Received: 20 January 2023 | Accepted: 4 May 2023

DOI: 10.1111/jfb.15427

JOURNAL OF FISH BIOLOGY fsbi

BRIEF COMMUNICATION

Ciona robusta macrophage migration inhibitory factor (*Mif1* and *Mif2*) genes are differentially regulated in the lipopolysaccharide-challenged pharynx

Francesca Dumas | **Manuela Mauro** | **Mirella Vazzana** | **Vincenzo Arizza** | **Aiti Vizzini**

Dipartimento di Scienze e Tecnologie Biologiche, Chimiche e Farmaceutiche-Università di Palermo, Palermo, Italy

Correspondence
Aiti Vizzini, Dipartimento di Scienze e Tecnologie Biologiche, Chimiche e Farmaceutiche-Università di Palermo, Via Archirafi 18, 90128 Palermo, Italy.
Email: aiti.vizzini@unipa.it

Funding information
Università degli Studi di Palermo, Grant/Award Numbers: FFR-D15-161211, FFR-D5-160403

Abstract

The effects of lipopolysaccharide (LPS) on *Mif* (macrophage migration inhibitory factor) gene expression in the pharynx (haemapoetic tissue) of *Ciona robusta* were investigated using quantitative reverse-transcription PCR (qRT-PCR) and *in situ* hybridisation (ISH). To verify the induction of an inflammatory response in the pharynx, a qRT-PCR analysis was performed to evaluate the change in the expression of proinflammatory marker genes such as *Mbl*, *Ptx-like*, *Tnf-α* and *Nf-kb*, which were shown to be upregulated 1 h post LPS challenge. The change in the expression of the two *Mif* paralogs in the pharynx was assessed before and after stimulation, and qRT-PCR and ISH results showed that, although *Mif2* and *Mif2* were expressed in clusters of haemocytes in pharynx vessels, only *Mif1* expression increased after LPS stimulation. This indicates that the *Mif* genes are differently regulated and respond to different ambient inputs that need further analysis.

KEYWORDS
Ciona robusta, inflammatory response, lipopolysaccharide, macrophage migration inhibitory factor

Macrophage migration inhibitory factor (MIF) is a pleiotropic cytokine (Bucala, 2000) involved in inflammatory and immune diseases (Bucala & Donnelly, 2007). It is produced and stored intracellularly in cells such as monocytes, macrophages and T cells of the immune system (Mun *et al*., 2014). The cellular response of MIF is mediated *via* the expression of pro-inflammatory genes such as *Tnf-α*, matrix metalloproteases and TLR 4 (Calandra & Roger, 2003; Sparkesa *et al*., 2017). The genes encoding MIF proteins have been found in prokaryotes and eukaryotes such as plants, vertebrates and invertebrates (Sparkesa *et al*., 2017).

Ciona robusta is a chordate invertebrate that has been studied in depth as a model for innate immunity (Delsuc *et al*., 2006; Tsagkogeorga *et al*., 2009; Vizzini, 2017). *C. robusta* has an innate immune system capable of both humoral and cellular responses (Vizzini, 2017). Exposure to pathogen-associated molecular patterns, such as gram-negative lipopolysaccharide (LPS), in the pharynx (haemopoietic tissue) induced an inflammatory reaction, expressing innate immune genes such as cytokines, galectins, mannose binding lectin (*Mbl*) and pentraxin (*Ptx-like*) (Bonura *et al*., 2009; Vizzini *et al*., 2012, 2015, 2016, 2021). Recently, it has been shown that *Mif* paralogs in *C. robusta* (*Mif1* and *Mif2*) are ancestral orthologues of the mammalian Mif superfamily genes and are involved in the inflammatory response in the pharynx (Vizzini *et al*., 2018), and a close interplay between Mif cytokines and Nf-κB signalling has also been shown (Arizza *et al*., 2020).

Francesca Dumas and Manuela Mauro are co-first authors.

