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RESEARCH REPORT

New data on C1qDC from the colonial ascidian *Botryllus schlosseri*

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Abstract

In the compound ascidian *Botryllus schlosseri*, we recently identified a novel C1q-domain-containing (C1qDC) protein expressed by circulating immunocytes, called BsC1qDC. It has two globular C1q domains and a signal peptide and can act either as an opsonin and facilitate the phagocytosis of nonself particles or as a cytokine and stimulate the degranulation of cytotoxic cells. In the present work, we used a commercial antibody raised against human CTRP4 (hCTRP4) to provide additional evidences of the involvement of this molecule in immune responses. The antibody was validated in immunoblot analysis and recognizes a band corresponding to the expected molecular weight inferred from the analysis of the amino acid sequence of BsC1qDC. The presence of the antibody in the culture medium in phagocytosis and degranulation assays significantly reduced the two responses.

In addition, the relationships between complement C3 activation and *bsc1qdc* transcription was studied using the injection of C3aR agonist in the colonial vasculature.

Key Words: colonial ascidians; *Botryllus*; C1qDC; complement

Introduction

C1q-domain-containing (C1qDC) proteins form an emerging family of proteins involved in immune responses in both vertebrates and invertebrates (Kishore *et al*., 2004; Ghai *et al*., 2007). They are characterized by the presence of globular C1q (gC1q) domain(s) (Kishore and Reid, 2000; Ghai *et al*., 2007) sharing a typical jelly roll topology with five pairs of anti-parallel β-strands organized in two parallel β-sheets (Gaboriaud *et al*., 2003).

In invertebrates, acting as lectins and opsonins (Gerlach *et al*., 2004; Zhang *et al*., 2008; Carland and Gerwick, 2010; Gerdol *et al*., 2011; Li *et al*., 2011; Yang *et al*., 2012; Jiang *et al*., 2015; Huang *et al*., 2017; Wang, 2017; Gorbushin, 2019). C1qDC proteins are involved in various responses, including microbial recognition (Kong *et al*., 2010; Wang *et al*., 2012a, 2015), agglutination (Kong *et al*., 2010; Wang *et al*., 2012b), phagocytosis promotion (Wang *et al*., 2012b), and cell migration (Tahtouh *et al*., 2009). They act as pattern recognition receptors and bind directly to pathogens by engaging a broad range of PAMPs (Medzhitov, 2002; Bohlson *et al*., 2007). Most of the C1qDC proteins, identified in invertebrate, contain only one gC1q domain

Most of the C1qDC proteins have only a gC1q domain, but the presence of molecules with multiple tandem C1q domains has been reported in both invertebrates and vertebrates (Gerdol *et al*., 2011; Wang *et al*., 2012b; Huang *et al*., 2017; Gorbushin, 2019). In vertebrates, in addition to the complement component C1q, with only one gC1q domain, a protein with two C1q domains, called C1q/TNF-related proteins 4 (CTRP4), has been described (Ghai *et al*., 2007).

The compound ascidian *Botryllus schlosseri* is a cosmopolitan tunicate easily found in shallow waters of all the oceans and seas. A colony can reproduce by both sexual and asexual reproduction. Three blastogenetic generations are usually present in a colony: the adult zooids, its palleal buds and budlets on buds. They share a common circulatory system in the form of a vessel network within the common tunic (Brunetti, 1969; Gasparini *et al*., 2007). A cyclical (weekly at 19 °C) generation change or takeover allows the replacement of adults zooids, that are progressively resorbed, by their buds. Therefore, it is possible to define a blastogenetic cycle as the period of time between a takeover and the following one. The colonial developmental phases lying more than one day

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from the previous and following TO (Lauzon *et al*., 1992; Manni *et al*., 2007) are collectively called mid-cycle (MC).

B. schlosseri is a reliable model organism for the study of immunobiology (Franchi and Ballarin, 2017). Its immune defences rely mainly on circulating immunocytes *i.e*., hemocytes directly involved in immune responses. They constitute the majority of circulating hemocytes and are represented by granular morula cells and phagocytes. Phagocytes can recognize and ingest foreign particles upon their recognition via surface receptors, such as Toll-like receptors (Peronato *et al*., 2020a), whereas morula cells, as a consequence of the recognition of nonself molecules, degranulate and trigger an inflammatory reaction involving various actors such as the enzyme phenoloxidase and the complement system. In addition, each immunocyte type can influence the activity of the other (Franchi and Ballarin, 2017).

