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STAT1 and STAT3 phosphorylation by porins are independent of JAKs but are dependent on MAPK pathway and plays a role in U937 cells production of interleukin-6

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Abstract

A group of transcription factors, termed signal transducers and activators of transcription (STATs), appears to orchestrate the downstream events propagated by cytokine/growth factor interactions with their cognate receptors. Similarly, cytoplasmic Janus kinases (JAKs) seem to play a critical role in diverse signal transduction pathways that govern cellular survival, proliferation, differentiation and apoptosis. In this work, we analysed the effects of the *Salmonella enterica* serovar Typhimurium porins on signaling by the JAK/STAT pathway and IL-6 release in U937 cells. Porins and LPS of membrane from Gram-negative bacteria are factors implicated in septic shock. In our assays porins induce interleukin-6 (IL-6) release (110 ± 2.6 pg/ml) 24 h after stimulation and STAT1/STAT3 tyrosine (Tyr701/Tyr705) and serine (Ser727) phosphorylation after 15 min. By using several selective inhibitors we demonstrate that porins modulate the activation of STAT1/STAT3 through mitogen activated protein kinases (MAPKs) and not JAKs. Furthermore, we demonstrated that STAT1 and STAT3 are not involved in the modulation of IL-6 release in U937 cells stimulated with porins. Inhibition of tyrosine/serine phosphorylation mediated by MAPKs of STAT1 and STAT3 decrease the IL-6 secretion following porin stimulation. Therefore, suggesting a key role of this pathway in phosphorylation of Ser 727 in STAT1 and STAT3. These results are confirmed by porin or LPS-induced nuclear translocation of STAT1 and STAT3 in U937 cells.

Keywords: Porins; IL-6 release; JAK/STAT pathway

1. Introduction

Signaling and transcriptional activity are important processes induced by several bacterial components which modulate many of the pathways involved in the host inflammatory response and in the innate and adaptative immune functions.

Mycobacterial parasites [1], peptidoglycan [2], lipopolysaccharide (LPS)[1] [3], outer membrane proteins [4,5] and other specific bacterial products besides their activity as

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[1] *Abbreviations used:* STAT, signal transducers and activators of transcription; JAK, Janus protein tyrosine kinase; MAPKs, mitogeni activated protein kinases; LPS, lipopolysaccharide; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence; TBS, Tris-buffered saline; TTBS, Tris-buffered saline Tween 20; BSA, bovine serum albumin; ELISA, enzyme-linked immunoassay; PBS, phosphate-buffered saline; PBM, polymixin B; PTK, protein tyrosine kinase; ERK, extracellular-signal-regulated kinase; AP-1, activate activating protein 1; NF-κB, nuclear factor kappa B.

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immunogens, on coming into contact with cells of the inflammatory and immune responses, induce the production of peptide and small peptide mediators such as cytokines, defensins, etc., which have different structures and functions. The molecular mechanisms which regulate the interactions of the small peptide mediators with host cells are well known, whereas there is little understanding of the mechanisms by which different bacterial products induce similar responses, releasing cytokines and defensins [6].

Porins from *Salmonella enterica* serovar Typhimurium, *Mannheimia haemolytica* and *Haemophilus influenzae* induce tyrosine phosphorylation of several proteins in THP-1 cells and in macrophages from C3H/HeJ mice [7].

Among the most prominent tyrosine phosphorylated bands in porin-stimulated cells there are a number of proteins with molecular masses similar to those of the tyrosine/serine/threonine protein kinase family. Mitogen-activated protein kinases (MAPKs) and their upstream kinases activate a number of transcription factors and induce transcription from a variety of inflammatory genes in response to LPS and cytokines [8]. MAPKs are kinases that signal the intracellular responses to an array of extracellular stimuli that include mitogens, growth factors, pathogen-derived products, and other physical stressors.

Additionally, porins activate the Raf-1-MEK1/2-MAPK pathway and transcriptional factors AP-1 and NF-κB [9].

The family of transcription factors called STATs (signal transducers and activators of transcription) have been found to be activated by the Janus kinases (JAKs) that are associated with the cytokine receptor components [10]. In resting cells the STATs, when phosphorylated by the JAKs, dimerize via their SH_2 domains and translocate to the nucleus, where they interact with specific DNA sequences and transactivate the associated genes.

