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Uptake of LPS/*E. coli*/latex beads via distinct signalling pathways in medfly hemocytes: the role of MAP kinases activation and protein secretion

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Abstract

In response to LPS/*E. coli* treatment, extracellular signal-regulated kinase (ERK) is activated in medfly hemocytes. To explore the molecular mechanisms underlying LPS/*E. coli*/latex beads endo- and phagocytosis, we studied the signalling pathways leading to p38 and c-jun N-terminal kinase (JNK) activation. JNK and p38-like proteins were initially identified within medfly hemocytes. Flow cytometry analysis revealed that mitogen-activated protein kinases (MAPK) are required for phagocytosis. Inhibition of specific MAPK signalling pathways, with manumycin A, toxin A, cytochalasin D and latrunculin A, revealed activation of p38 via Ras/Rho/actin remodelling pathway and activation of JNK that was independent of actin cytoskeleton reorganization. ERK and p38 pathways, but not JNK, appeared to be involved in LPS-dependent hemocyte secretion, whereas all MAPK subfamilies seemed to participate in *E. coli*-dependent secretion. In addition, flow cytometry experiments in hemocytes showed that the LPS/*E. coli*-induced release was a prerequisite for LPS/*E. coli* uptake, whereas latex bead phagocytosis did not depend on hemocyte secretion. This is a novel aspect, as in mammalian monocytes/macrophages LPS/*E. coli*-triggered release has not been yet correlated with phagocytosis. It is of interest that these data suggest distinct mechanisms for the phagocytosis of *E. coli* and latex beads in medfly hemocytes.

Keywords: MAP kinase; Protein secretion; Phagocytosis; LPS; *E. coli*; Insect immunity

1. Introduction

Hemocytes are the primary mediators of cell-mediated immunity in insects, as monocytes/macrophages are in mammals. Responses to external stimuli appear to be performed by similar signalling molecules in both insect hemocytes and mammalian monocytes. The LPS/*E. coli*-stimulated signalling pathways appear to mediate, among others, in the regulated release of biologically active molecules in monocytes/macrophages [1,2] and hemocytes [3], in rapid morphological changes [4–6] and the LPS/*E. coli* uptake [7,8].

The mitogen-activated protein kinase (MAPK) family is a well-characterized intracellular evolutionary conserved phosphorylation cascade and is implicated in the regulation of many cellular events including control of differentiation, cell proliferation, development, inflammatory response and apoptosis [9,10]. Three major MAPK subfamilies have been characterized, the extracellular signal-regulated kinases (ERKs), the c-jun N-terminal kinases (JNKs), also known as stress-activated protein kinases (SAPKs), and the p38 mitogen-activated protein.

Abbreviations: MAPK, mitogen-activated protein kinase; ERK, extracellular-signal regulated kinase; MEK, MAPK/ERK Kinases; GRB2, growth factor receptor-bound protein 2; JNK, c-jun N-terminal kinase

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Phagocytosis is a key event in the immune response to pathogen infection in insect hemocytes. However, the relationship between phagocytosis and the associated intracellular signalling pathways remains unclear. Recently, it has been demonstrated that medfly hemocytes respond to LPS by changing their morphology, inducing the activation of several signalling pathways, such as Ras/MEK/ERK, PI-3K and Rho, thus, contributing to LPS uptake [6]. Furthermore, it has been observed that phagocytosis of *E. coli* by medfly hemocytes is mediated by an integrin-dependent process, via the activation of FAK/Src complex [7,11]. In a mosquito cell line (C6/36), it was observed that phagocytosis involves the JNK but not ERK or p38 pathway [8].

An interesting relationship between LPS/*E. coli* uptake and hemocyte secretion has also been observed [6,7]. Such a relationship has not yet been observed in monocytes/macrophages. In particular, genistein, a protein tyrosine phosphorylation inhibitor, as well as brefeldin and SITS, inhibitors of cell secretion, block the LPS/*E. coli*-dependent hemocyte secretion and the LPS/*E. coli* uptake, denoting that secretion is a protein tyrosine phosphorylation-dependent process and evidently a prerequisite for LPS/*E. coli* uptake. This has been further supported by the fact that Drk, a homologue protein to the mammalian GRB2, which acts upstream of Ras/MAP pathway, blocks the LPS-dependent secretion [3,7]. These results strongly recommend the involvement of Ras/MAP kinase pathway in LPS/*E. coli* uptake.

The present work is a first attempt to study the relationship between the MAP kinases subfamilies ERK, p38, JNK signalling pathways and LPS/*E. coli*-dependent secretion with the consequent LPS/*E. coli* uptake, and in parallel with latex bead phagocytosis.

2. Materials and methods

2.1. Antibodies, inhibitors and activators

Monoclonal and polyclonal antibodies against dually phosphorylated p38 and JNK mammalian sequences were obtained from Cell Signalling Technology (Beverly, MA, USA). Affinity-purified rabbit polyclonal antibodies to p38 and JNK were purchased from Cell Signalling Technology. PD 098059 and U0126, MEK1/2 inhibitors, were obtained from Cell Signalling Technology. LPS–FITC conjugate, manumycin A (from *Streptomyces parvulus*, a potent inhibitor of Ras farnesyltransferase), *Clostridium difficile* Toxin A (inhibitor of Rho family small GTPases), cytochalasin D (from *Zygosporium masonii*, potent inhibitor of actin polymerization), latrunculin A (from *Latruncula magnifica*, disrupts microfilament-mediated processes), brefeldin A (from *Penicillium brefeldianum*, inhibitor of cell secretion), SITS (4-acetamido-4′-isothiocyanatostilbene-2, 2′-disulfonic acid disodium salt hydrate), SB 203580 and SB 202190, inhibitors of p38, and protein A-Sepharose 4B Fast Flow were from Sigma (St. Louis, MO, USA). SP 600125, a JNK inhibitor, was obtained from Calbiochem (USA). Other reagents were obtained as indicated. C6 cell line was purchased from Cell Signalling.

2.2. FITC-labelled LPS, E. coli, and latex beads

Fluorescein isothiocyanate (FITC)-labelled LPS and carboxy-modified latex beads were obtained from Sigma. FITC-labelled *E. coli* (DH10B) was prepared after incubation of 10^8 heat-killed bacteria with 1-mg FITC, in 0.5-ml 0.5 M Na_2CO_3/0.5 M $NaHCO_3$ pH 9.5, for 30 min in the dark. FITC conjugated *E. coli* was rinsed three times with PBS, resuspended in Grace's medium and stored in aliquots at −20 °C.