In the present paper, whether LPS modulates the expression of *Mif1* and *Mif2* in the pharyngeal tissue of *C. robusta* was investigated using quantitative reverse-transcription PCR (qRT-PCR) and *in situ* hybridisation (IHS) assay. Furthermore, a qRT-PCR analysis was performed to verify whether LPS induced an inflammatory response in the pharynx by modulating the expression of pro-inflammatory marker genes such as *Mbl*, *Ptx-like*, *Tnf-α* and *Nf-kb*.

TABLE 1 Specific sets of primers used for qRT-PCR analysis

Gene	Primer sequence (5′-3′)
Ptx-like	5′-TGGATCCAAGACAGGCTTTCA-3′
	5′-TCGCGGCCAAATTAGAAAGT-3′
Mbl	5’-AGCCTTGACGTTCGCAGAGT-3′
	5’-AGCCTTGACGTTCGCAGAGT-3′
Tnf-α	5′-GCCTCCCATAGACCGTTGTTAA-3′
	5′-CGGGACACCTTCAGCACAT-3′
Nf-kB	5’-GCCGACGTACTGCTTTGCA-3′
	5’-GCCAGCCACCACGATGTT-3’
Actinβ	5’-TGATGTTGCCGCACTCGTA-3’
	5’-TCGACAATGGATCCGGT-3’

Abbreviation: qRT-PCR, quantitative reverse-transcription PCR.

C. robusta, formerly known as *Ciona intestinalis* (Linnaeus, 1767) (Brunetti *et al*., 2015; Caputi *et al*., 2007; Iannelli *et al*., 2007; Pennati *et al*., 2015), were collected from Sciacca Harbour (Sicily, Burghausen, Italy) and treated with a 100 µg LPS concentration (*Escherichia coli* 055:B5, LPS, Sigma-Aldrich, Germany), as previous described (Vizzini *et al*., 2021).

Ascidian tissue fragments were treated for total RNA extraction using an RNAqueousTM-Midi, Kit purification system (Ambion, Austin, TX, USA) following Vizzini *et al*. (2021).

The expression of *C. robusta* genes *Ptx-like*, *Mbl*, *Tnf-α*, *Mif1*, *Mif2* and *Nfkb* was studied using qRT-PCR with specific sets of primers (Table 1) using an Applied Biosystems 7500 real-time system, as previously described by Arizza *et al*., 2020. To examine the time course of the response, LPS-treated ascidians ($N = 4$) were examined at post-inoculation time points (1, 2, 4, 24 and 48 h). Untreated ascidians ($N = 4$) were used as controls.

Fragments of *C. robusta* tunic and pharynx tissue were excised from the control and LPS-injected ascidians at 1 h for ISH studies. Body wall fragments were cut serially (6 µm, Leica RM2035 microtome, Solms, Germany) (Vizzini *et al*., 2021).

The ISH assays were carried out on histological sections of the pharynx, with *Mif1* and *Mif2* digoxigenin-11-UTP-labelled riboprobes (Roche Diagnostics, 1 µg/ml final concentration, Basilea, Switzerland), on control ascidians and ascidians at 1 h post LPS stimulation, as

