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Research Report

Exogenous IL-8 induces phagocyte activation in the compound ascidian *Botryllus schlosseri*

A Menin, L Ballarin

Department of Biology, University of Padova, Padova, Italy

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Abstract

We studied the responses of *Botryllus schlosseri* phagocyte to human recombinant IL-8 (hrIL-8) at three different concentrations (10, 25 and 50 ng/ml). Both spreading ability and phagocytosis were significantly enhanced by the exogenous chemokine at 25 and 50 ng/ml in the culture medium and the effects are coupled to modifications of the actin cytoskeleton. The addition of the signal transduction inhibitors suramin, calphostin C and H-89 to the incubation medium, inhibits the above-reported effects and suggests that exogenous IL-8 acts via protein kinase (PK) C and PKA pathways through its binding to a G protein-coupled receptor.

Key Words: *Botryllus schlosseri*; immunocytes; IL-8

Introduction

Interleukin-8 (IL-8, CXCL8) is an inducible chemokine released during infections and inflammation in response to bacteria toxin or inflammatory cytokines like interleukin-1 (IL-1) and tumour necrosis factor alpha (TNF-α) (Rollins, 1997; Baggiolini, 2001; Casilli *et al.*, 2005; Esche *et al.*, 2005). It causes rapid changes in the morphology of leucocytes, such as neutrophils, T-lymphocytes and natural killer cells (NK), as the consequence of a re-organisation of the actin cytoskeleton. In addition, IL-8 induces the up-regulation and activation of integrins on leucocytes required for their adhesion to both endothelial cells, prior to extravasation, and extracellular matrix, during their way to inflammation sites (Luster, 1998; Imhof and Aurrand-Lions, 2004; Casilli *et al.*, 2005). As a chemokine, IL-8 exerts its action by interacting with seven transmembrane, G protein-coupled receptors which, upon ligand binding, activate a heterotrimeric G-protein which dissociates into the GTP-bound α and the $\beta\gamma$ subunits. The latter, in turn activates phospholipase C (PLC) leading to the production of inositol triphosphate (IP3) and diacylglycerol (DAG). In the case of IL-8, the $\beta\gamma$ subunit of the G-protein also recruits and activates phosphatidylinositol-3-kinase (PI3K) which has a central role in inflammation as it allows the recruitment and activation of phagocytes and NK cells (Smith *et al.*, 1992; Hirsh *et al.*, 2000; Naccache *et al.*, 2000; Fuhler *et al.*, 2005 Maghazachi, 2005).

As far as invertebrates are concerned, although the existence of molecules sharing homology with vertebrate cytokines is still a matter of debate (Beschin *et al.*, 2001, 2004), the presence of endogenous immunoregulatory cytokines has been indirectly suggested in various phyla (Beck and Habicht, 1991; Hughes *et al.*, 1990, 1991; Ouwe-Missi-Oukem-Boyer *et al.*, 1994; Granath *et al.*, 1994; Cooper *et al.*, 1995; Ottaviani *et al.*, 1995, 1996; Franchini *et al.*, 1996; Beck, 1998). According to Beschin *et al.* (2001, 2004), these molecules share with vertebrate cytokines some lectin domains which can explain the observed effects of mammalian cytokines on invertebrate immunocytes (Kletsas *et al.*, 1998; Ottaviani *et al.*, 2000, 2004).

In Tunicates, the presence of endogenous chemotactic molecules, is known from many studies. They are represented by either homologues of the

Corresponding author:
Loriano Ballarin
Department of Biology, University of Padova
Via U. Bassi 58/B, 35100 Padova, Italy
Email: loriano.ballarin@unipd.it

mammalian complement C3, as in *Styela plicata* (Raftos *et al.*, 2002), *Halocynthia roretzi* (Nonaka *et al.*, 1999), *Pyura stolonifera* (Raftos *et al.*, 2003) and *Ciona intestinalis* (Pinto *et al.*, 2003), or different molecules cross-reacting with anti-IL-1 antibodies as in *Styela clava* (Raftos *et al.*, 1998).