2.3. Collection of hemocytes and cell viability test

C. capitata was reared as described previously [12]. Isolation and homogenization of 3rd instar larva hemocytes were performed according to Charalambidis et al. [3]. In brief, hemolymph was collected and centrifuged at 200×*g* for 10 min at 4 °C. Sedimented hemocytes were washed three times with Ringer's solution (128 mM NaCl, 18 mM $CaCl_2$, 1.3 mM KCl, and 2.3 mM $NaHCO_3$, pH 7.0). The viability of hemocytes was assessed by exclusion of trypan blue dye (Sigma) under a microscope.

2.4. Immunoprecipitation

Hemocytes were lysed in lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM sodium orthovanadate, 5 mM NaF, 1 mM PMSF, 10 μg/ml leupeptin and 10 units/ml aprotinin) at 4 °C. Insoluble material was removed by centrifugation (16,000×*g* for 15 min at 4 °C) and supernatant was collected. For immunoprecipitation, 400 μg of crude extract protein was incubated with 2-μg agarose-conjugated phospho-SAPK/JNK (Thr183/Tyr185) G9 or phospho-p38 (Thr180/Tyr182) 28B10 monoclonal antibody (Cell Signalling) for 2 h at 4 °C and then for an additional 1 h at 25 °C with protein A–Sepharose 4B Fast Flow (Sigma). The immune complexes were washed four times with lysis buffer. Proteins were eluted from the beads by boiling samples for 3 min in 50 μl of electrophoresis sample buffer. Immunoprecipitated proteins were analyzed on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with phospho-SAPK/JNK or phospho-p38 polyclonal antibodies (Cell Signalling).

2.5. Cell secretion

Hemocytes (0.5×10^6 cells/ml) were preincubated in Grace's cultured medium, with specific MAP kinases

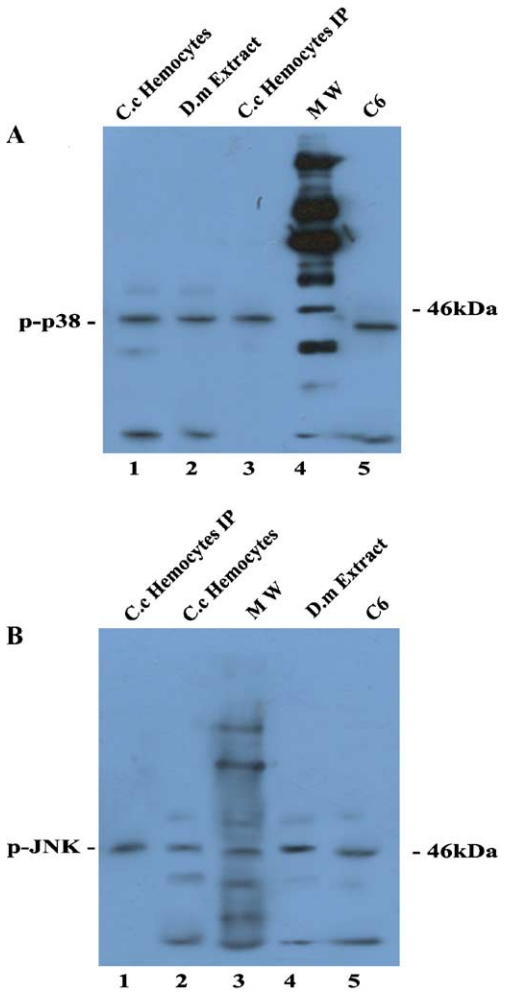

Fig. 1. Identification of phospho-p38 and phospho-JNK homologues in medfly hemocytes. Phospho-p38 and phospho-JNK were immunoprecipitated from *C. capitata* larva hemocytes crude extracts. The immune precipitant (IP) as well as protein extracts from *Drosophila melanogaster* (D.m), *Ceratitis capitata* (C.c) hemocytes and C6 cells were immunoblotted with anti-phospho-p38 (A) and anti-phospho-JNK (B) antibodies. Phospho-p38 and phospho-JNK are present in all samples as indicated.

inhibitors 0.4 μM SB 203580 and SB 202190 for p38, 4 μM PD 098059 and U0126 for MEK1/2, 4 μM SP 600125 for JNK or secretion inhibitors 20 μM SITS and 35 μM brefeldin for 30 min. Cells were then stimulated with LPS (50 μg/ml), *E. coli* (10 bacteria per cell) or latex beads (10 beads per cell) for 45 min in 30 °C. Samples with no preincubation with MAPK or secretion inhibitors were stimulated with LPS/*E. coli*/latex beads to define 100% protein secretion. The concentration of the secreted proteins was determined in the incubation medium.

2.6. Protein determination

Proteins were determined according to Bradford [13] with a modified solution containing 10% (w/v) Coomassie G250 (Merck, Germany) in 5% (v/v) ethanol, 10% (v/v) H_3PO_4. O.D. was recorded at 595 nm.

2.7. SDS-PAGE and immunoblot analysis

SDS-PAGE was performed on 10% acrylamide and 0.10% bisacrylamide slab gels, according to Laemli [14]. Samples with equal amounts of protein were analysed and electroblotted onto Immobilon P polyvinylidene fluoride membranes (Millipore Corp., USA). Membranes were incubated in SuperBlock™ Blocking Buffer (Pierce, USA) for 1 h at room temperature. Subsequently, membranes were incubated overnight at 4 °C with primary antibody diluted 1:1000 in TBS (10 mM Tris–HCl, pH 7.5, 100 mM NaCl), 10% (v/v) SuperBlock™ Blocking Buffer and 0.05% (v/v) Tween-20. Membranes were washed and incubated with horseradish peroxidase-linked secondary antibody (Transduction Laboratories, USA) for 1 h at room temperature. Immunoreactive proteins were visualized on X-ray film by enhanced chemiluminescence (ECL) methodology (Amersham, UK). Stripping of membranes was performed according to the manufacturer's instructions.

