TNFα-producing inflammatory haemocytes, we propose that the

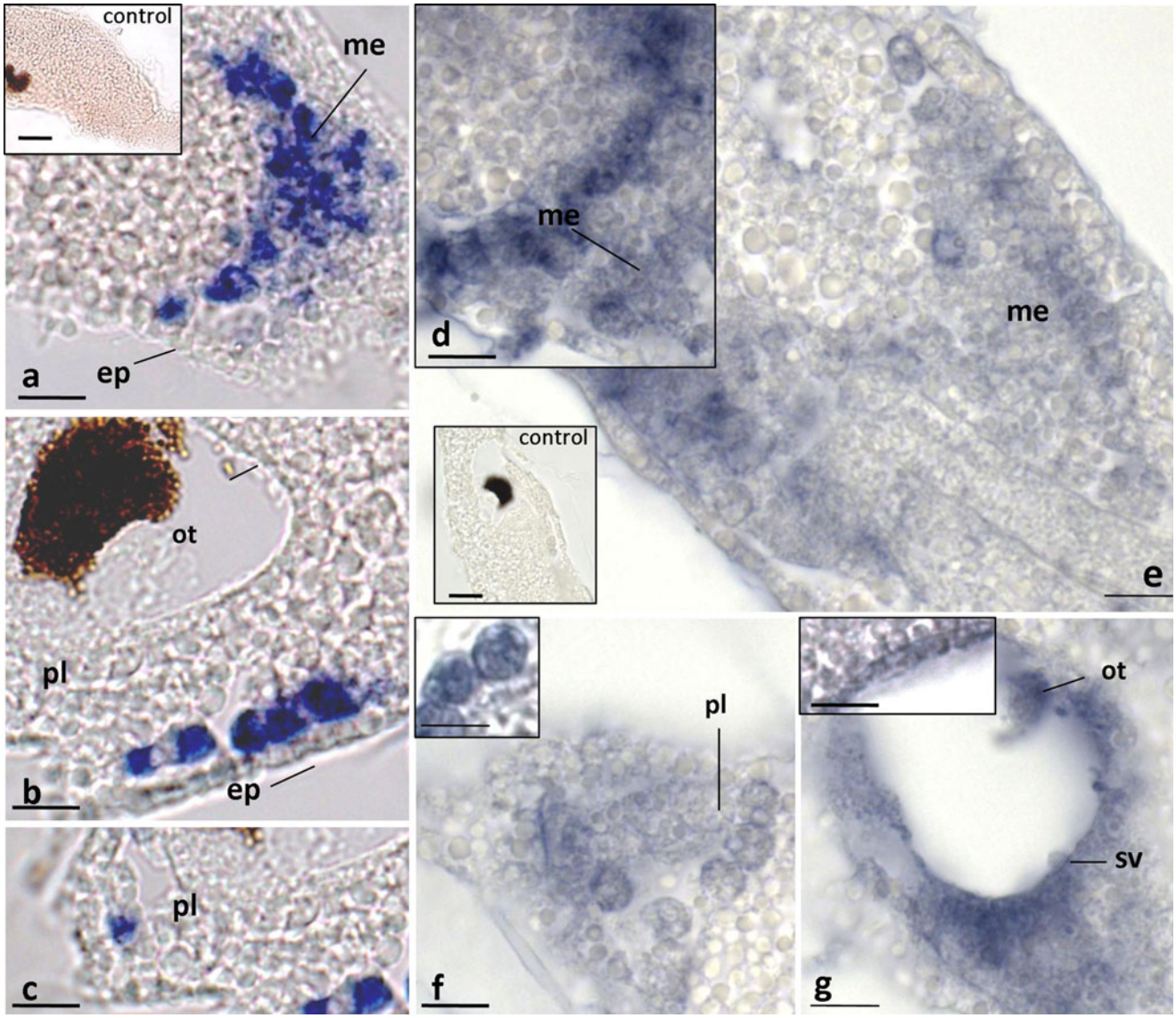

Fig. 5 *Ci*TNFα-producing cells in longitudinal histological sections of *C. intestinalis* mid-late swimming larva (3–4 h post-hatching). **a–c** Mesenchymal cells stained with the riboprobe. **a** Mesenchyme (*me*) in the posterior part of the trunk (*ep* epidermis). **b** Anterior trunk, migrating mesenchymal cells close to the ventral epidermis (*pl* preoral lobe, *ot* otolith). **c** Large compartment/morula cell marked by the riboprobe. **d–g** Mesenchymal cells stained with the antibody. **d**, **e** Mesenchyme in the posterior part of the trunk. **f** Antibody-stained compartment/morula cells (*inset*) in the pre-oral lobe. **g** Antibody-stained cells lining the sensory vesicle (*sv* sensory vesicle). *Inset* in **a** Control with sense strand (*control*). *Inset* in **e** Control by omitting the primary antibody (*control*). *Bars* 10 μm (**a–g**), 10 μm (*insets* in **f**, **g**), 25 μm (*controls*)

cells represent various stages of maturation of a cell lineage stimulated by LPS (Arizza and Parrinello 2009). Accordingly, these cells express *Ci*C3-1 (a homologue of the third component of the complement cascade, C3; Pinto et al. 2003), a *Ci*C3-receptor (Melillo et al. 2006) and type-IX-like collagen genes in response to LPS (Vizzini et al. 2008). Presumably, these cells are provided with a complex array of surface receptors enabling them to be efficient effectors in the *C. intestinalis* inflammatory response.

The free nodules in the vessel lumen are formed by tightly packed cells, including compartment/morula cells and small cells similar to LLCs, and these cells are observed mainly at the outer edge of the nodule. Small nodules, composed mainly of LLCs, are linked or closely associated with the inner surface of the endothelium. These cells, which contain *Ci*TNFα transcripts in their large nuclei and the protein at the periphery of their cytoplasm, might be retained as activated cells engaged in the inflammatory response both in the pharynx and in the haemolymph. As LLCs have been shown to be retained as proliferating cells (Ermak 1982; Sawada et al. 1994; Peddie et al. 1995; Peddie and Smith 1995), the possibility exists