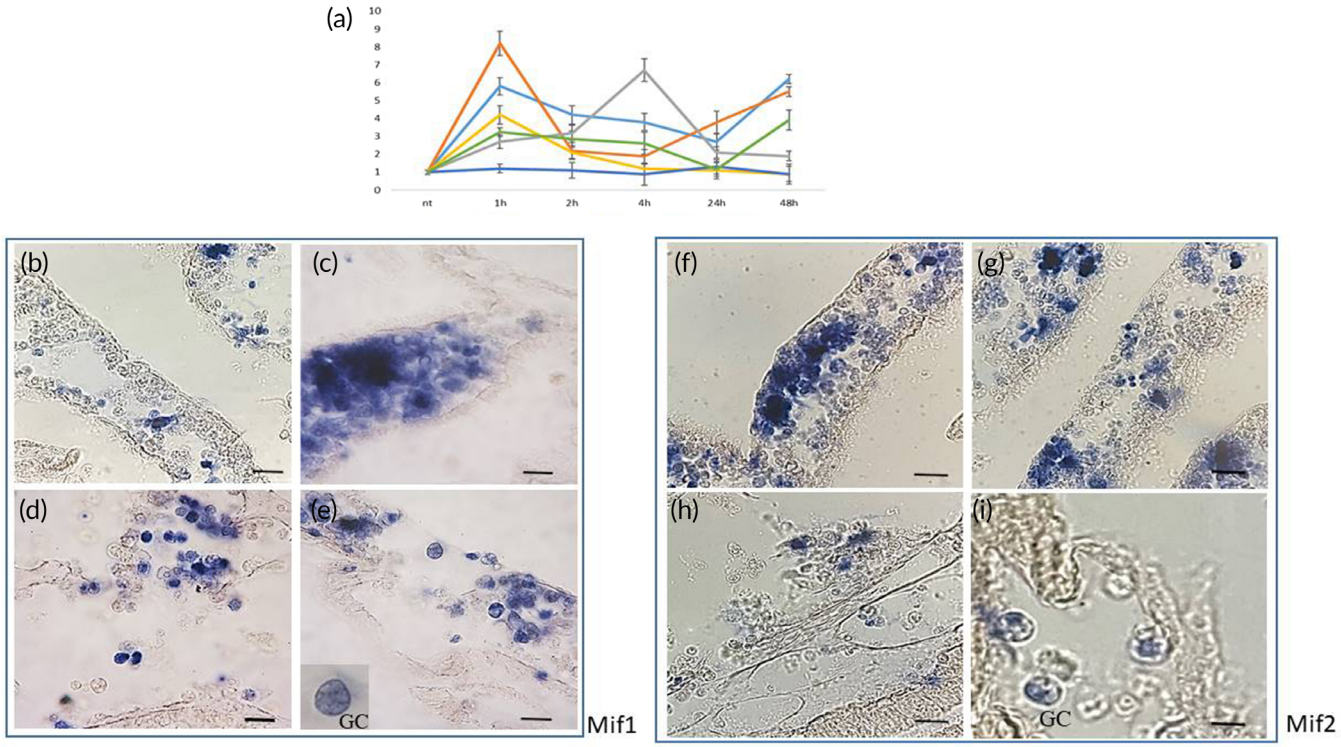

FIGURE 1 (a) Time course of *Ptx-like*, *Mbl*, *Tnf-α*, *Nf-kb* and *Mif1*, *Mif2* gene expression in *Ciona robusta* exposed to LPS (lipopolysaccharide), compared with gene expression in untreated ascidians (nt). Values are presented as means ± S.D. Statistical differences were estimated using one-way ANOVA and Tukey's *t*-test. The level of significance was set at *P*-value ≤ 0.05 ($N = 4$). (b–i) *In situ* hybridisation with *Mif1* and *Mif2* riboprobes in *C. robusta* (b, f) control pharynx vessels, (c, g) pharynx vessels and (d, e, h, i) lacunae at 1 h after LPS inoculation. Bar sizes: a and b: 50 µm, c–f 5 µm. GC: granular cells. —, Ptx; —, Mbl; —, Tnf; —, Mif1; —, Mif2; —, Nfkb

previously described (Dumas *et al*., 2015; Dumas & Sineo, 2014; Vizzini *et al*., 2021). The *Mif1* probe was generated using PCR amplification and cloning of a 410 bp cDNA fragment covering the 3′ region from nucleotides 248 to 658. The *Mif2* probe was generated using PCR amplification and cloning of a 534 bp cDNA fragment covering the 3′ region from nucleotides 244 to 779.

qRT-PCR showed enhanced *Ptx-like*, *Tnf-α*, *Mbl*, *Nf-kb* and *Mif1* mRNA levels after LPS inoculation (Figure 1a). *Ptx-like*, *Mbl* and *Mif1* gene expression increased significantly at 1 h, *Nf-kb* gene expression increased significantly at 1–4 h and *Tnf-α* gene expression increased significantly at 1–4 h. On the contrary, the inoculation procedure did not significantly modulate *Mif2* mRNA expression (Figure 1a), whose levels remained almost constant.