The ability of STATs to homo-or-hetero-dimerize allows cytokine-specific cellular responses [11]. Several polypeptide ligands use the JAK-STAT molecules in signal transduction [12]. Four JAKs have been identified in mammals: JAK1, JAK2, JAK3 and Tyk2. They have molecular masses ranging from 120 to 140 kDa and a conserved structure with distinct regions. Two JAKs of the same or different class must be in close proximity for phosphorylation to occur. Seven STAT proteins have currently been identified in mammalian cells, with molecular masses between 75 and 95 kDa. Many different models have been used to investigate the role of JAK/STAT in response to infection *in vivo* [13]. The models range from polymicrobial peritonitis [14] and *Klebsiella pneumoniae* lung infection [15], to intraperitoneal injection of pathogens or their components, such as endotoxin [16].

In the present study, we investigated the ability of porins to directly activate the JAK/STAT signal transduction pathway and stimulate IL-6 release from U937 cells. The results will contribute to our current knowledge on the mechanism and function of JAK/STAT signaling in sepsis.

2. Materials and methods

2.1. Cell lines

U937 monocytes (ATCC CRL-1593.2) were grown at 37 °C in 5% CO_2 in RPMI 1640 and differentiated as previously described [17]. Cells were tested every 2 weeks by a PCR-based detection assay for mycoplasma contamination [18].

2.2. Bacterial strain

The bacterial strain used was *S. enterica* serovar Typhimurium SH5014 grown in nutrient broth for 18–24 h at 37 °C under agitation; cells were harvested at the end of the exponential growth phase.

2.3. Preparation of porins and LPS

Porins were isolated from the lysozyme–EDTA extracted envelopes of *S. enterica* serovar Typhimurium strain SH5014 as previously reported [9]. The protein content of the porins preparation was determined by the method of Lowry et al. [19] and checked by SDS–PAGE according to Laemmli [20]. SDS–PAGE revealed two bands with molecular weights of 34 and 36 kDa, confirming the purity of the preparation. LPS was isolated using the phenol/chloroform/ether method described previously [9]. All possible traces of LPS were revealed on SDS–PAGE stained with silver nitrate as described by Tsai and Frasch [21] and by the *Limulus*-amoebocyte-lysate assay (*Limulus* test, pbi international, Milan Italy) according to Yin et al. [22].

2.4. Cell stimulation with S. enterica serovar Typhimurium porins and LPS for preparation of cell lysates

U937 cells (3×10^6 cells/ml) were stimulated with different concentrations (1, 5 and 10 μg/ml) of stimuli for different time periods (15, 30 and 60 min) in 96-well polypropylene plates. After incubation, the cells were prepared as previously reported [9] and used for enhanced chemiluminescence's (ECL) Western Blot analysis.

2.5. Kinase phosphorylation

Cell lysates were immunoprecipitated and used for western blotting. Immunoprecipitation was carried out with the appropriate antibodies (anti-JAK1, -JAK2, -JAK3, -Tyk2, -STAT1, -STAT2, -STAT3, -STAT4, -STAT5, -STAT6) (Santa Cruz Biotechnology, Inc.). Blots were blocked for 1 h at room temperature in Tris-buffered saline (TBS [150 mM NaCl, 20 mM Tris–HCl, pH 7.5]) containing 1% bovine serum albumin (BSA; Sigma–Aldrich S.r.l., Milano, Italy) plus 1% blotting grade blocker non-fat milk (Bio-Rad Laboratories) and subsequently membranes were washed twice with TBS containing 0.05% Tween 20 (TTBS) before incubation for 1 h at room

temperature with anti-phosphoaminoacid monoclonal antibodies (anti-phosphotyrosine, anti-phosphoserine or anti-phosphothreonine antibody; Calbiochem, Novabiochem Corporation, GmbH) diluted in TBS containing 1% BSA. After being washed six times with TTBS for 3 min, PVDF membranes were incubated at room temperature for 2 h with anti-mouse or anti-rabbit IgG HRP secondary antibodies diluted 1:3000. They were then washed six times with TTBS and twice with PBS for 5 min. In some experiments, the same blot was used for detection of p(Tyr701)STAT1 (Santa Cruz Biotechnology, Inc.), p(Tyr705)STAT3 (Santa Cruz Biotechnology, Inc.), p(Ser727)STAT1 Upstate Biotechnology (Lake Placid, NY), p(Ser727)STAT3 (New England BioLabs), p(Tyr1007/1008)JAK2 Upstate Biotechnology (Lake Placid, NY) and p(TyrTyr1054/1055)Tyk2 (Cell Signaling Technology Inc., Beverly, MA) after stripping of the previous antibody with Restore Western blot stripping buffer (Pierce).