Recently, in the same organism, we identified the transcript for a novel C1qDC protein, called BsC1qDC (Peronato *et al*., 2021). It contains two gC1q domains and a signal peptide and resembles human CTRP4 (hCTRP4); the *bsc1qdc* gene is transcribed by circulating immunocytes. The knockdown of *bsc1qdc* resulted in a reduction of phagocytosis and degranulation processes, so we can suppose that BsC1qDC is able to recognize and bind non-self molecules acting as both an opsonin, favoring phagocytosis, and a chemokine, activating the inflammatory reaction. (Peronato *et al*., 2021).

In the present work, we continued to study the expression of BsC1qDC and its role in *Botryllus* innate immunity. using an antibody raised against human CTRP4 (hCTRP4). The obtained results confirm what suggested by previous molecular studies and stress the important role of this molecule in *Botryllus* immune responses.

Materials and methods

Animals

Colonies of *Botryllus schlosseri* were collected in the lagoon of Venice, near the Marine Station of the Department of Biology in Chioggia. They were transferred to glass slides and kept in aerated aquaria in thermostatic rooms at the Department of Biology, University of Padova. Colonies were reared at 16 °C and fed with living unicellular algae (*Tetraselmis chuii*) and Phyto Marine (Oceanlife, Bologna, Italy).

Hemocyte collection

To prevent haemocytes clumping, colonies were previously rinsed in 14.7 mM Na citrate in filtered seawater (FSW), pH 7.5. Then, the peripheral vessels were punctured with fine tungsten needles and the flowing hemolymph was collected with a glass micropipette. It was then centrifuged at 800 xg for 10 min and pelleted hemocytes were re-suspended in FSW to a final concentration of 10^6 cells/mL.

Primer design, RNA extraction, cDNA synthesis, cloning and sequencing

Total RNA was isolated from colonies with the RNA NucleoSpin RNA XS kit (Macherey-Nagel, Düren, Germany) and its quality was determined by the A260/280 ratio. RNA integrity was checked by the visualization of rRNAs in Midori green (Nippon genetics, Düren, Germany)-stained 1.5 % agarose gels. The first strand of cDNA was reverse transcribed from 1 µg of total RNA at 42°C for 1 h in a 20 µL reaction mixture containing 1 µL of ImPromII Reverse Transcriptase (Promega, Madison, WI, USA) and 0.5 µL of random primers (Promega, Madison, WI, USA).

PCR reactions were carried out in a 25-µL reaction volume containing 100 ng of cDNA from *B. schlosseri* colonies, 2.5 µL of 10x incubation buffer (PCRBIO Classic Taq, PCR BIOSYSTEMS, London, UK) with 15 mM MgCl2, 0.25 µM of each primer, 10 mM of each of the deoxynucleotide triphosphates, and 2 units of Taq polymerase. PCR was performed on a MyCycler (BioRad, Hercules, CA, USA) thermocycler as follows: 94 °C for 2 min, then 42 cycles of 94 °C for 30 s, 55-60 °C for 30 s, 72 °C for 40 s, and 72 °C for 10 min. Amplicons were subjected to electrophoresis on 1.5 % agarose gel and the corresponding bands were purified with ULTRAPrep Agarose Gel Extraction MiniPrep kit (AHN Biotechnologie, Nordhausen, Germany), ligated in pGEM-T Easy Vector (Promega, Madison, WI, USA) and cloned in DH-5α *Escherichia coli* cells (Tang *et al*., 1994). Positively screened clones were sequenced at Eurofins Genomics (Ebersberg, Germany) on a Sanger sequencing with the ABI 3730XL.