The pharynx, the haematopoietic organ of *C. robusta*, consists of two epithelial monolayers aligned in rows of elongated elliptical and ciliated stigmata enclosed in a net of vessels with haemolymph and abundant mature and immature haemocytes (Figure 1b,c,f,g), as well as lacunae (Figure 1d,e,h,i). Histological sections of the pharynx were examined from control ascidians (Figure 1b,f) at 1 h after LPS inoculation for *Mif1* and *Mif2* expression (Figure 1c,d, e and Figure 1g, h, i) using ISH. *Mif-1* and *Mif-2* signal probes were present in tightly packed haemocyte clusters within the vessel lumen (Figure 1b,c,f,g). Granulocytes (GR) were the main haemocytes that expressed *Mif1* and *Mif2* transcripts (Figure 1b,c,f,g). Transcript-expressing GRs were also found in the lacunae of the circulatory system in injected ascidians (Figure 1d,e,h,i). Controls with the sense strand probe were negative (data not shown). A *Mif1* transcript probe showed an increase after LPS stimulation, whereas *Mif2* expression levels were similar before and after LPS stimulation. LPS induced *Mif1* gene upregulation (Figure 1c–e), whereas it did not affect the rates of *Mif2* transcription (Figure 1g–i) relative to levels in control ascidians (Figure 1f).

These data indicate that *Mif1* is involved in *C. robusta*'s immune defence systems against bacterial infection, in agreement with previous observations (Arizza *et al*., 2020; Vizzini *et al*., 2018); indeed, its transcription was modulated by LPS. Instead, as previously shown, *Mif2* has a peculiar 3′-UTR mRNA Cytoplasmic Polyadenylation Element (CPE) element (Weill *et al*., 2012) that is not present in *Mif13′-UTR mRNA* (Vizzini *et al*., 2018). The CPE element is involved in the mechanism of the poly(A) tail elongation of mRNA after it is exported into the cytoplasm and increases protein expression by the translational activation of stored mRNAs, allowing the rapid expression of proteins for a prompt response (Weill *et al*., 2012).

In conclusion, qRT-PCR analysis and ISH assays showed that only *Mif1* was transcriptionally upregulated during the inflammatory process induced by LPS inoculation, whereas *Mif2* was already expressed but not modulated by LPS. These data show that, in *C. robusta*, different transcriptional and post-transcriptional control mechanisms are involved in responses to ambient stimuli, which warrant further investigation.

ORCID

Francesca Dumas https://orcid.org/0000-0001-7570-7578

REFERENCES

Arizza, V., Bonura, A., La Paglia, L., Urso, A., Pinsino, A., & Vizzini, A. (2020). Transcriptional and in silico analyses of MIF cytokine and TLR signalling interplay in the LPS inflammatory response of *Ciona robusta*. *Scientific Reports*, *10*, 1–16.

Bonura, A., Vizzini, A., Salerno, G., Parrinello, N., Longo, V., & Colombo, P. (2009). Isolation and expression of a novel MBL-like collectin cDNA enhanced by LPS injection in the body wall of the ascidian *Ciona intestinalis*. *Molecular Immunology*, *46*, 2389–2394.

Brunetti, R., Gissi, C., Pennati, R., Caicci, F., Gasparini, F., & Manni, L. (2015). Morphological evidence that the molecularly determined *Ciona intestinalis* type a and type B are different species: *Ciona robusta* and *Ciona intestinalis*. *Journal of Zoological Systematics and Evolutionary Research*, *53*, 186–193.

Bucala, R. (2000). Signal transduction. A most interesting factor. *Nature*, *408*, 146–147.

Bucala, R., & Donnelly, S. C. (2007). Macrophage migration inhibitory factor: A probable link between inflammation and cancer. *Immunity*, *26*, 281–285.

Calandra, T., & Roger, T. (2003). Macrophage migration inhibitory factor: A regulator of innate immunity. *Nature Reviews Immunology*, *3*, 791–800.

Caputi, L., Andreakis, N., Mastrototaro, F., Cirino, P., Vassillo, M., & Sordino, P. (2007). Cryptic speciation in a model invertebrate chordate. *Proceedings of the National Academy of Sciences of the United States of America*, *104*, 9364–9369.

Delsuc, F., Brinkmann, H., Chourrout, D., & Philippe, H. (2006). Tunicates and not cephalochordates are the closest living relatives of vertebrates. *Nature*, *439*, 965–968.