2.8. Endocytosis and phagocytosis assays

Hemocytes (5×10^5) were suspended in 100-μl Grace's medium. Hemocyte monolayers were prepared on glass slides and incubated with LPS–FITC (50 μg/ml) or *E. coli*–FITC (10 bacteria per hemocyte) or latex beads–FITC

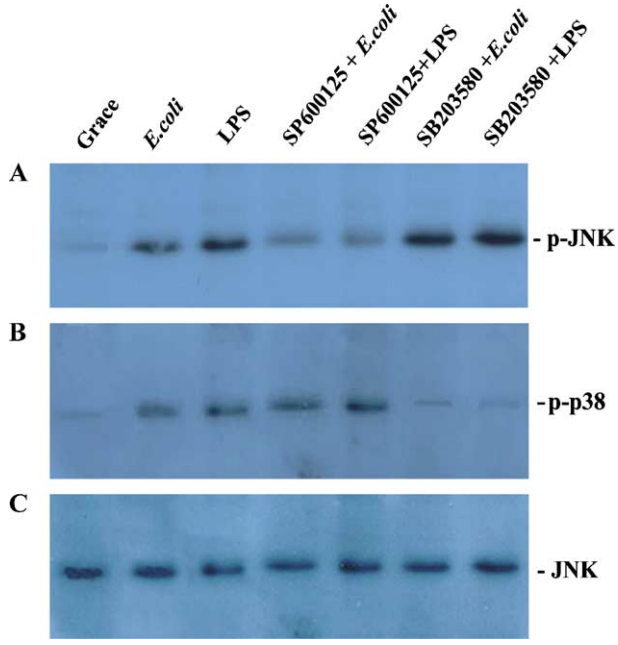

Fig. 2. Inhibition of JNK and p38 activation by their specific inhibitors SP 600125 and SB 203580, respectively. Hemocytes in suspension were incubated with 4 μM SP 600125 or 0.4 μM SB 203580 for 30 min, followed by the presence of *E. coli* (10 bacteria per cell) or LPS (30 μg/ml) for 20 min. Cells incubated in plain medium and cells treated under the same conditions only with *E. coli* or LPS were used as controls. Hemocyte lysates were immunoblotted with anti-phospho-JNK (A). Membrane was stripped and reprobed with anti-phospho p38 (B), and once more with anti-JNK antibody (C), to detect total JNK as loading control.

conjugates (10 beads per hemocyte) for 1 h at 25 °C. Free LPS, *E. coli* and latex beads were removed by rinsing slides three times with Ringer's solution. Trypan blue 2% (w/v) was added to the monolayers for 10 min to quench extracellular fluorescence [11]. Slides were washed and cells were fixed in 4% formaldehyde for 10 min. Endo- and phagocytosis was observed under fluorescence microscope.

2.9. Immunofluorescence microscopy

Hemocyte monolayers were prepared on glass slides and treated with 0.1% Triton X-100 for membrane permeabilization. Cells were incubated with 0.5 μg/ml phalloidin-FITC (Sigma) for 45 min at RT. Hemocyte monolayers were mounted with an aqueous mounting medium (Dako Patts, UK) and observed under fluorescence microscope to examine the distribution of F-actin.

2.10. Flow cytometry analysis

Larval hemocytes (5×10^5 cells) were incubated in 100-μl Grace's insect medium containing either LPS–FITC (10 μg/ml) or *E. coli*–FITC (10 bacteria per hemocyte) or latex beads–FITC (10 beads per hemocyte)