2.6. Cytokine release

All assays were carried out using 3×10^6/ml U937 cells stimulated with different concentrations of stimuli (5 μg/ml of porins or 1 μg/ml of LPS) for 24 h at 37 °C in 5% CO_2; the time points and concentrations have been determined in preliminary experiments as that giving maximum release. After incubation the samples were centrifuged at 1800 rpm at 4 °C for 10 min and the supernatants were collected and stored at −70 °C. IL-6 release was measured by ELISA using pair-matched monoclonal antibodies, according to the manufacturer's recommendations (Roche Diagnostic SpA, Milan, Italy).

2.7. Inhibitors of signal transduction

In some experiments, before stimulation, U937 cells were pretreated with PD-098059 (New England Biolabs, Inc.), a selective inhibitor of MEK1 activator [23]; SB203580 (Calbiochem, Novabiochem Corporation, GmbH), a specific inhibitor of the p38 pathway [24]; AG490 (Calbiochem–Novabiochem Corp., CA), a specific JAK inhibitor [25]; H7, a serine/threonine kinase inhibitor [26]; genistein, a broad-range tyrosine kinase inhibitor [27] for 60 min.

2.8. STATs trans-activation analysis

To detect and quantify STATs activation in U937 cells, we used ELISA-based Trans-Am transcription factor kits (Active Motif, Carlsbad, USA) that exploited a patented technology to attach an oligonucleotide containing the STAT consensus binding site (5′-TTCCCGGAA-3′) to a 96-well plate according to the transcription factors analysed [28]. The active form of STAT contained in cell extract specifically binds to this oligonucleotide. The primary antibodies used to detect STAT recognize an epitope on STAT1, STAT3, STAT5A or 5B that accessible only when the STAT is activated and bound to its target DNA. Preparation of nuclear cell extracts was done according to the manufacturer's instructions. The specificity of the assays was checked by measuring the ability of soluble wild-type or mutated STAT oligonucleotide to inhibit binding. In preliminary assays, the Trans-Am kits showed a good correlation with an EMSA in detecting the DNA binding capacity of STATs. The optimal time of stimulation (1 h) and amount of porins (5 μg/ml) or LPS (1 μg/ml) used in the STAT ELISA were determined in preliminary experiments.

2.9. Endotoxin test

All materials and solutions were tested for LPS by a *Limulus* test as described by Yin et al. [22]. The results of this test was compared with a standard LPS solution which was *Limulus* test positive at 0.1 EU/ml.

2.10. Lactate dehydrogenase (LDH) assay

LDH assays were carried out according to manufacturer's instructions using a cytotoxicity detection kit (Roche Diagnostic SpA, Milan, Italy).

2.11. Reproducibility

Gels were scanned for densitometry analysis by Sigma Gel software (Sigma–Aldrich S.r.l., Milano, Italy). The results shown are from a single experiment typical of at least three giving similar results. The results were expressed as mean values ± standard errors of three independent observations. Comparisons between cytokine test was done by Student's *t*-test, with statistical significance considered to $P \leqslant 0.01$ and $P \leqslant 0.05$ for STATs trans-activation analysis and densitometry analysis of band intensity.

3. Results

3.1. Purity of porin preparations

The purification of the porin preparation and the eventual contamination by LPS have been amply discussed in previous works [29,30]. Using the *Limulus* test, the LPS contamination in the porin preparations, used in this study, was estimated to be <0.005% (w/w) compared with a standard *S. enterica* serovar Typhimurium LPS solution. The concentration of porins used in our experimental procedures, contains a biologically insufficient percentage of LPS (about 50 pg/μg of porins), which did not induce any enzyme phosphorylation or cytokine release.

3.2. Porin and LPS induce release of IL-6 in U937 cells

It has been extensively documented that porins have the ability to stimulate the synthesis and release of pro- and anti-inflammatory cytokines [31].

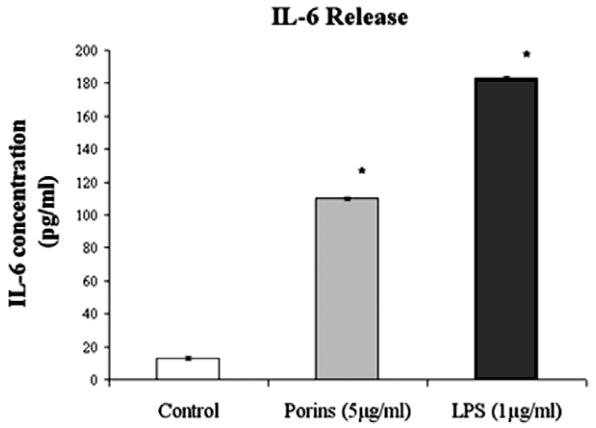

Fig. 1. IL-6 production by U937 cells stimulated with porins or LPS of *S. enterica* serovar Typhimurium. U937 cells (3×10^6/ml) were not stimulated (Control) or stimulated with porins (5 μg/ml) or LPS (1 μg/ml). The IL-6 level was measured by ELISA 24 h after stimulation. Data presented are averages from three independent experiments, and the error bars indicate the standard errors of the means (*$P \leqslant 0.01$; Student's *t*-test).