qRT-PCR

To compare the total amount of *bsc1q* mRNA, mRNA was extracted from control subclones and subclones injected with C3aR agonist. Then, we carried out qRT-PCR with the SYBR green method (QPCRBIO sygreen mix separate rox, PCR BIOSYSTEM, London, UK). Specific primers (Table 1) for BsC1qDC and for the elongation factor 1α (BsEF1α) as housekeeping gene were synthesized by Sigma-Aldrich (St. Louis, MO, USA). All the designed primers contained parts of contiguous exons, so to exclude contamination by genomic DNA; a qualitative PCR was also carried out before

Table 1 Primers used for qRT PCR analysis

BsC1qDC-Forward	CGTCATAAAGGTCACCGTCA
BsC1qDC-Reverse	GTGAGTCCCGAAAGTGCCCC
BsEF Forward	GCCGCCATACTCTGAAGC
BsEF Reverse	GTCCAACTGGCACTGTTCC

qRT-PCR. Furthermore, analysis of the qRT-PCR dissociation curve gave no indications of the presence of contaminating DNA. qRT-PCR analyses were performed using an Applied Biosystem (Foster City, CA, USA) 7900 HT Fast Real-Time PCR System, using the following cycling parameters: 3 min at 95 °C (denaturation), 20 s at 95 °C plus 1 min at 60 °C, 45 times (extension/annealing), 15 s at 95 °C, 1 min at 60 °C. Each set of samples was run three times and each plate contained cDNA from three different biological samples (n = 3) and negative controls. The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used to estimate the total amount of mRNA. The levels of transcripts in different conditions were normalized to that of BsEF1α to compensate for variations in the amounts of cDNA.

Predicted 3D structure of BsC1qDC

The structure of BsC1qDC was predicted as described in Peronato *et al*. (2021). As already reported, the two gC1q domains have a typical jelly roll topology.

Immunoblot analysis

In order to validate the anti-hCTRP4 polyclonal antibody (ThermoFisher scientific, Waltham, MA, USA), we carried out an immunoblot analysis on colony homogenates. Briefly, colonies (5-6 systems in size) were transferred in lysis buffer (50 mM Tris-HCl, 0.25 M sucrose, 1 % SDS, 1 mg/mL pepstatin, 1 mg/mL leupeptin, 40 mg/mL PMSF, 2 mm Na-orthovanadate, 10 mM NaF, 0.1 % NP-40, 5 mM EDTA, 5 mM N-ethylmaleimide), in a 1.5 mL vial, disaggregated with a glass pestle, subjected to sonication at 4 °C in a Branson (Emerson Corporate, London, UK) 1200 sonifier at 50 % duty cycles for.5 min, and centrifuged at 10,000 xg for 10 min. The protein content of the supernatants was determined according to Bradford (1976). SDS polyacrylamide (15 %) slab gel electrophoresis was performed according to the method of LaemmLi (1970). Proteins were transferred to 0.45 mm Electran nitrocellulose membrane (BDH, Poole, UK) according to Towbin *et al*. (1979), with 25 mM Tris, 160 mM glycine, 20 % methanol, 0.7 mM SDS as transfer buffer. After blotting, membranes were thoroughly washed in Tris-buffered saline (TBS: 50 mM Tris-HCl, 150 mM NaCl, pH 7.4), incubated for 30 min in TBS containing 5 % powdered milk and probed overnight with 10 µg/mL anti-hCTRP4 polyclonal antibody. After further extensive washing in TBS containing 0.05 % Tween 20 (TTBS), membranes were overlaid for 1 h with goat anti-rabbit IgG conjugated with peroxidase (BioRad, Hercules, CA, USA), 10 µg/mL in TTBS. Immunogenic bands were revealed by incubation for 10 min in 0.025 % 3,3'-diaminobenzidine (DAB) and 0.04 % H_2O_2 in TBS for 15 min. In controls, primary antibodies (20 µg/mL) were pre-incubated overnight with an equal volume of hemocyte lysate from colonies at MC as a source of BsC1qDC. The mixture was then used for immunoblot analysis. Primary antibodies were replaced with rabbit pre-immune serum, at the same dilution in negative controls.