In the colonial ascidian *Botryllus schlosseri*, we have recently demonstrated that activated immunocytes release cytokines (i.e. immunomodulatory molecules, in the broad sense of the term) able to enhance yeast phagocytosis (Menin *et al.*, 2006). The above molecules are recognised by antibodies raised against mammalian IL-1α and TNF-α and are mainly produced by cytotoxic morula cells upon the recognition of non-self molecules (Ballarin *et al.*, 2001). These cells are known to be involved in the non-fusion reaction which occurs when genetically incompatible colonies contact each other and shares many features, such as chemotaxis, extravasation, degranulation and cytotoxicity, with vertebrate inflammation where chemokines play a key role (Ballarin *et al.*, 1995, 1998; Rinkevich *et al.*, 1998; Cima *et al.*, 2004). Recently, we undertook a new research aimed to identify and characterise chemokines in our model. As a preliminary approach, we studied the effects of human recombinant IL-8 (hrIL-8) on the activity of *B. schlosseri* immunocytes. Results indicate that ascidian immunocytes can sense the presence of exogenous IL-8 and consequently modify their activity.

Materials and methods

Animals

Colonies of *Botryllus schlosseri* from the Lagoon of Venice were used. They were kept in aerated aquaria, attached to glass slides and fed with Liquifry Marine (Liquifry Co., Dorking, England) and algae (*Dunaliella* sp.).

Blood plasma preparation

Blood was collected with a glass micropipette after puncturing, with a fine tungsten needle, the tunic marginal vessels of colonies previously blotted dry. Blood was centrifuged at 780 x g and the supernatant was referred as blood plasma (BP). The terms “autologous” and “heterologous” refer to BP from the same colony used for hemocyte collection or from a different, non-fusible colony, respectively.

Hemocyte collection and culture

Blood was collected, as described above, from colonies previously rinsed in 0.38 % Na-citrate in filtered sea water (FSW), pH 7.5, as anti-clotting agent. It was then centrifuged at 780 x g for 10 min and pellets were finally resuspended in FSW to give a concentration of 5 x 10^6 cells/ml. Sixty µl of hemocyte suspension were placed in the centre of culture chambers prepared as described elsewhere (Ballarin *et al.*, 1994) and left to adhere to coverslips for 30 min at room temperature.

Light microscopy of hemocytes

After the adhesion of hemocytes to the coverslips, cells were exposed for 60 min to FSW containing hrIL-8 (PeproTech EC; 10, 25 and 50 ng/ml) in the presence or in the absence of 0.7 mM suramin, an antagonist of G protein (Huang *et al.*, 1990; Ottaviani *et al.*, 2000); FSW alone was used in controls. Cells were then fixed for 30 min at 4 °C in 1 % glutaraldehyde in FSW containing 1 % sucrose, rinsed in phosphate-buffered saline (PBS: 1.37 M NaCl, 0.03 M KCl, 0.015 M KH_2PO_4, 0.065 M Na_2HPO_4, pH 7.2) and stained with 10 % Giemsa solution for 5 min. The morphology of hemocytes was observed under a Leitz Dialux 22 light microscope at a magnification of 1250 and the cell-spreading index, i.e. percentage of hemocytes with amoeboid shape, was finally calculated after counting at least 300 cells per coverslip.

In another series of experiments, calphostin C, a specific inhibitor of protein kinase C (PKC; Tamaoki, 1991) and H-89, a specific inhibitor of protein kinase A (PKA; Chijiwa *et al.*, 1990) were added to the incubation media, in the presence or in the absence of hrIL-8, at the sublethal concentration of 0.1 and 1 µM, respectively. These concentrations were already used in experiments with invertebrate immunocytes (Ottaviani *et al.*, 2000).