Salmonella enterica serovar Typhimurium porins (5 μg/ml or 0.2 μM) induced IL-6 production (110 ± 2.6 pg/ml) 24 h after stimulation, representing at least 60% of the production obtained with 1 μg/ml (0.5 μM) of LPS (Fig. 1). Time points chosen for IL-6 production were previously optimized (data not shown). Porin-mediated IL-6 induction by U937 cells is not affected by adding the specific LPS-inhibiting oligopeptide Polymyxin B (PMB) (Sigma–Aldrich S.r.l., Milano, Italy) (10 μg/ml) [32] to porins prior to stimulation, while LPS-mediated stimulation was abolished in the presence of PMB. This confirms that IL-6 induction by porins in U937 cells is not due to contamination by LPS (data not shown).

3.3. JAK/STAT phosphorylation by porins

In order to examine the role of JAK/STAT signaling pathways for IL-6 release induced by porins in U937 cells, we performed Western blot analyses of JAK/STAT activation. U937 cells were treated with either porins (5 μg/ml) or LPS (1 μg/ml). The time points and concentrations chosen for phosphoaminoacid analysis of JAKs and STATs were previously optimized (data not shown). The extracts were examined for the presence of tyrosine phosphorylated JAKs, and threonine, serine or tyrosine phosphorylated STATs by Western blot analysis.

LPS stimulation induced rapid tyrosine phosphorylation (1 min, optimal stimulation time) of JAK2 (Tyr1007/1008) and Tyk2 (Tyr1054/1055) corresponding to about 80% and 50% of signaling induced by interferon (IFN)-γ (100 U/ml) (Roche Diagnostic SpA, Milan, Italy) used as positive control of JAK/STAT activation [12]. On the other hand LPS stimulation resulted in no detectable tyrosine phosphorylation of JAK1 or JAK3 (Fig. 2).

Following 1 min stimulation (optimal stimulation time) with *S. enterica* serovar Typhimuirum porins, we could not observe any significant immunoprecipitation band using anti-JAK1, anti-JAK2, anti-JAK3 or anti-Tyk2 antibodies for tyrosine phosphorylation analysis (Fig. 2).

Western blotting analysis of protein extracts derived from U937 cells treated for 15 min with either porins (5 μg/ml) or LPS (1 μg/ml) revealed several STAT phosphorylated proteins (Fig. 3). The time points and concentrations chosen were previously optimised. The lysates obtained were immunoprecipitated by antibodies that specifically recognised STAT1, STAT2, STAT3, STAT4, STAT5 or STAT6 enzymes. Immunoblot analyses were performed using anti-phosphothreonine, anti-phosphoserine or anti-phosphotyrosine antibodies. After stimulation with porins or LPS, tyrosine phosphorylation levels of STAT1 (Tyr701) or STAT3 (Tyr705) were comparable to levels obtained using IFN-γ (Fig. 3a). Serine phosphorylation analysis of STATs showed immunoprecipitation bands with anti-STAT1 and -STAT3 antibodies (Fig. 3b). The levels of STAT1 (Ser727) serine phosphorylation were comparable after stimulation with porins, LPS or IFN-γ. The immunoprecipitation with anti-STAT3 followed by anti-phosphoserine immunoblotting demonstrated a marked increased serine phosphorylation (about 70% respect to IFN-γ) after LPS stimulation. Porin stimulation induced an evident serine phosphorylation of STAT3 (Ser727) (about 56% respect to IFN-γ).

No phosphorylation of STAT threonine residues was detected after porins or LPS stimulation (data not shown).

When the immunoprecipitating antibodies used were specific for STAT2, 4, 5 or 6 no bands were observed for analysis of threonine, serine or tyrosine residue phosphorylation after porin or LPS stimulation (data not shown).