Immunocytochemical assays

Sixty µl of hemocyte suspension were placed in the center of culture chambers prepared as described elsewhere (Ballarin *et al*., 2008) and left to adhere to Superfrost Plus (Menzer Glaser) slides for 30 min at room temperature. They were then incubated for 60 min in fixed in in 4 % paraformaldehyde plus 0.1 % glutaraldehyde in 0.4 M cacodylate buffer containing 1.7 % NaCl and 1 % sucrose, at 4 °C for 30 min. Fixed haemocytes were incubated for 30 min in 3 % hydrogen peroxide in methanol, to block endogenous peroxidase, washed in PBS and treated for additional 30 min with 3 % powdered milk in PBS, to prevent unspecific binding. Cells were then incubated overnight in polyclonal anti-human CTRP4 (ThermoFisher scientific; 50 µg/mL in PBS), washed and treated for 1 h with goat anti-rabbit secondary antibody, conjugated with biotin. They were finally incubated for 30 min in ABC complex (Vector) and exposed for 10 min to DAB to reveal positive sites.

Phagocytosis assay

Hemocytes, collected as described above from colonies at MC, were left to adhere for 30 min on clean coverslips. They were then incubated for 60 min at room temperature with 100 µl of a suspension of yeast (*Saccharomyces cerevisiae*) cells (yeast:hemocyte ratio = 10:1) in FSW in the presence or in the absence (control) of 50 µg/mL of anti-hCTRP4 antibody (Thermofisher scientific, Waltham, MA, USA). Anti-rabbit IgG (50 µg/mL; Calbiochem, San Diego, CA, USA) was used as a negative control. Slides were then washed several times in FSW to eliminate uningested yeast and cells were fixed in 4 % paraformaldehyde in 0.2 M Na-cacodylate buffer containing 1 % NaCl and 1 % sucrose, washed in PBS, stained with 10 % Giemsa. Finally, hemocyte monolayers were mounted on glass slides with Acquovitrex (Carlo Erba, Milano, Italy). Slides were thenobserved under the light microscope (LM), at 1250 x and the percentage of phagocytosing cells, i.e., cells with ingested yeast cells was evaluated.

Degranulation assays

Hemocytes were collected and as described above from colonies at MC and left to adhere for 30 min on clean coverslips. They were then incubated for 60 min at room temperature with 100 µl of a suspension of *Bacillus clausii* (4×10^5 cells/mL) in FSW, in the presence or in the absence (control) of 50 µg/mL of anti-hCTRP4 or anti-rabbit IgG antibody, the latter used as negative control. Previous experiments (Peronato *et al*., 2021) demonstrated that, in the presence of *B. clausii*, most of morula cell rapidly degranulated and changed their morphology.

Effects of C3aR agonist on bsc1qdc transcription

In another series of experiments, three colonies were split in three subclones of 3-4 systems each and, after an acclimation period of 5 days, one of them was injected with 5 µL of C3aR agonist (Santa Cruz Biotechnology; 0.3 µM in DMSO). The second one received 5 µL of DMSO (control for C3aR