In addition, computer-assisted image analysis (Casting Image NT) was performed on fixed hemocyte monolayers, previously treated as described above, to evaluate the phagocyte shape factor, defined as in Ottaviani *et al.* (1997). Lower shapes factors indicate larger perimeters with respect to the areas and, therefore, an increased amoeboid shape.

Immunocytochemical assays for cytoskeleton

Hemocyte monolayers on coverslips, treated as described above, were fixed for 30 min at 4 °C in 4 % paraformaldehyde (Serva electrophoresis GmbH, Heidelberg, Germany) and 1 % sucrose in isotonic salt solution (ISO: 20 mM Tris, 0.5 M NaCl, pH 7.5; Edds, 1985), washed in PBS plus 1 % sucrose and permeabilised with 0.1% Triton X-100 (Merck KGaA, Darmstadt, Germany) in PBS for 5 min. For the specific detection of F-actin, the monolayers were then incubated for 30 min at 25 °C in FITC-labelled phalloidin (Sigma-Aldrich Co, St Louis, MO, USA), 1 µg/ml in PBS. Lastly, the coverslips were rinsed in 0.1 M carbonate buffer, pH 9.5, to amplify the fluorescent signal, mounted with Vectashield (Vector Laboratories Inc., Burlingame, CA, USA) on glass slides, and observed at a magnification of 1250 under a Leitz Dialux 22 light and fluorescence microscope equipped with I2/3 filter block for FITC excitation.

Phagocytosis assay

After adhesion, hemocytes were incubated with 60 µl of a suspension of yeast cell (yeast:hemocyte ratio = 10:1) in FSW containing hrIL-8 at the same concentrations indicated above, in the presence or

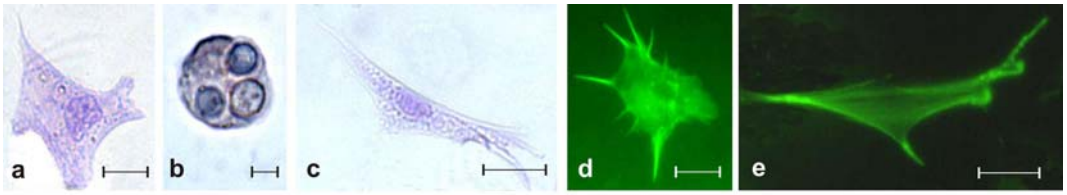

Fig. 1 Fixed *B. schlosseri* phagocytes. **a**, **c-e**) hyaline amoebocytes; **b**) univacuolated macrophage-like cell. **a-c**) Giemsa's stain; **d-e**) treatment with fluorescent falloidin to reveal F-actin. Scale bar = 3 µm.

absence of 0.7 mM suramin; FSW alone was used in controls. Cultures were kept upside down for 60 min at room temperature. Hemocyte monolayers were then washed by dipping the coverslips repeatedly in a large volume (100 ml) of FSW, fixed in a solution of 1 % glutaraldehyde and 1 % sucrose in FSW for 30 min at 4 °C and stained with 10 % Giemsa for 5 min.