The specificity of the effect was demonstrated using a different protein (BSA) as a stimulus; no phosphorylation of the proteins investigated in our study was observed. The possibility that traces of LPS contaminating the preparation of porins could be responsible for the observed effect can be excluded. The minimum concentration of LPS able to induce phosphorylation of STAT1 and STAT3 in our *in vitro* model is 100 ng/ml, which is much more than the quantity of LPS present in our preparation (about 50 pg/μg of porins) (data not shown).

3.4. Effect of inhibitors on STAT1 and STAT3 tyrosine/serine phosphorylation in U937 cells stimulated by porins

Since JAKs are not involved in phosphorylation induced by porin, to further examine the signaling pathways involved in STAT1 and STAT3 tyrosine and serine phosphorylation by porins, we investigated if other signaling pathways are involved in both STAT1 and STAT3 phosphorylation in response to a porin stimulus. We tested the effect of different inhibitors: PD-098059 (inhibitor of MEK1 activator and MAPK pathway), SB203580 (inhibitor of p38 pathway), AG490 (JAK inhibitor), H7 (serine/threonine kinase inhibitor) (Fig. 4).

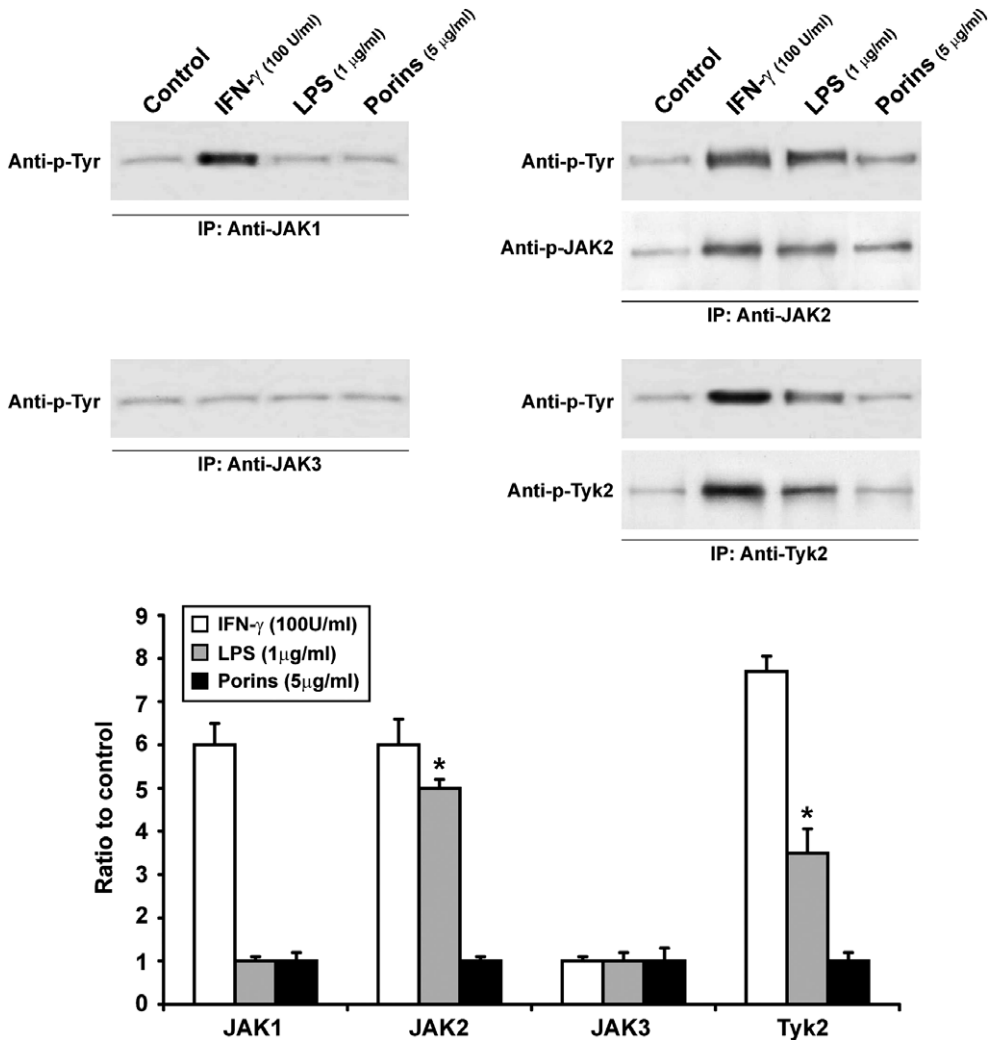

Fig. 2. Western blot analysis of JAKs activation. U937 cells (3×10^6/ml) were stimulated with either porins, LPS or IFN-γ (positive control) for 1 min. The immunoprecipitated proteins for JAK1, JAK2, JAK3 and Tyk2 were separated by electrophoresis, transferred to PVDF membrane and probed with Abs specific for anti-phosphotyrosine. In some experiments, the same blot was used for detection of p(Tyr1007/1008)JAK2 and p(Tyr1054/1055)Tyk2 after stripping of the previous antibody. Gels were scanned for densitometry analysis with the Sigma Gel Software, and the ratio of the value for each stimulation time to the value for an unstimulated control is shown. The data are averages from three different experiments, and the error bars indicate the standard errors of the means. Band intensity was quantified densitometrically. *$P \leqslant 0.05$ indicates statistically significant difference compared to IFN-γ stimulated cells.