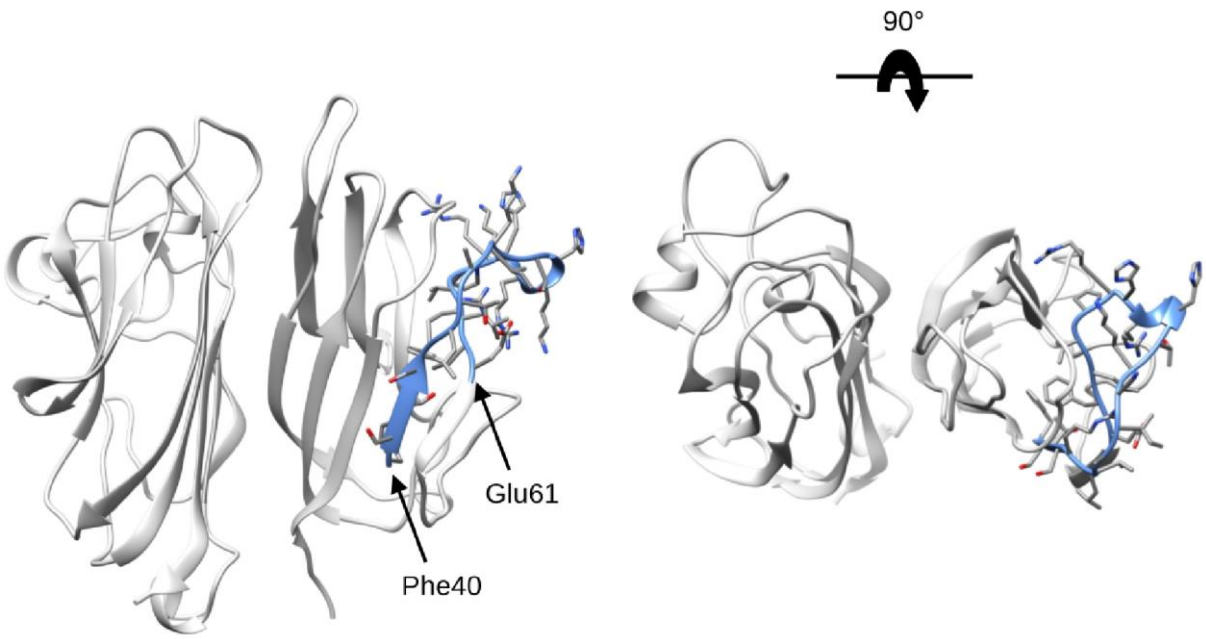

Fig. 1 predicted 3D conformation of BsC1qDC. The colored part corresponds to the amino acid sequence corresponding to the epitope in hCTRP4 used to raise the antibody

agonist), whereas the third was injected with the same amount of FSW. After 24 h, mRNA was extracted from treated and control subclones, reverse transcribed to cDNA and the transcription of bsc1qdc and bsef1α was followed by qRT PCR.

Statistical analysis

Each experiment was replicated at least three times (n = 3) with three independent blood samples; data are expressed as mean ±SD. At least cells in 10 optical fields at 1250 were counted under the LM for each experiment. Indexes were compared with the χ^2 test. qPCR experiments were replicated three times (n = 3) with three independent samples. One-way ANOVA was followed by the Student-Newman-Keuls test to assess significant differences with respect to colonies injected with DMSO in FSW. Statistical analyses were performed with the PRIMER statistical program.

Results

Analysis of the anti-hCTRP4 antibody

The commercial antibody used in our assays was develop against the epitope represented by the sequence of the amino acids form 32 to 61 of hCTRP4. It corresponds to the amino acid sequence from 32 to 62 of BsC1qDC (Genbank accession number: MT783425). The alignment of the two sequences shows 33 % of similarity, the full conservation of 7 amino acids and additional 5 with highly similar properties. In addition, according to our 3-D reconstruction, the sequence appears exposed to the external milieu (Fig. 1), which supports our idea that the antibody raised against the corresponding human sequence can recognize our protein both in the native and denatured form.

Anti-hCTRP4 antibody recognizes a band of 37 kDa in immunoblot analysis and labels circulating immunocytes

In immunoblot analysis of colony homogenates, the anti-hCTRP4 antibody recognized a single band of 37 kDa, in accordance with the predicted value of 37.19 kDa (Peronato *et al*., 2021). An additional band, of 75 kDa, is clearly visible. In samples treated with the antibody pre-incubated with hemocyte lysate, the intensity of the 37-kDa band was highly (67 %) reduced whereas the band was not visible when the preimmune serum was used (Fig. 2).

Anti-hCTRP4 antibody recognizes B. schlosseri *immunocytes*

The anti-hCTRP4 antibody, when used for immunocytochemical analyses, confirmed the result obtained with ISH: only morula cells and phagocytes resulted immunopositive (Fig. 3a,c), with a frequency of about 70 % of morula cells and 34 % of phagocytes, respectively (Fig. 4a).

Anti-hCTRP4 antibody inhibits yeast phagocytosis and morula cell degranulation

When hemocytes were exposed to yeast cells under control conditions, the fraction of phagocytes able to ingest yeast cells (Fig. 3c) amounted to 87 %. The incubation of the hemocytes with anti-hCTRP4 antibody significantly ($p < 0.01$) decreased the number of cells with ingested yeast; no effects were observed in the presence of the anti-rabbit IgG

antibody (Fig. 4b). The percentage of degranulated morula cells (Fig. 3e) amounted to 80 % in cells exposed to *B. clausii*; in the presence of anti-hCTRP4, the extent of degranulated cells was ($p < 0.001$) reduced, although still significantly ($p < 0.001$) higher than in controls. The anti-rabbit IgG antibody did not induce any inhibition of degranulation (Fig. 4c).