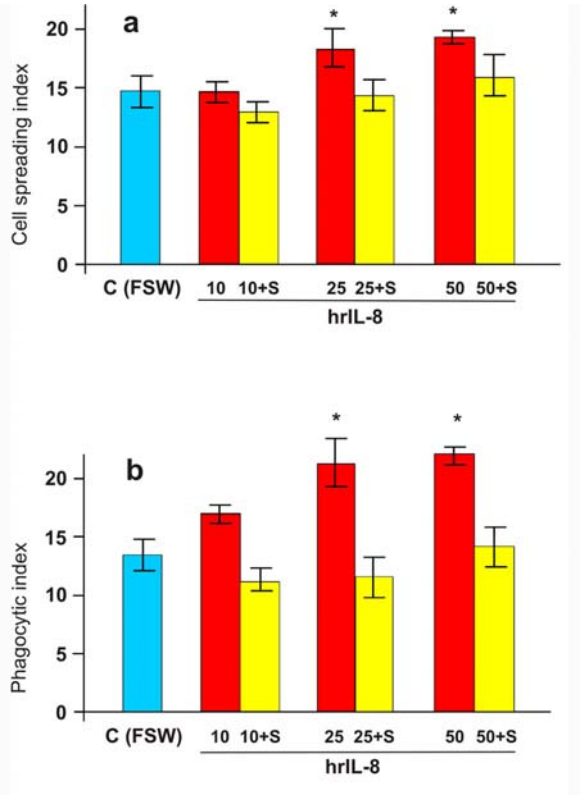

Fig. 2 a) Cell-spreading index of *Botryllus* hemocytes left to adhere for 60 min in FSW containing hrIL-8 in the presence or in the absence of 0.7 mM suramin; FSW alone was used in controls; **b**) phagocytic index of *Botryllus* hemocytes exposed to yeast-containing FSW and hrIL-8 in the presence or in the absence of 0.7 mM suramin; FSW alone was used in controls. Significant differences with respect to controls are marked by asterisks. * = $p < 0.05$.

The coverslips were then mounted on glass slides with the aqueous medium "Acquovitrex" (Carlo Erba Reagenti SpA, Milan, Italy) and at least 300 hemocytes were observed under the light microscope, in ten optical fields at a magnification of 1250, to determine the phagocytic index, i.e. the percentage of hemocytes with ingested yeast cells.

In another series of experiments, calphostin C and H-89 were added to the incubation media, in the presence or in the absence of hrIL-8, at the sublethal concentration of 0.1 and 1 µM, respectively, as reported above.

Statistical analysis

Experiments were replicated at least three times; data are expressed as mean ± SD. At least 300 cells, in 10 optical fields at a magnification of 1250, were counted for each experiment aiming to determine the frequencies of amoeboid or yeast-containing cells; frequencies were subjected to angular transformation. The shape factor was calculated on 20 hyaline amoebocytes for each treatment. Means were compared with the Duncan's test.

Results

Phagocyte spreading

B. schlosseri phagocytes are represented by hyaline amoebocytes and macrophage-like cells. The former are motile cells able to spread on the substrate (Fig. 1a) and actively engulf foreign particles. As a consequence of the ingestion, they change their morphology to globular, vacuolated macrophage-like cells (Fig. 1b). In our controls, we had about 15 % of hemocytes with spreading morphology (Fig. 2). Similar results were obtained with the protein kinase inhibitors (Fig. 3a).

In the presence of hrIL-8 at the concentrations of 25 and 50 ng/ml, a significant ($p < 0.05$) increase in the cell spreading index was observed (Fig. 1c) which was abolished by the presence of the G protein inhibitor suramin (Fig. 2a). No significant effects were observed in the presence of 10 ng/ml hrIL-8 or suramin alone (data not shown).

The shape factor of hyaline amoebocytes resulted significantly ($p < 0.05$) decreased by hrIL-8 at 25 and 50 ng/ml (Table 1). No significant variation in the shape factor was observed in the presence of the protein kinase inhibitors.

Control spreading phagocytes had a typical cytoskeletal organisation, with actin filaments organized in bundles of stress fibres without a preferential orientation. The same was true for pseudopodia which radiated from most of the cell contour and resulted strongly fluorescent (Fig. 1d). In the presence of hrIL-8, phagocytes appeared remarkably spread with stress fibres oriented according to a well defined major axis and their cytoplasmic projections, rich in fluorescent actin, emerging from the apical endings (Fig. 1e).

Phagocytosis

About 15 % of hemocytes were able to ingest yeast cells. This fraction increased significantly ($p < 0.05$) in presence of hrIL-8 at the concentrations of 25 and 50 ng/ml, while the chemokine effect was suppressed by suramin (Fig. 2b).

In the absence of the chemokine, both calphostin C and H-89 significantly ($p < 0.05$) inhibit phagocytosis. The protein kinase inhibitors were able to significantly ($p < 0.05$) reduce the hrIL-8-stimulated phagocytosis when the chemokine was used at the concentration of 10 ng/ml. The inhibitory effect of H-89 only was still detectable at 25 ng/ml, while at the concentration of 50 ng/ml the phagocytic index was not influenced by none of the inhibitors used (Fig. 3b).