Cells were pretreated for 60 min with PD-098059 (25 μM) and then stimulated with either porins or LPS. From cell lysates a decrease in intensity of the immunoprecipitation bands with anti-STAT1 or anti-STAT3 antibodies for tyrosine phosphorylation analysis was observed. Using porins the decrease in intensity of the immunoprecipitation bands with anti-STAT1 or anti-STAT3 antibodies for tyrosine phosphorylation (Tyr701 and Tyr705, respectively) analysis was about 35% (Fig. 4a) and using LPS it was about 40% (Fig. 4b).

Pretreatment with SB203580 (10 μM for 60 min) prior to stimulation with porins or LPS resulted in a considerable decrease in intensity of STAT1 and STAT3 immunoprecipitation bands for tyrosine phosphorylation analysis. The intensity of the bands was reduced of about 50% with either porins or LPS.

When using AG490, at a concentration of 10 μM for 60 min, we could not observe any change in the immunoprecipitated bands for tyrosine phosphorylated of STAT1 or STAT3 after stimulation with porins (Fig. 4a), while LPS reduced activation of about 20% (Fig. 4b). The cell pretreatment with H7 (25 μM for 60 min) modulated both porin or LPS-induced STAT1 and STAT3 tyrosine phosphorylation. For maximal transcriptional activity, STAT1 and STAT3 need to be phosphorylated on both tyrosine and serine residues [33]. To verify STAT1 and STAT3 serine phosphorylation, U937 cells were pretreated with PD-098059, SB203580 or H7 prior to the addition of porins or LPS. The results, presented in Fig. 5, indicate that PD-098059, reduced the phosphorylation status of STAT1 or STAT3 serine (about 30% for porins and 35% for LPS) as well as the pretreatment with SB203580 (about 50% for