C3aR agonist influences bsc1qdc transcription

In colonies injected with the C3aR agonist, a significant ($p < 0.05$) increase in the level of transcription of *bsc1qdc* was observed with respect to DMSO-injected subclones (Fig. 5).

Discussion

According to our previous results (Peronato *et al*., 2021), BsC1qDC is a novel soluble receptor, acting either as an opsonic lectin able to enhance the phagocytosis of nonself particles, and as a cytokine able to facilitate morula cell degranulation. The transcription of *bsc1qdc* is significantly enhanced by the injection of foreign microorganisms, such as *B. clausii* and *Saccharomyces cerevisiae*, in the colonial circulatory system and its knock-down by RNA interference influences both phagocytosis and degranulation (Peronato *et al*., 2021). We also noticed the high predicted structural similarity and residue conservation of BsC1qDC and hCTRP4, in particular of C-terminal gC1q domains (Peronato *et al*., 2021). Therefore, in the present work, we also used a commercial antibody raised against hCRTP4, to collect additional information on the expression and function of BsC1qDC. We are well aware that the antibody specificity can be directed towards other, still unrelated or undiscovered *Botryllus* proteins; however, we were not able to find additional C1qDC proteins in our transcriptome and, although the antibody is not specific to our protein, it recognizes an electrophoretic band in colony homogenate with an estimated molecular weight around 37 kDa, comparable to the expected molecular weight of BsC1qDC, that was greatly reduced after its pre-absorption with the lysate itself.

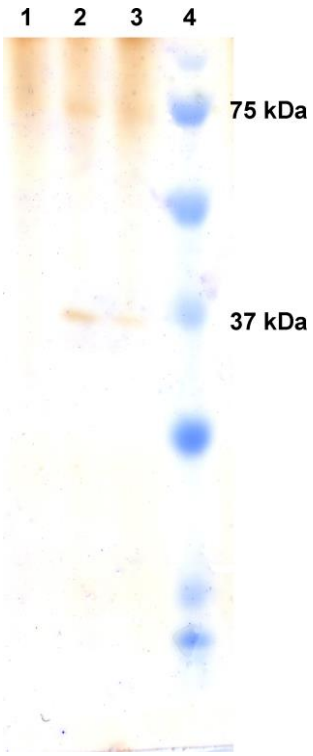

Fig. 2 Immunoblot analysis of lysates of colonies at MC. Lane 1: pre-immune serum; lane 2: anti hCTRP4 antibody; lane 3: pre-absorbed anti-hCTRP4 antibody; lane 4: molecular weight standards.