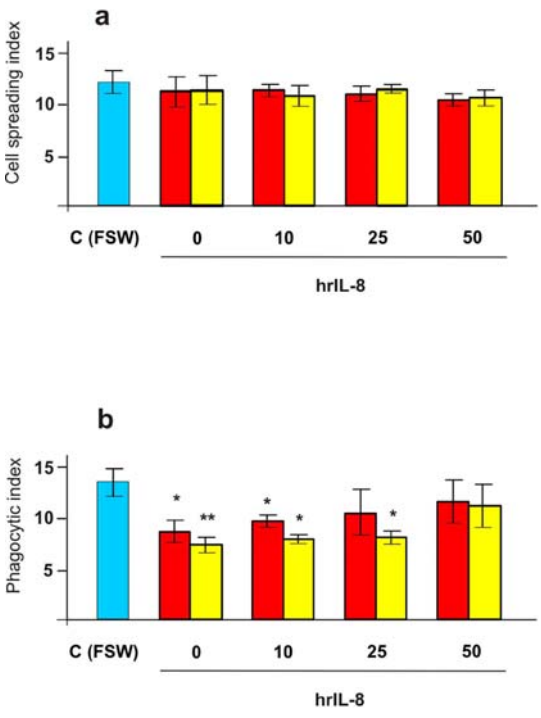

Fig. 3 Effects of 0.1 μM calphostin C (red) and 1 μM H-89 (yellow) on cell spreading index (**a**) and phagocytosis (**b**) in the presence or in the absence of hrIL-8. Asterisks mark significant differences with respect to controls (incubation in yeast-containing FSW). * = $p < 0.05$.

Table 1 Shape factors of hyaline amoebocytes in various experimental conditions

Treatment	Shape factor
FSW (control)	0.21 ± 0.10
FSW + hrIL-8 10 ng/ml	0.19 ± 0.06
FSW + hrIL-8 25 ng/ml	0.14 ± 0.07 *
FSW + hrIL-8 50 ng/ml	0.12 ± 0.04 ***

Asterisks mark significant differences with respect to controls. *: $p < 0.05$; ***: $p < 0.001$

Discussion

Circulating immunocytes represent an important feature of coelomate metazoans as they represent the main component of the immune system. Once sensed a non-self molecular pattern, immunocytes must be able to leak from the circulation into the tissues and migrate towards the source of the stimulus in order to kill and/or engulf it in the course of an inflammatory reaction. Cytokines are immunoregulatory soluble molecules which modulate the activity of immunocytes (Rubinstein *et al.*, 1998; Borish and Steinke, 2003), whereas chemokines represent chemotactic cytokines, released by activated immunocytes and damaged tissues, which mediate immunocytes recruitment to the site of inflammation. Several chemokines were identified in vertebrates, grouped in four families on the basis of their primary sequence (Esche *et al.*, 2005). The situation is less clear in invertebrates, even if the presence of chemotactic factors was reported (Alvarez *et al.*, 1995; Nonaka *et al.*, 1999; Raftos *et al.*, 2002; Pinto *et al.*, 2003; Raftos *et al.*, 2003; Ottaviani *et al.*, 2004).

IL-8, one of the most extensively studied vertebrate chemokine, is a key mediator in polymorphonuclear leucocyte, T lymphocyte and NK cell recruitment and activation (Baggiolini, 2001; Casilli *et al.*, 2005; Esche *et al.*, 2005). It induces changes in the organisation of the actin cytoskeleton and expression of adhesion molecules on leucocytes required for their migration to the inflammation sites (Luster, 1998; Imhof and Aurrand-Lions, 2004; Casilli *et al.*, 2005).