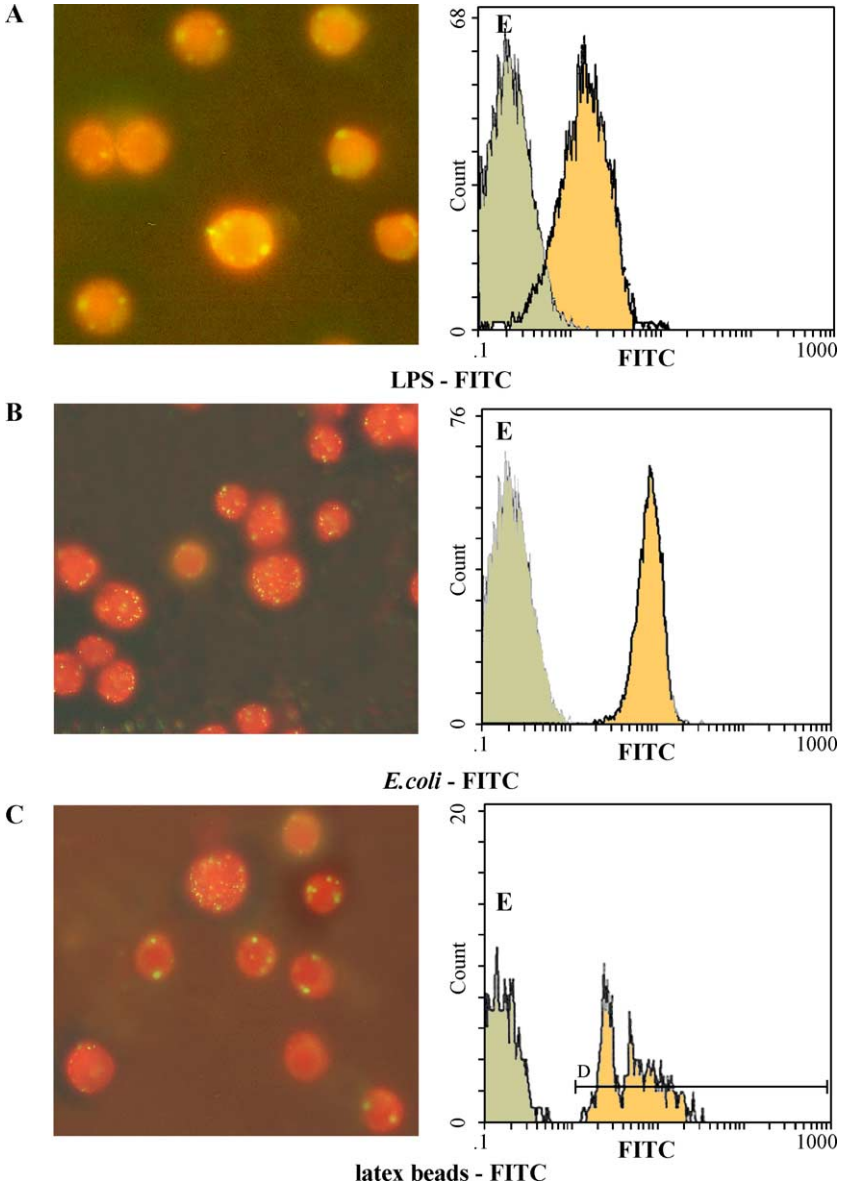

Fig. 3. Uptake of LPS/*E. coli*/latex beads by medfly hemocytes. Hemocytes from third instar larvae were incubated at 25° C for 60 min with FITC-labelled either LPS or latex beads or with FITC-labelled *E. coli* at 30 °C for 60 min. After trypan blue quenching of extracellular bound LPS, bacteria or beads, their uptake by hemocytes was evaluated by flow cytometry and observed under fluorescence microscope. Endogenous fluorescence (E) was presented by analysing hemocyte suspension in plain medium. LPS (A) and *E. coli* (B) are taken up by all hemocytes, while latex beads (C) by approximately 30% of the hemocyte population under the experimental conditions used.

for 60 min at 30 °C in the presence or absence of MAPK or cell secretion inhibitors. Internalized LPS/*E. coli*/latex beads–FITC were measured by quenching surface-exposed FITC–LPS/*E. coli*/latex beads with trypan blue 4% in Ringer's solution. Approximately 20,000 cells from each sample were analysed by flow cytometry using a Coulter EPICS-XL-MCL cytometer (Coulter, Miami, FL, USA), and the data were processed using the XL-2 software.

3. Results

3.1. Identification of hemocyte JNK and p38

ERK-like proteins have been recently identified in medfly hemocytes [6]. *Drosophila* JNK, and p38 are 80% and 66% identical in amino acid sequence to their mammalian counterparts [15,16]. In the present paper, we identified p38 and JNK homologues in medfly hemocytes.

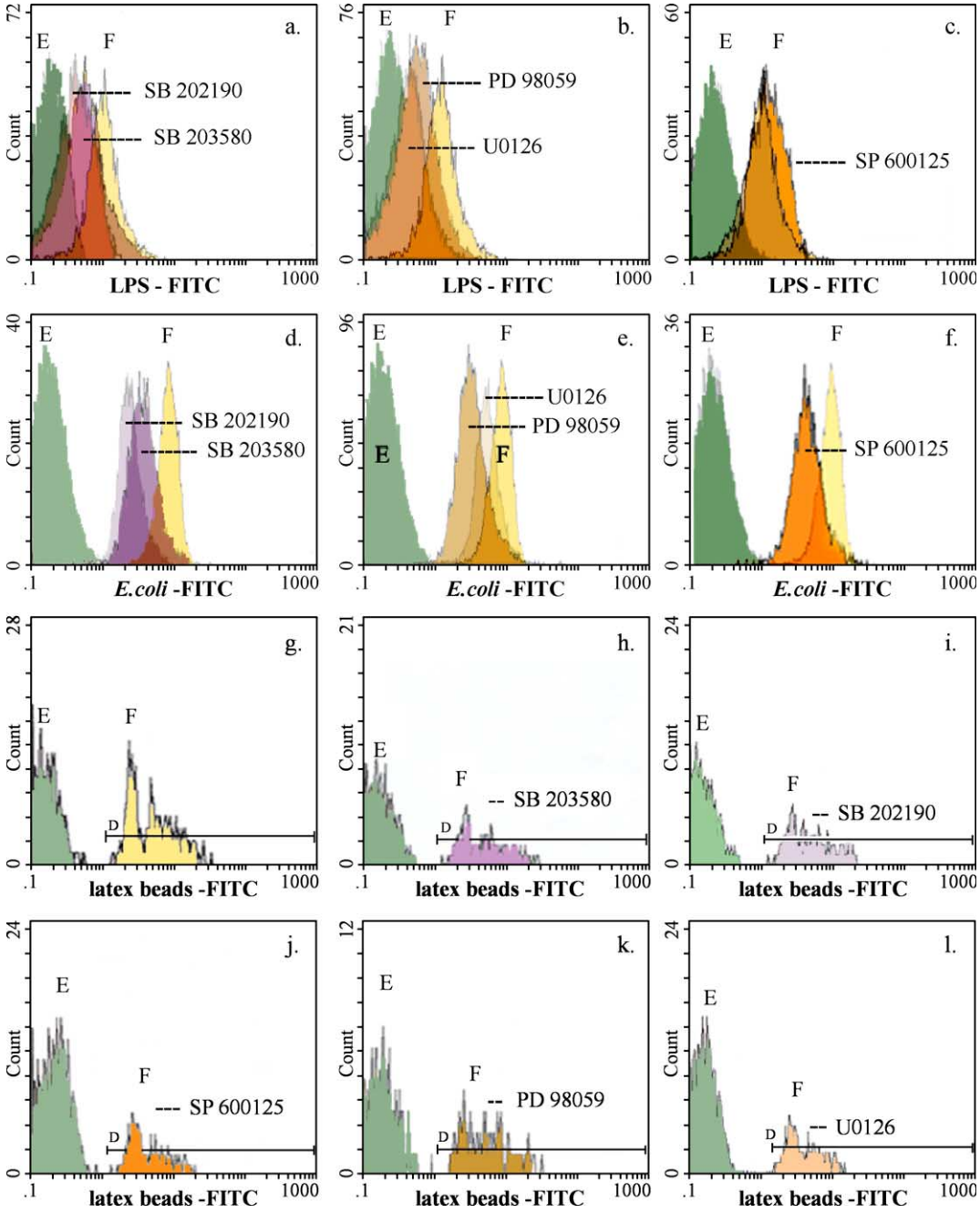

Fig. 4. MAP kinases regulate the endo- and phagocytosis of LPS/*E. coli*/latex beads by medfly hemocytes. Hemocytes were incubated with either FITC-labelled LPS (a, b, c) or *E. coli* (d, e, f) or latex beads (g, h, i, j, k, l) alone (F) or after preincubation with MAP kinase inhibitors 0.4 μM SB 203580 or 10 μM SB 202190 for p38, 4 μM PD 98059 or 10 μM U0126 for MEK1/2 and 4 μM SP 600125 for JNK. After trypan blue quenching, hemocyte suspensions were analysed with flow cytometry. Endogenous fluorescence (E) was presented by analysing hemocyte suspension in plain medium.