Dumas, F., & Sineo, L. (2014). The evolution of human synteny 4 by mapping sub-chromosomal specific probes in primates. *Caryologia*, *67*, 281–291.

Dumas, F., Sineo, L., & Ishida, T. (2015). Taxonomic identification of *Aotus* (Platyrrhinae) through cytogenetics. *Journal of Biological Research*, *88*, 65–66.

Iannelli, F., Pesole, G., Sordino, P., & Gissi, C. (2007). Mitogenomics reveals two cryptic species in *Ciona intestinalis*. *Trends Genetics*, *23*, 419–422.

Mun, S. H., Oh, D., & Lee, S. K. (2014). Macrophage migration inhibitory factor Down-regulates the RANKL–RANK signaling pathway by activating Lyn tyrosine kinase in mouse *models*. *Arthritis & Rheumatology*, *66*, 2482–2493.

Pennati, R., Ficetola, G. F., Brunetti, R., Caicci, F., Gasparini, F., Griggio, F., … Manni, L. (2015). Morphological differences between larvae of the *Ciona intestinalis* species complex: Hints for a valid taxonomic definition of distinct species. *PLoS ONE*, *10*(5), e0122879.

Sparkesa, A., De Baetseliera, P., Roelantsc, K., De Treza, C., Mageza, S., Van Ginderachtera, J. A., … Stijlemansa, B. (2017). The non-mammalian MIF superfamily. *Immunobiology*, *222*, 473–482.

Tsagkogeorga, G., Turon, X., Hopoft, R. R., Tilak, M. K., Feldstein, T., Shenkar, N., & Delsuc, F. (2009). An updated 18S rRNA phylogeny of tunicates based on mixture and secondary structure models. *BMC Evolutionary Biology*, *9*, 1–16.

Vizzini, A. (2017). Gene expression and regulation of molecules involved in pharynx inflammatory response induced by LPS in *Ciona intestinalis*. *Invertebrate Survival Journal*, *14*, 119–128.

Vizzini, A., Di Falco, F., Parrinello, D., Sanfratello, M. A., & Cammarata, M. (2016). Transforming growth factor β (CiTGF-β) gene expression is induced in the inflammatory reaction of *Ciona intestinalis*. *Developmental & Comparative Immunology*, *55*, 102–110.

Vizzini, A., Di Falco, F., Parrinello, D., Sanfratello, M. A., Mazzarella, C., Parrinello, N., & Cammarata, M. (2015). *Ciona intestinalis* interleukin 17-like genes expression is upregulated by LPS challenge. *Developmental & Comparative Immunology*, *48*, 129–137.

Vizzini, A., Dumas, F., Di Falco, F., & Arizza, V. (2021). Evolutionary and transcriptional analyses of a pentraxin-like component family involved in the LPS inflammatory response of *Ciona robusta*. *Fish & Shellfish Immunology*, *111*, 94–101.

Vizzini, A., Parisi, M. G., Di Falco, F., Cardinale, L., Cammarata, M., & Arizza, V. (2018). Identification of CPE and GAIT elements in 3UTR of maophage migration inhibitory factor (MIF) involved in inflammatory response induced by LPS in *Ciona robusta*. *Molecular Immunology*, *99*, 66–74.

Vizzini, A., Parrinello, D., Sanfratello, M. A., Salerno, G., Cammarata, M., & Parrinello, N. (2012). Inducible galectins are expressed in the inflamed pharynx of the ascidian *Ciona intestinalis*. *Fish & Shellfish Immunology*, *32*, 101–109.

Weill, L., Belloc, E., Bava, F. A., & Méndez, R. (2012). Translational control by changes in poly (a) tail length: Recycling mRNAs. *Nature Structural & Molecular Biology*, *19*, 577–585.

How to cite this article: Dumas, F., Mauro, M., Vazzana, M., Arizza, V., & Vizzini, A. (2023). *Ciona robusta* macrophage migration inhibitory factor (*Mif1* and *Mif2*) genes are differentially regulated in the lipopolysaccharide-challenged pharynx. *Journal of Fish Biology*, 1–4. https://doi.org/10.1111/jfb.15427