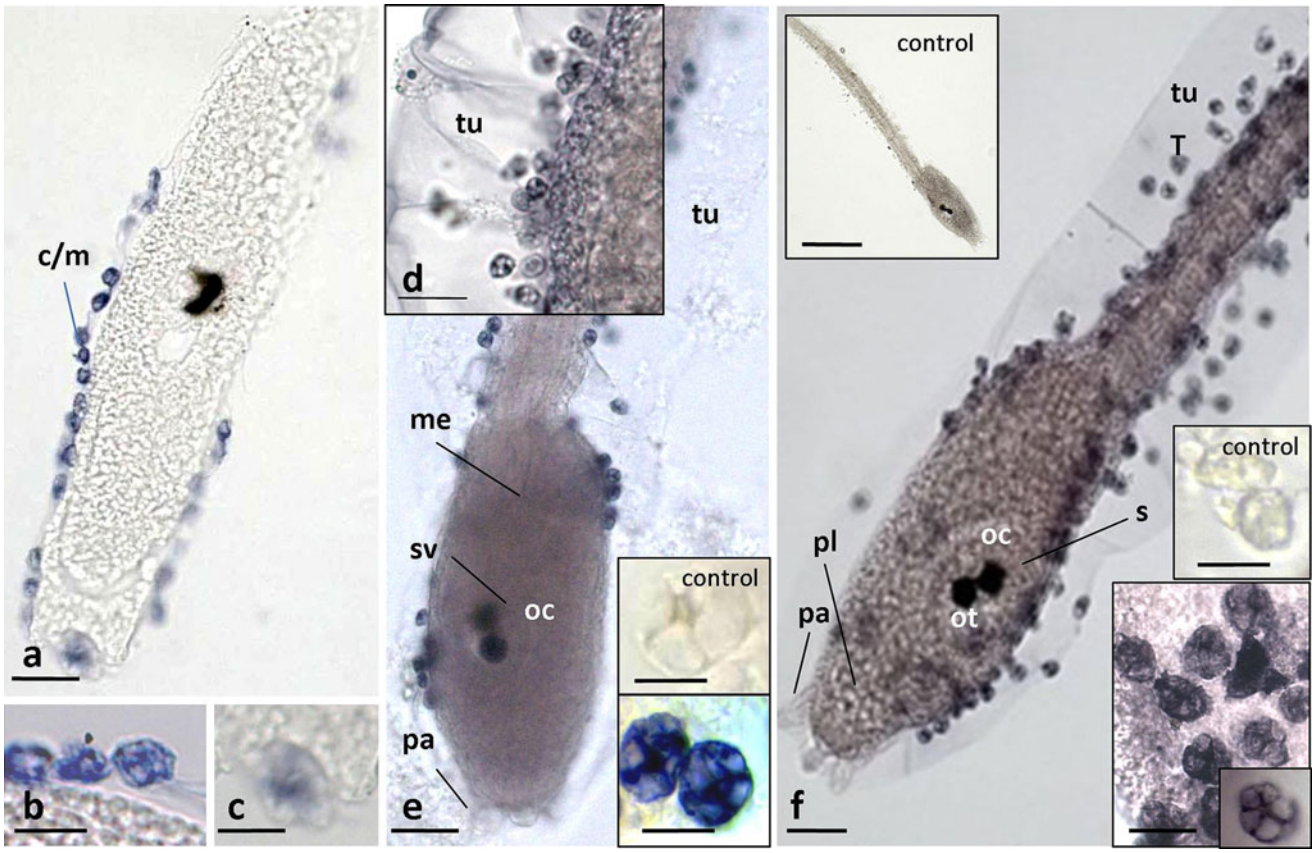

Fig. 6 **a–c** Longitudinal histological section of *C. intestinalis* mid-late swimming larva (3–4 h post-hatching). **a**, **b** Compartment/morula cells (*c/m*) lining the outer side of the epidermis. **c** Large compartment/morula cell at the most anterior part of the trunk. **d**, **e** Whole-mount ISH. **d** Riboprobe-marked cells migrating into the tunic (*tu*). **f** Antibody reaction. *Insets* in **e**, **f** Compartment/morula cells (*me* mesenchyme, *pa* papillae, *pl* preoral lobe, *sv* sensory vesicle, *oc* ocellum, *ot* otolith). Controls: compartment/morula cells after treatment with sense strand (*inset* in **e**, *control*) and secondary antibody (*inset* in **f**, *control*). *Bars* 25 μm (**a**,**d**,**e**), 10 μm (**b**,**c**), 10 μm (*insets* in **e**, **f**)

that endothelium-associated hematopoietic nodules develop following the LPS stimulus. Indeed, hematopoietic nodules have been described in ascidians by Ermak (1982). Nodular stem cells might therefore differentiate into active LLCs and granulocyte cell lineages that circulate in the haemolymph and are recruited into the inflamed tunic matrix. The vessel endothelium is also involved in the response; although no continous staining has been seen within the endothelial tissue, several cells in the endothelium express the *Ci*TNFα transcript and contain the protein in their cytoplasm. Furthermore, the *Ci*TNFα protein has been localised at the endothelium basal membrane, which has been described by De Leo et al. (1987). These results show that pharynx tissues can be challenged by an inflammatory stimulus and that they might participate in immunity and inflammation via the vessel endothelium and nodules that potentially differentiate into inflammatory haemocytes.

*Ci*TNFα is also expressed in naive swimming larvae, supporting the notion that the evolutionary conservation of immune-related genes might be connected to the role of these genes in development. Other genes known to be involved in immunity have been recognised during ascidian development (Davidson and Swalla 2002). For example, genes encoding small protein domains, known as complement control proteins or short consensus repeats, have been discovered to exist in a wide variety of complement and adhesion proteins. A lectin (MBL)-dependent complement response has been shown to activate larval metamorphosis just before settlement and development into the juvenile stage (Davidson et al. 2003; Roberts et al. 2007). Another group shows C3 complement factor expression during *C. intestinalis* embryogenesis (Marino et al. 2002). Products of these immune-related genes have been suggested to coordinate a massive wave of apoptosis during metamorphosis.