Like mammalian JNK and p38, insect homologues are activated by dual phosphorylation of the TPY and TGY motifs, respectively [17]. Therefore, antibodies, which specifically detect activated (phosphorylated) mammalian JNK and p38, were used to analyze JNK and p38 activities in medfly hemocytes. Immunoblot analysis with antiphospho-JNK and antiphospho-p38 antibodies recognizes only a single band in medfly hemocyte protein extracts, similar to that of *Drosophila* JNK and p38 and mammalian C6 cell extracts (Fig. 1). It is also evident that the same protein band is also recognized in medfly hemocyte protein immunoprecipitant (Fig. 1). Therefore, JNK and p38 are activated by dual phosphorylation of the TPY and TGY motifs in medfly hemocyte, just like in mammals and *Drosophila*. These results demonstrate for the first time the presence of JNK and p38 in insect hemocytes, strongly supporting a role for JNK and p38 in mediating immune and inflammatory responses.

Supporting evidence that these proteins are phospho-JNK and phospho-p38 and that the antibodies specifically recognize JNK and p38 comes from the use of inhibitors. LPS and *E. coli* induced JNK and p38 phosphorylation in medfly hemocytes (Fig. 2). Treatment of suspended hemocytes with the JNK and p38 phosphorylation inhibitors, SP 600125 and SB 203580, respectively, in the presence of LPS/*E. coli* almost completely blocked the induced phosphorylation (Fig. 2). Therefore, JNK and p38 can be stimulated in insect hemocytes by LPS or Gram-negative bacteria, as is the case for mammalian macrophages [18]. Since the same protein band is recognized in immunoprecipitants and crude extracts (Fig. 1), the next experiments were performed using immunoblotting analysis of protein crude extracts.

3.2. Phagocytosis depends on MAP kinases phosphorylation

Although well documented in vertebrate immunity, it remains unclear how insect hemocytes phagocytoze pathogens. Flow cytometry analysis and fluorescence microscopy revealed extensive endo- and phagocytosis of LPS, *E. coli*, and latex beads by plasmatocytes of 3rd instar larvae, the major hemocyte type that constitute more than 90% of total circulating hemocytes (Fig. 3). It was clearly showed that endo- and phagocytosis of LPS and *E. coli* were performed by all hemocytes (Fig. 3A and B), while endocytosis of latex beads was performed by the 30% of the hemocytes (Fig. 3C).

To explore the involvement of MAPK pathways in LPS/*E. coli*/latex beads uptake, the ability of several inhibitors to affect phagocytosis was tested. For this purpose, hemocytes were pretreated with either PD 098059 or U0126 for MEK1/2, or SP 600125 for JNK, or SB 203580 or SB 202190 for p38 followed by the addition and incubation 30 min of either soluble LPS–FITC or *E. coli*–FITC or latex beads–FITC. The uptake of LPS/*E. coli*/latex beads was monitored by flow cytometry (Fig. 4). It was clearly demonstrated that all MAP kinases are required for the phagocytosis of *E. coli* and inert latex beads. On the contrary, it appeared that ERK and p38, but not JNK, pathways are required for the LPS uptake. The inhibitor of JNK repeatedly showed an induction rather than inhibition of the uptake of LPS, indicating that it acts as a negative regulator, as is the case for p38 inhibitor in *Drosophila*, which induces genes responsible for antibacterial peptides [19]. Consequently, MAP kinases are required for the endo- and phagocytosis processes.

3.3. LPS/E. coli/latex beads-challenged hemocytes and signalling pathways leading to JNK and p38 activation

Exploring the molecular mechanisms underlying LPS/*E. coli*/latex beads endo- and phagocytosis, the signalling pathways leading to p38 and JNK were studied. ERK signalling pathway has already been elucidated [6]. We examined the effects of inhibitors on these signalling pathways, such as manumycin A, which blocks Ras activity, and toxin A, which inactivates Rho family proteins. The obtained results clearly showed that preincubation of suspended hemocytes with manumycin A strongly inhibited JNK and p38 activation, supporting the mediation of Ras in the JNK and p38 pathways (Fig. 5). The Rho family of small GTPases is a key mediator of actin cytoskeletal remodelling induced by extracellular signals and also regulates the formation of cell–cell and cell–substratum adhesions [20,21]. Based on the results for toxin A, which

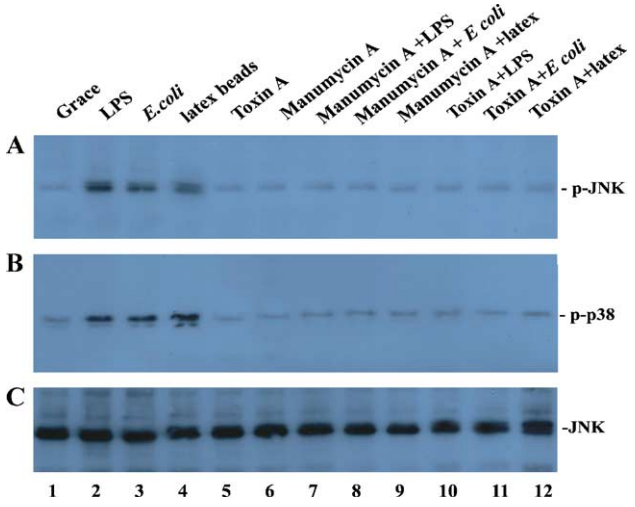

Fig. 5. Effect of inhibitors of Rho family proteins on JNK and p38 activation, induced by LPS/*E. coli*/latex beads. Hemocytes in suspension were incubated with 300 nM toxin A (lanes 5, 10, 11, 12) and 20 μM manumycin A (lanes 6, 7, 8, 9), followed by stimulation with LPS (lanes 7, 10), *E. coli* (lanes 8, 11) and latex beads (lanes 9, 12). Cells treated with LPS, *E. coli* or latex beads in the absence of inhibitors (lane 2, 3, 4, respectively) were used as positive controls for JNK and p38 activation. Hemocytes incubated in Grace's medium (lane 1) were used to show any minor activation due to the isolation procedure. Cell lysates were subjected to immunoblotting analysis with anti-phospho JNK (A). Membrane was stripped and reprobed with anti-phospho p38 (B) and once more with an anti-JNK antibody to detect total JNK as loading control (C).