Invertebrate immunocytes can change their functionality in the presence of exogenous mammalian cytokines (Hughes *et al.*, 1990; Beck and Habicht, 1991; Granath *et al.*, 1994, 2001; Ottaviani *et al.*, 1995; Barcia *et al.*, 1999). This study demonstrates that *B. schlosseri* immunocytes can respond to exogenous hrIL-8 with an increase in both phagocyte spreading, as indicated by the higher haemocyte cell spreading index and the reduced amoebocyte shape factor, and phagocytic index.

In vertebrates IL-8-induced leucocyte modifications are mediated by both changes in the organisation of the actin cytoskeleton and up-regulation of integrin expression (Luster, 1998; Imhof and Aurrand-Lions, 2004; Casilli *et al.*, 2005), the latter required for adhesion to substrata during migration and for the establishment of close contacts with foreign particles in the course of phagocytosis (Matricon-Gondran and Letocart, 1999). We have already demonstrated the importance of cytoskeletal modifications and integrin expression in phagocyte spreading and phagocytosis in *Botryllus* (Ballarin *et al.*, 2002). Our results confirm previous data and indicate that exogenous IL-8 can induce a cytoskeletal reorganisation which is related to the reported increase in phagocyte spreading and phagocytosis. IL-8-induced modifications in immunocytes activity were also reported by Ottaviani *et al.* (2000) in the bivalve mollusc *Mytilus galloprovincialis*, suggesting that responsiveness to exogenous IL-8 can be common among invertebrate immunocytes.

In vertebrates, it is known that IL-8 exerts its activity through its interaction with G protein-coupled receptors with seven α-helix transmembrane domains (Esche *et al.*, 2005). Although no sequences sharing homology with vertebrate chemokine receptor genes have been identified in invertebrates so far (De Vries *et al.*, 2006), the presence of G protein-coupled receptors is firmly established and orthologues of the vertebrate receptors have been identified in both Radiata and Bilateria (Brody and Cravchik, 2000; Bouchard *et al.*, 2003; Keating *et al.*, 2003; Kawada et al., 2004); in the compound ascidian *Ciona intestinalis* a G protein-coupled receptor involved in intercellular signalling was recently described (Elphick *et al.*, 2003).

The inhibition of the IL-8-induced increase in cell spreading and phagocytosis by suramin, which is known to disrupt receptor-G protein coupling (Chung and Kermode, 2005), in *Botryllus* (this report) and *M. galloprovincialis* (Ottaviani *et al.*, 2000), suggests that the chemokine interacts with some G protein-coupled receptor in invertebrate immunocytes, unrelated to vertebrate cytokine receptors, which can be the natural target of endogenous chemotactic molecules.

Vertebrate G protein-coupled receptors mainly act through two well known signal transduction pathways: the cyclic AMP and the phosphoinositide pathways (Gomberts *et al.*, 2002). In the first case, the adenylate cyclase is activated with the consequent production of cyclic AMP which, in turn, activates PKA. In the phosphoinositide pathway, a PLC is activated upon ligand-receptor interaction which results in the production of IP3 and DAG, the former mobilising Ca^{2+} from intracellular stores, the latter activating PKC.

As suggested by experiments with calphostin C and H-89, both the transduction pathways are involved in IL-8-induced *Botryllus* cell spreading and phagocytosis. The observed decrease of the phagocytic index in the presence of the protein kinase inhibitors in the absence of the exogenous chemokine or in presence of IL-8 at the ineffective concentration of 10 ng/ml indicates that both PKA and PKC are routinely required for phagocytosis. In addition, in the presence of 25 ng/ml of IL-8, H-89, but not calphostin C, reduces the phagocytic index to levels lower than the controls suggesting that the PKA pathway is more important than that mediated by PKC in *Botryllus* phagocytosis. Similar effect were reported for IL-8-induced changes in cell shape in bivalve molluscs (Ottaviani *et al.*, 2000).

Further studies are now required to better characterise invertebrate G protein-coupled receptors, their natural ligands and the signal transduction pathways involved in immunocyte activation.

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