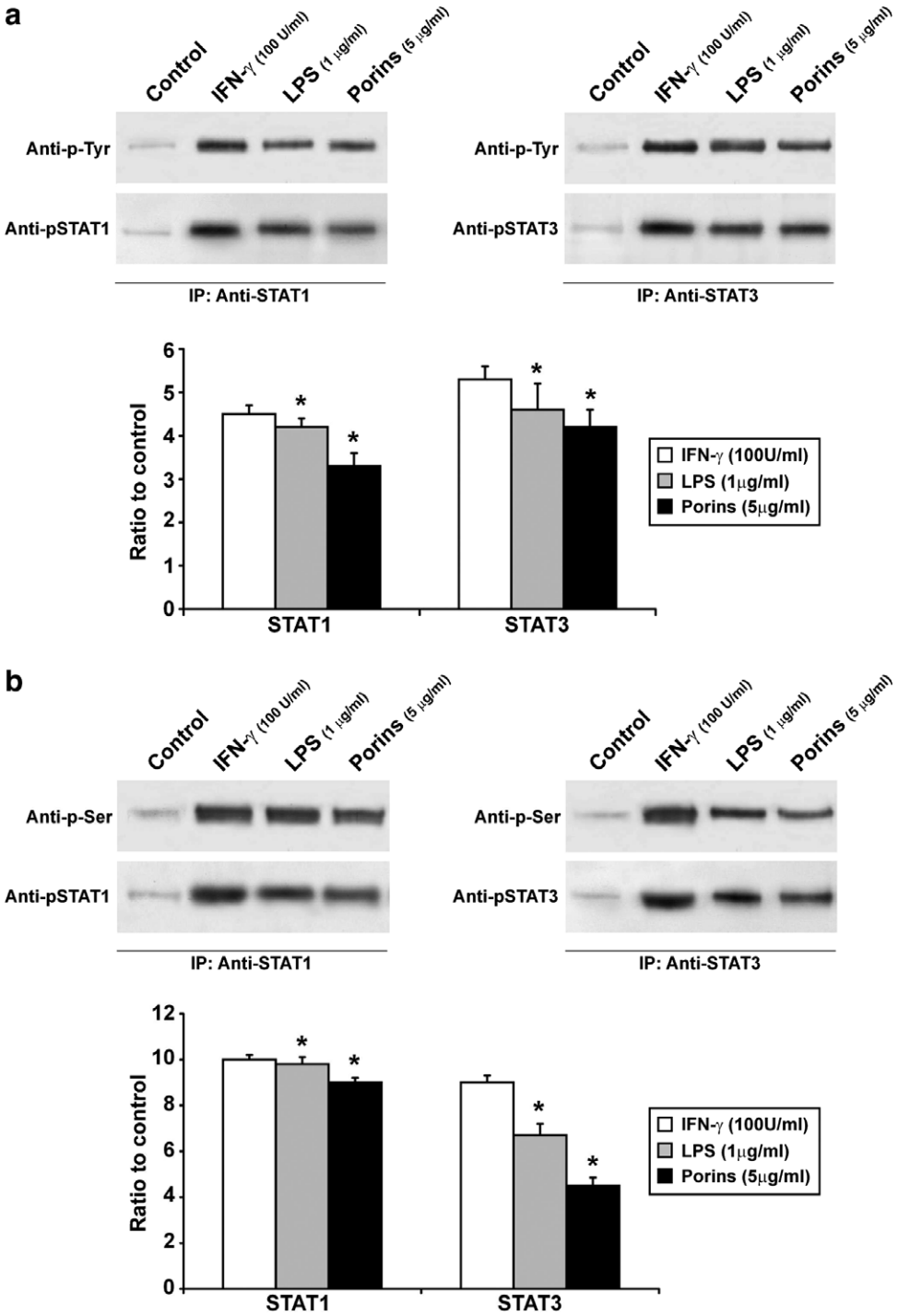

Fig. 3. Western blot analysis of STAT1 and STAT3 activation. U937 cells (3×10^6/ml) were stimulated with either porins, LPS or IFN-γ (positive control) for 15 min. The immunoprecipitated proteins for STAT1 and STAT3 were separated by electrophoresis, transferred to PVDF membrane and probed with Abs specific for phosphotyrosine (a) and phosphoserine (b). In some experiments, the same blot was used for detection of phosphorylated forms of STAT1(Tyr701), STAT3(Tyr705), STAT1(Ser727) and STAT3(Ser727) after stripping of the previous antibody. Gels were scanned for densitometry analysis with the Sigma Gel Software, and the ratio of the value for each stimulation time to the value for an unstimulated control is shown. The data are averages from three different experiments, and the error bars indicate the standard errors of the means. Band intensity was quantified densitometrically. *$P \leqslant 0.05$ indicates statistically significant difference compared to IFN-γ stimulated cells.

porins and 42% for LPS); the inhibitor H7 completely abrogated serine phosphorylation of STAT1 and STAT3 induced by porins or LPS stimulation.

These data suggest that activation of MAPK by porins or LPS is required for modulation of maximal STAT1 and STAT3 activation.

3.5. Porin-induced nuclear translocation of STAT1 and STAT3

Experiments investigate the dimerization by porin or LPS-activated STAT1 and STAT3 and the concomitant nuclear translocation of dimers in U937 cells were carried out. The results obtained showed that increased specific binding of active STAT1 or STAT3 (Fig. 6) were detected in U937 lysates 1 h after stimulation with porins (5 μg/ml) or LPS (1 μg/ml).

Further experiments were performed to ascertain whether MAPKs had a role in the binding of STAT1 and STAT3 to DNA by porins. Specifically, we looked at the effects of the PD-098059, SB203580, AG490 and H7 inhibitors on binding of STAT1 and STAT3 to a consensus binding site. PD-098059 pretreatment was effective in decreased STAT1 or STAT3 porin or LPS-mediated nuclear translocation (about 37% for porins and about 45% for LPS). The pretreatment with the p38 inhibitor SB203580 decreased porin or LPS stimulated STAT1 or STAT3 binding to DNA (50%), suggesting that the functionality of these proteins requires the activation of p38 pathway. Furthermore, the results presented in Fig. 6, indicate that AG490, had a decreasing effect on STAT1 and STAT3 DNA binding induced by LPS (22%). On the contrary, no effect was evidenced on STAT1 or STAT3 nuclear translocation following porin stimulation.