In ascidian development, within a few hours of hatching, the swimming larvae establish competency, which is the ability to respond to external factors by initiating metamorphosis (Davidson and Swalla 2002; Kimura et al. 2003; Nakayama et al. 2005). Histological sections of the mid-late swimming larva (3–4 h post-hatching) have shown that mesenchymal cells produce *Ci*TNFα. More specifically, the

position of the *Ci*TNFα-positive cells (containing transcript and protein) in the posterior part of the trunk has allowed us to identify these cells as components of the mesenchyme. In this region, *Ciona* larva contains four pockets (two pairs) of mesodermal cells (mesenchyme; Satou et al. 2001; Nakayama et al. 2002; Jeffery et al. 2008). The localisation of *Ci*TNFα-containing cells mainly in the lateral part of the mesenchyme, as observed in some histological sections, presumably can be related to distinct cell lineages. For example, components of A-line trunk lateral cells are fated to become blood cells, with two of the three B-lineages giving rise to tunic cells (Nishide et al. 1989; Hirano and Nishida 1997; Passamaneck and Di Gregorio 2005).

Because mesenchyme-specific genes are known also to be expressed in the preoral lobe (Kusakabe et al. 2002), the presence of *Ci*TNFα-expressing cells lining the inner side of the ventral epidermis and at the anterior edge of the trunk indicates that mesenchymal cells migrate to the preoral lobe and papillae. The migration of mesenchymal cells is also indicated by the localisation of the cells close to the posterior portion of the sensory vesicle in which *Ci*TNFα can be released and by the positive compartment/morula cells that, via the epidermis, populated the larval tunic matrix.

The overall results show that the inflammatory response and larval development of *C. intestinalis* have in common the expression of *Ci*TNFα by compartment/morula-like cells. In addition, according to previous reports (Hirano and Nishida 1997; Passamaneck and Di Gregorio 2005), cell migration of early tunic cells and larval tunic colonisation through the epidermis are similar to the recruitment of haemocytes to the inflamed tunic matrix (Parrinello and Patricolo 1984; Parrinello et al. 1990; Di Bella and De Leo 2000). *Ci*TNFα expression during these various processes can be understood in the light of the "ontogenic inflammation" model (Kohchi et al. 1991, 1994). The presence of *Ci*TNFα in the swimming larval stage that precedes metamorphosis might be associated with inflammatory events, including apoptosis (Cloney 1982; Vaux and Korsmeyer 1999; Davidson and Swalla 2002; Baghdiguian et al. 2007; Roberts et al. 2007; Nakayama-Ishimura et al. 2009). Interestingly, three possible TNF receptor (TNFR)-related genes in the *C. intestinalis* genome are capable of initiating signal transduction that culminates in caspase activation and programmed cell death (Terajima et al. 2003). Moreover, the *Ciona* genome contains eight TNFR-associated factor (TRAF)-related genes, which are the major signal transducers for the TNFR superfamily (Arch et al. 1998). Finally, Chambon et al. (2007) have shown that extracellular-signal-regulated kinase (ERK) and Jun NH2-terminal kinase (JNK) signalling, both of which regulate gene networks, are involved in stimulating *C. intestinalis* larval metamorphosis and apoptosis in tail tissues. Further research on the expression of *Ci*TNFα in early developmental stages and during metamorphosis should contribute to our understanding of the involvement of this multifunctional molecule in developmental mechanisms.

According to Davidson and Swalla (2002), the activation of innate immunity at the larval stage, including the upregulation of cytokine-like genes, might represent the programmed maturation of the adult immune system and is probably necessary for signalling and the resorption and restructuring of larval tissues. Furthermore, according to the danger model (Gallucci and Matzinger 2001; Matzinger 2002), innate immune mechanisms during metamorphosis might occur as a response to stress associated with the death and reorganisation of larval tissues.

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