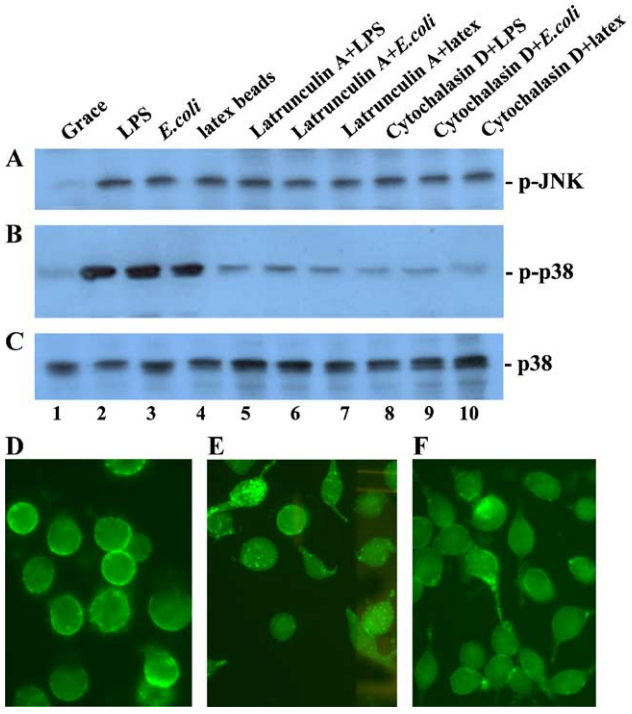

Fig. 6. Actin cytoskeleton remodelling and activation of JNK and p38 in LPS/*E. coli*/latex beads-challenged hemocytes. Hemocytes in suspension were incubated with 60 nM latrunculin A (lanes 5, 6, 7) or 10 μM cytochalasin D (lanes 8, 9, 10) followed by either LPS or *E. coli* or latex beads stimulation. Challenged hemocytes with LPS, *E. coli* or latex beads in the absence of inhibitors were used as positive controls for JNK and p38 activation (lanes 2, 3, 4), respectively. Hemocytes incubated in Grace's medium (lane 1) were used to show any minor activation due to the isolation procedure. Hemocyte lysates were subjected to immunoblotting analysis with anti-phospho JNK (A). Membrane was stripped and reprobed with anti-phospho p38 (B) and once more with anti-p38 antibody to detect total p38 as loading control (C). Remodelling of cytoskeletal F-actin was detected immunocytochemically and observed under fluorescence microscope, in hemocytes suspended in plain medium (D) and after treatment with 10 μM cytochalasin D (E) or 60 nM latrunculin A (F).

inactivates Rho family proteins, it appears that LPS/*E. coli*/latex beads-induced Rho family proteins are involved in both JNK and p38 activation (Fig. 5).

It is known that LPS or *E. coli* induce morphological changes leading to the formation of filopodia and lamellipodia in medfly hemocytes [3,6]. To test whether or not hemocyte JNK and p38 phosphorylation is dependent upon the integrity of actin filaments, suspended hemocytes were treated with cytochalasin D or latrunculin A and then stimulated with LPS, *E. coli* or latex beads. It was shown that cytochalasin D and latrunculin A had no effect on LPS, *E. coli* or latex beads-mediated phosphorylation of JNK (Fig. 6A), as they do in *Salmonella*-infected macrophages [22], suggesting that this kinase activity does not depend upon the integrity of actin filaments. Similarly, hemocyte ERK activation appeared to be independent of the integrity of actin filaments [6]. On the contrary, both inhibitors of actin polymerization block p38 phosphorylation (Fig. 6B and C). Consequently, p38 is the only MAP kinase and its phosphorylation depends on cytoskeletal remodelling. Additional experiments confirming that inhibitors of F-actin polymerization work in these assays were performed. Incubation of hemocyte suspension with 60 nM latrunculin A or 10 μM cytochalasin D caused changes on the shape of hemocytes and a redistribution of F-actin (Fig. 6E and F) compared with the untreated hemocytes (Fig. 6D). Notably, a variable number of bright spots were observed in hemocytes treated with either latrunculin A or cytochalasin

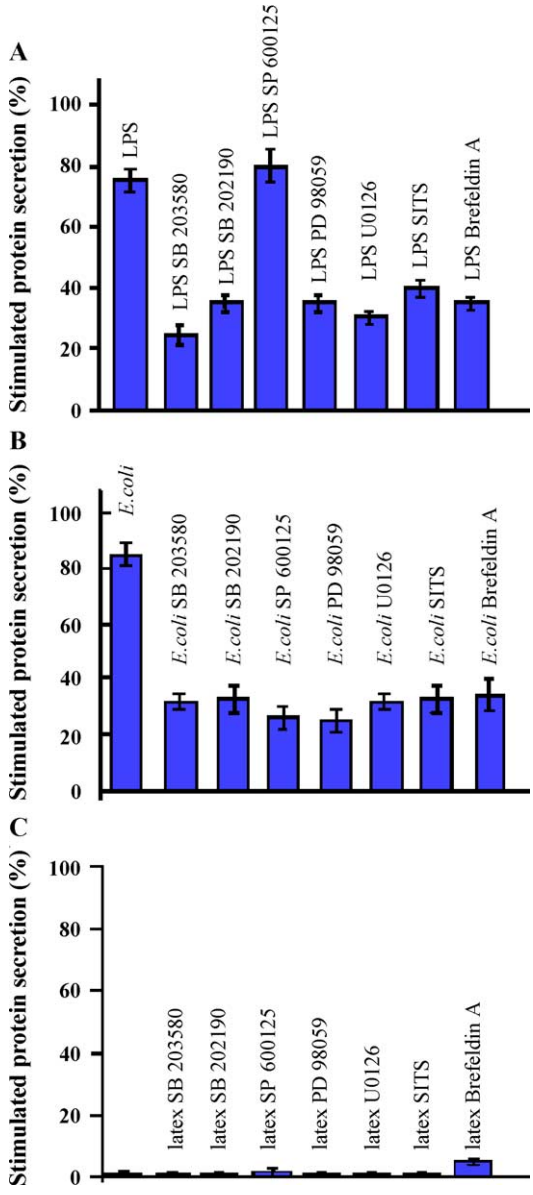

Fig. 7. MAP kinases regulate hemocyte protein secretion in response to LPS/*E. coli*/latex beads stimuli. Hemocytes were incubated with MAP kinases inhibitors 0.4 μM SB 203580 or 10 μM SB 202190 for p38, 4 μM PD 098059 or 10 μM U0126 for MEK1/2 and 4 μM SP 600125 for JNK or secretion inhibitors 20 μM SITS and 35 μM brefeldin A for 30 min. Cells were then stimulated with LPS (A) or *E. coli* (B) or latex beads (C) for 45 min at 30 °C. Stimulated protein secretion in the incubation medium was determined as the percentage (%) of the stimulated over the basal secretion.