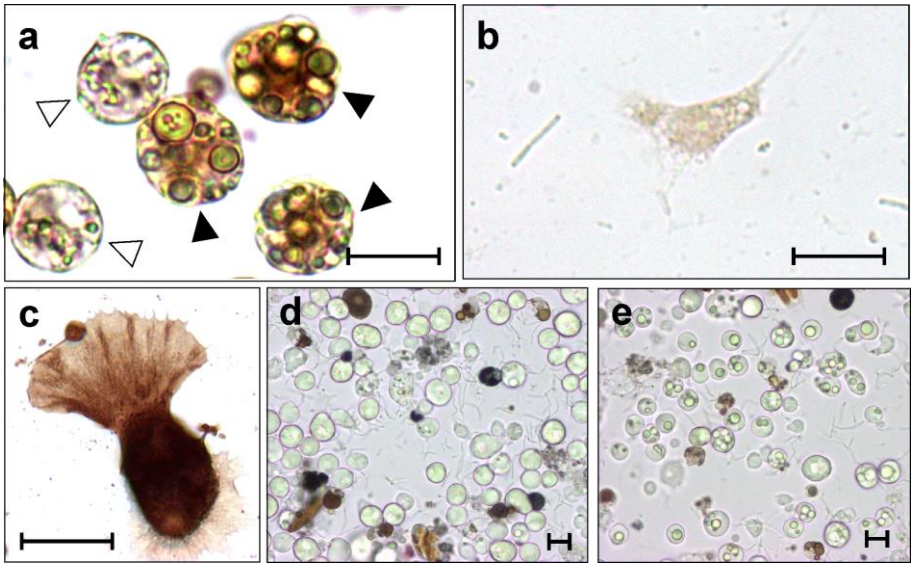

Fig. 3 Staining of fixed hemocytes with anti-hCTRP4 antibody. **A**: morula cells (dark arrowheads: labeled cells; white arrowheads: unlabeled cells); **B**: fixed phagocytes with (**H**) and without (**I**) ingested yeast cells. **C-D**: healthy (**D**) and degranulated (**E**) living morula cells. Scale bars: 10 μm

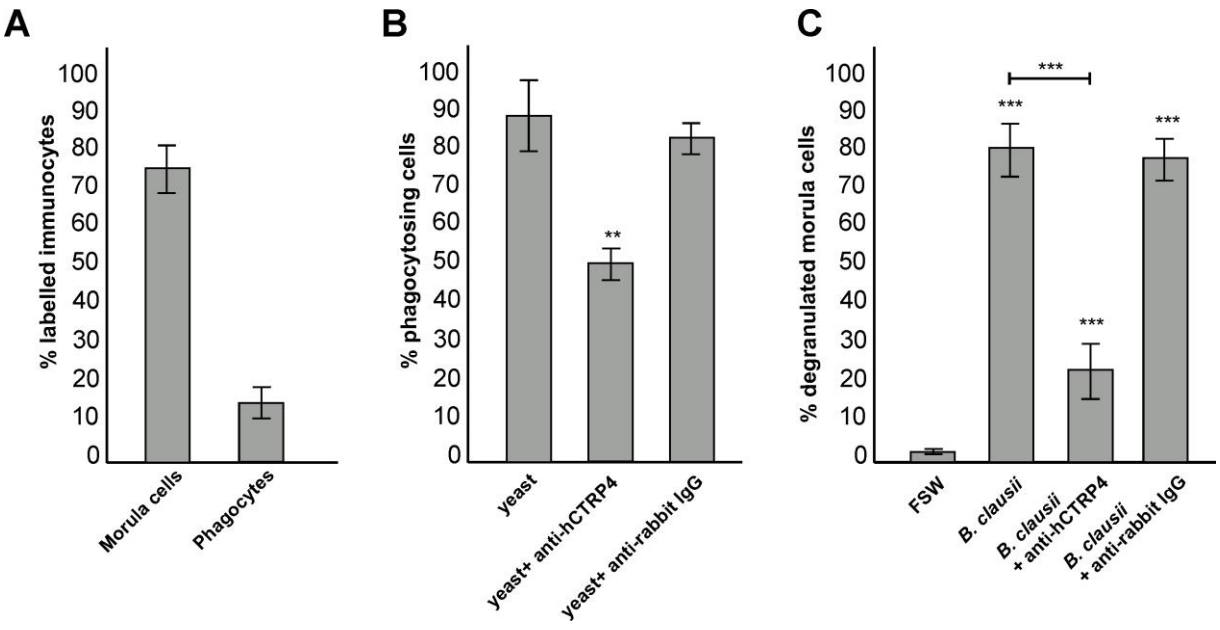

Fig. 4 A: fraction of phagocyte and morula cells labelled by the anti-hCTRP4 antibody. B: mean percentages of phagocytosis cells in hemocyte monolayers incubated with yeast cells in the absence and in the presence of either anti-hCTRP4 or anti-rabbit IgG antibody. C: degranulation of morula cells triggered by *B. clausii* in the absence or in the presence of either anti-hCTRP4 or anti-rabbit IgG antibody. Significant differences with untreated cells are marked asterisks. **: $p < 0.01$; ***: $p < 0.001$

In addition, the human amino acid sequence against which the antibody was raised has a corresponding sequence in our protein which, in the 3-D reconstruction, appears exposed to the external environment so to justify its recognition by the antibody in both the native and denaturated form. In addition, the comparison of the human epitope used for immunization and the corresponding sequence of our protein shows 33 % of similarity and various conserved amino acids which can represent recognition sites for the antibody. The presence of a second band, of approximately 75 kDa, is likely due to the presence of dimers: the presence of a putative multimerization surface was already suggested by the analysis of the predicted 3D-structure of BsC1qDC (Peronato *et al*., 2021).