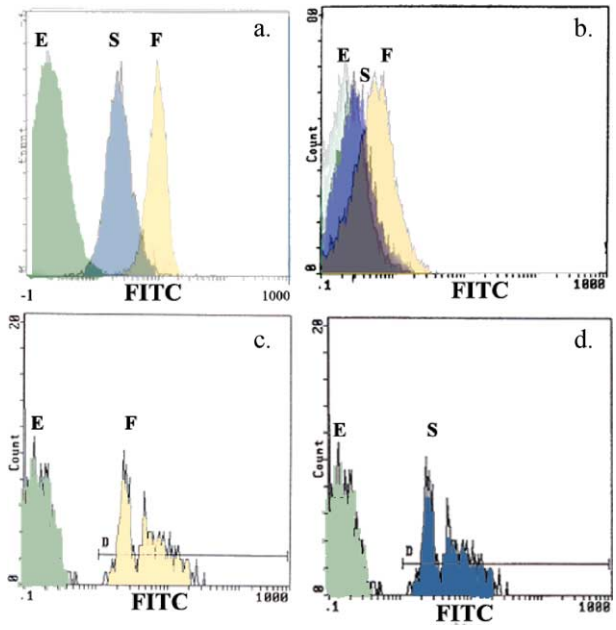

Fig. 8. Hemocyte secretion and endo- and phagocytosis in medfly hemocytes. Hemocytes in suspension were incubated with either FITC-labelled LPS (a), or *E. coli* (b), or latex beads (c, d) in the presence (S) or absence (F) of the secretion inhibitor SITS and analysed with flow cytometry. Endogenous fluorescence (E) was presented by analysing hemocyte suspension in plain medium.

D. In untreated cells F-actin was mainly localized as a peripheral ring or appeared to be diffusely distributed throughout the cell. Therefore, it appears that actin cytoskeleton remodelling is the point where the MAP kinase pathways diverge.

3.4. LPS/E. coli-dependent secretion, MAP kinase phosphorylation and endo- and phagocytosis

LPS/*E. coli* activate ERK, p38 and JNK in monocytes/macrophages [23], as well as in medfly hemocytes (Ref. [6]; present results). The activation of MAP kinases appears to regulate LPS/*E. coli*-dependent secretion of TNF-α and IL-1β in macrophages [24]. LPS also stimulates the secretion of medfly hemocytes in a protein tyrosine phosphorylation-dependent manner [3], and Drk (GRB2 homologue), an upstream protein of Ras/MAP kinase pathway, blocks LPS/*E. coli*-dependent release in medfly hemocytes [7]. These results prompted us to clarify initially the role of MAP kinases in LPS/*E. coli*-dependent secretion of hemocytes by using several inhibitors for ERK, p38 and JNK. Pretreatment of hemocytes with PD 098059 and U0126, inhibitors of MEK1/2, followed by 30-min incubation with LPS at room temperature, efficiently suppressed LPS-dependent hemocyte secretion (Fig. 7A). Similarly, pretreatment of hemocytes with SB 203580 and SB 202190, inhibitors of p38 kinase, substantially suppressed LPS-dependent hemocyte secretion. On the other hand, SP 600125, an inhibitor for JNK, did not block the release due to LPS triggering (Fig. 7A). Brefeldin A and SITS were used as positive controls for the inhibition of secretion due to LPS triggering (Fig. 7A). With pretreatment of hemocytes with the same MAPK and secretion inhibitors, *E. coli*-challenged hemocytes also resulted in a rather strong blockage of protein release (Fig. 7B). Latex beads did not affect hemocyte secretion (Fig. 7C). Consequently, MAP kinases appeared to be involved in LPS/*E. coli*-induced hemocyte protein secretion.

To strengthen our previous observation for a relationship between LPS/*E. coli* uptake and hemocyte secretion [6,7], LPS/*E. coli*/latex beads-challenged hemocytes were treated with protein secretion inhibitor SITS followed by flow cytometry analysis. Results showed that the presence of this inhibitor blocked endo- and phagocytosis of LPS/*E. coli* (Fig. 8a and b) but did not affect inert latex beads uptake (Fig. 8c and d). Thus, it appeared that (a) protein secretion is an essential step for endo- and phagocytosis of LPS/*E. coli* and (b) distinct mechanisms are responsible for bacterial and abiotic components uptake.

Furthermore, to test whether hemocyte secretion is related to MAP kinase phosphorylation, hemocytes were treated with SITS and Brefeldin A, followed by the addition of LPS/*E. coli*/latex beads. Immunoblot analysis of lysates demonstrated that secretion inhibitors did not inhibit MAP kinase phosphorylation (Fig. 9). These results suggest that protein secretion, a prerequisite of endo- and

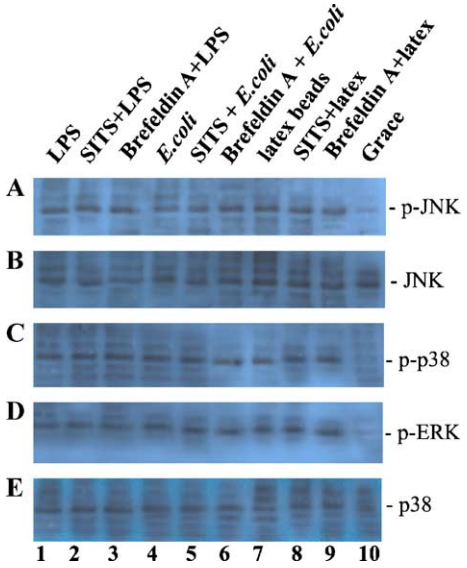

Fig. 9. Effect of hemocyte secretion on JNK and p38 activation. Hemocytes in suspension were incubated with 20 μM SITS (lanes 2, 5, 8) or 35 μM Brefeldin A (lanes 3, 6, 9) for 30 min, followed by the presence of LPS (30 μg/ml) (lanes 2, 3) or *E. coli* (10 bacteria per cell) (lanes 5, 6) or latex beads (10 beads per cell) (lanes 8, 9) for 30 min. Cells incubated in plain medium (lane 10) and cells treated under the same conditions only with LPS, or *E. coli* or latex beads (lanes 1, 4, 7) were used as controls. Hemocyte lysates were immunoblotted with anti-phospho-JNK (A). Membrane was stripped and reprobed with anti-JNK antibody (B) to detect total JNK as loading control. Hemocyte lysates were immunoblotted with anti-phospho-p38 (C). Membrane was stripped and reprobed with anti-phospho ERK (D), and once more with anti-p38 antibody (E), to detect total p38 as loading control.

phagocytosis, appears to be a downstream target of MAP kinases.

4. Discussion

The signalling pathways involved in vertebrate immunity are well documented. The signal transduction pathways involved in immune responses such as phagocytosis by insect hemocytes have been largely uncharacterized. In mammalian monocytes/macrophages, LPS activates ERK, p38 and JNK in a Ras/Rho-dependent manner [23]. This activation regulates, among others, the LPS-dependent secretion of TNF-α and IL-1β as well as phagocytosis [24,25]. Other mammalian cell types also show similar behavior. For example, the regulation of matrix metalloproteinase secretion in fibroblasts depends on ERK and p38 activation [26]. In insect hemocytes, LPS also activates the Ras/Rho/MEK1/2/ERK pathway [6], and this activation stimulates the release of certain proteins by medfly hemocytes in a protein tyrosine phosphorylation-dependent manner [3]. Similarly, challenge of molluscan hemocytes with LPS resulted in a transient activation of ERK that is vital for efficient phagocytosis [27].

In this study, freshly isolated hemocytes from medfly were used as a model system to explore the regulation of the MAP kinase-signalling pathway, challenged by LPS/*E. coli*/latex beads. The role of these pathways in phagocytosis was examined. The data presented in this paper indicate unequivocally for the first time that insect hemocytes respond to LPS/*E. coli*/latex beads challenge by activation of both JNK and p38, similarly to mammalian monocytes/macrophages. Concerning the pathways that lead to JNK and p38 activation, the use of inhibitors succeeded in demonstrating that the upstream regulators of the pathways leading to JNK and p38 activation include, among others, Ras and Rho small GTPases (Fig. 5), as is the case for ERK pathway [6]. However, downstream of Rho kinases regulation, the JNK and p38 pathways must be diverged, as p38 activation depends upon the integrity of actin filaments, whereas JNK activation does not (Fig. 6).

Since activation of the MAP kinase pathways plays a key regulatory role in immune responses including endo- and phagocytosis in mammalian macrophages, this study was also set out to establish whether this was evident in medfly hemocytes, as well. Inhibiting the MAP kinases with inhibitors, the hemocyte phagocytic activity was significantly reduced as demonstrated by flow cytometry experiments (Fig. 3). In addition, it appears that ERK and p38, but not JNK, pathways are required in LPS uptake. The inhibitor of JNK induces, rather than inhibits, the uptake of LPS, indicating that it acts as a negative regulator, as is the case of p38 inhibitor in *Drosophila* where it induces genes responsible for antibacterial peptides [19]. Consequently, MAP kinases activation is vital for efficient endo- and phagocytosis processes in insect hemocytes as is the case for mammalian counterparts. The comparable responses of the MAPK pathways to LPS/*E. coli* challenge between these two kinds of cells suggest that MAPK signalling in innate immunity may have been conserved through evolution. Convincing evidence, however, concerning the role of MAP kinases on LPS/*E. coli*/latex beads uptake possibly can be obtained with RNA interference experiments, which specifically alter gene function by silencing gene expression, thereby creating conditions in which a gene functions rather poorly or not at all.

The above data as well as our previous studies, supporting that in insect hemocytes the regulated secretion in response to LPS/*E. coli* is a prerequisite for LPS/*E. coli* uptake (Refs. [6,7], present data), prompted us to further explore whether any relationship exists between MAP kinase-dependent secretion and phagocytosis. Indeed, our data indicated that both ERK and p38 kinases are involved in LPS/*E. coli*-dependent hemocyte secretion. Thus, secretion appears to be a critical downstream target of MAP kinases, required for endo- and phagocytosis, since MAP kinase phosphorylation is not inhibited by secretion inhibitors. JNK activation is involved in *E. coli* but not in LPS-dependent hemocyte secretion (Fig. 7). This may be due to different signaling pathways involved in LPS and *E. coli* uptake. As we have shown earlier, phagocytosis of *E. coli*, but not LPS uptake, by medfly hemocytes is mediated by an integrin-dependant process via the activation of FAK/Src complex [7,11]. Therefore, ERK- and p38-dependent secretion in response to LPS/*E. coli* both in mammalian cell systems and insect hemocytes may be an evolutionary conserved intracellular response of monocytes/macrophages and hemocytes (Ref. [26], present data). Furthermore, using flow cytometry, a more convincing procedure than fluorescence microscopy used in the past [7], we observed that inhibitors of cell secretion significantly reduced LPS/*E. coli* endo- and phagocytosis. To our knowledge, this is a novel aspect, as in mammalian monocytes/macrophages it has not yet been correlated their regulated release upon LPS/*E. coli* triggering and phagocytosis. Evidently, one or more of the secreted bioactive molecules from hemocytes in response to LPS/*E. coli* are somehow actively involved in the internalization of LPS/*E. coli*. On the other hand, the latex beads, although they stimulate the activation of MAP kinases, do not induce hemocyte secretion and they are internalized into hemocytes in a secretion-independent way (Figs. 7 and 8). In other words, latex phagocytosis events are independent of hemocyte secretion. The role of regulated release in LPS/*E. coli* uptake is not yet known.

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