In the hemolymph of *B. schlosseri*, immunocytes represent the majority of circulating hemocytes and are represented by phagocytes and granular morula cells. As suggested by their name, phagocytes can recognize and ingest foreign particles in order to clear them from the circulation. Conversely, morula cells, upon the recognition of foreign molecules, trigger an inflammatory reaction consequent to their degranulation leading to cell recruitment and cytotoxicity (Franchi and Ballarin, 2017).

Among the vertebrate C1qDC proteins, the mammalian C1q is a pattern-recognition receptor involved in the recognition of antigen antibody complexes and in the consequent triggering of the complement classical activation pathway (Carland and Gerwick, 2010). However, it was demonstrated that it also binds directly to the surface of microbes and modulate their phagocytosis (Bohlson *et al*., 2007). As demonstrated in our previous work (Peronato *et al*., 2021), the transcription of *bsc1qdc* is significantly upregulated following the injection of nonself particles or molecules in the colonial circulation and both phagocytosis and morula cell degranulation are significantly impaired by the knock-down of the transcription. This suggests a role of the molecule as opsonin in phagocytosis and as cytokine in modulating morula cell degranulation. Our results, showing that the anti-hCTRP4 antibody recognizes the same circulating cells that actively transcribe *bsc1qdc*, *i.e*., phagocytes and morula cells, support the idea that immunocytes are the sites of synthesis of the BsC1qDC protein that, once released, can act in an autocrine or paracrine way to enhance phagocytosis and morula cell degranulation. Indeed, the presence of the antibody in the incubation medium can significantly reduce yeast cell phagocytosis and degranulation of morula cells in the presence of *B*. *clausii*, a reliable inducer of degranulation (Peronato *et al*., 2021).

As for the relationships of BsC1qDC with the components of the complement activation pathways, we observed an increase in the transcription of *bsc1qdc* in colonies injected with the C3aR agonist. From previous experiments we know that the injection of C3aR agonist induces a significant increase in the transcription of BsC3 (Peronato *et al*., 2020b), synthesized and released by morula cells (Franchi *et al*., 2014). Therefore, we can suppose that the observed results are the consequence of a positive autocrine loop between BsC3 and BsC1qDC so that the rising level of BsC3

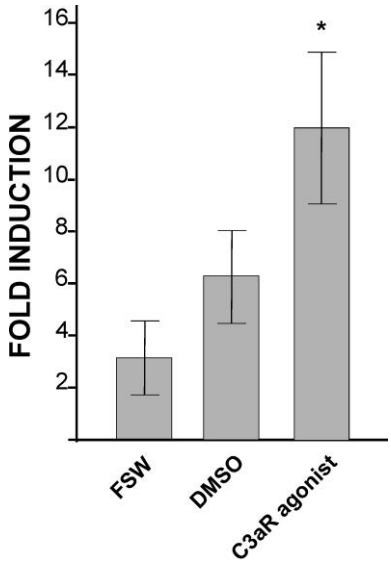

Fig. 5 Effect of the injection, in the colonial vasculature, of FSW (control), DMSO (negative control) and 3aR agonist on the transcription of *bsc1qdc*

in the circulation fosters the synthesis and release of BsC1qDC as a strategy to reinforce the immune responses against the microbial invaders. A rise in human CTRP6, another C1qDC-protein was indeed observed in mice with rheumatoid arthritis, characterized by enhanced C3 activation (Murayama *et al*., 2015). However, differently from what expected, we see a decrease of BsC1qDc at TO, during which we previously reported an increase in the transcription of BsC3 (Peronato *et al*., 2020c). We can suppose a delay in the synthesis of the BsC3 protein so that the activation of BsC1qDC transcription is postponed to the beginning of the new blastogenetic cycle, as reported in the present paper, so that BsC1qDC can exert an immunosurveillance role during the MC. Future studies will help us to better clarify the role of BsC1qDC in *Botryllus* immune responses.

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