We finally investigated the effect of H7 on the induction of porin- or LPS-mediated nuclear translocation of STAT1 or STAT3. Treatment of U937 cells with H7 partially suppressed porin or LPS induction of STAT1 binding to a consensus binding site (30%) (Fig. 6). Moreover, H7 pretreatment caused an evident inhibition of STAT3 LPS-mediated DNA binding (70%), while modulated the porin effect on STAT3 nuclear translocation (Fig. 6).

3.6. MAPK and not JAKs are involved in IL-6 production in U937 cells stimulated by porins

To further examine the signaling pathways involved in IL-6 release by porins and LPS, U937 cells were incubated with specific inhibitors of ERK1/ERK2 (PD-098059, 25 μM for 60 min), p38 MAPKs (SB203580, 10μM for 60 min), JAKs (AG490, 10 μM for 60 min), a serine/threonine kinase inhibitor (H7, 25 μM for 60 min) or a total PTKs inhibitor (genistein, 50 μM for 60 min) before being

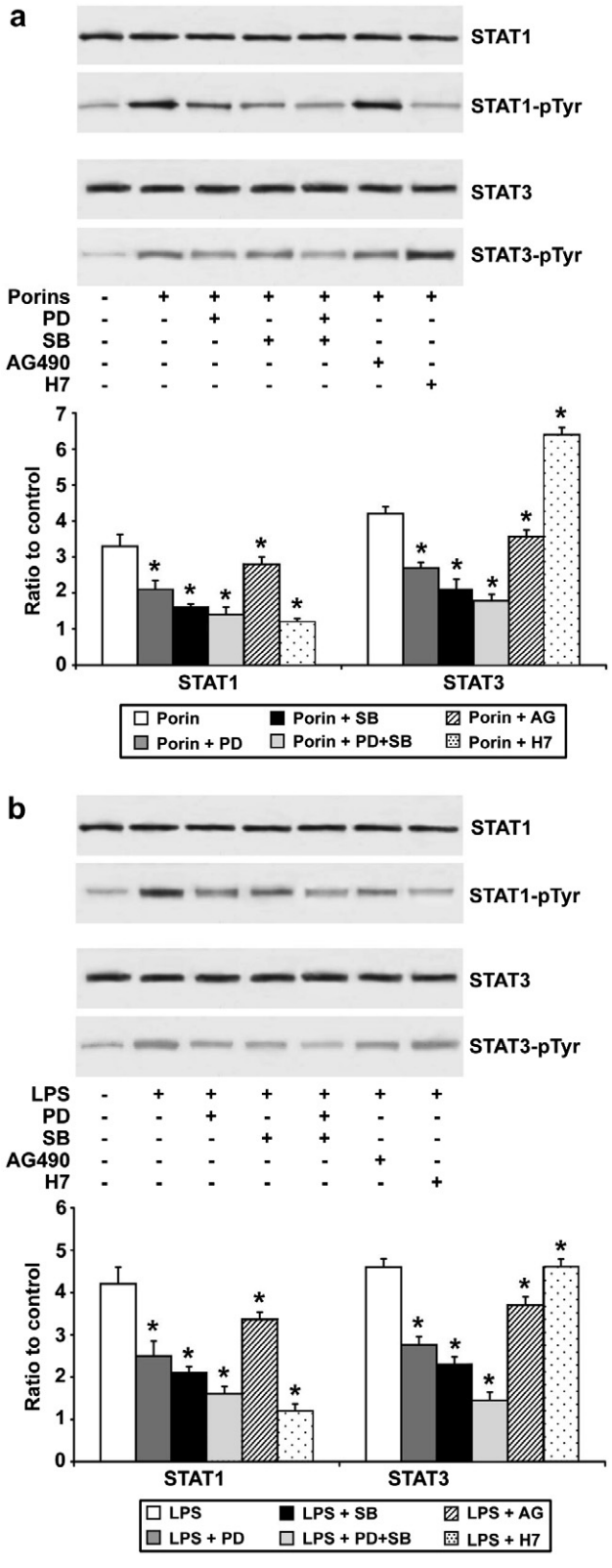

Fig. 4. Effect of PD-098059, SB203580, AG490 and H7 inhibitors on STAT1 and STAT3 tyrosine phosphorylation in response to *S. enterica* serovar Typhimurium porins or LPS. U937 cells (3×10^6/ml) were incubated with PD-098059 (25 μM for 60 min, MEK1 inhibitor) (PD), SB203580 (10 μM for 60 min, p38 inhibitor) (SB), AG490 (10 μM for 60 min, JAK/STAT pathway inhibitor) (AG) or H7 (a serine/threonine kinase inhibitor, 25 μM for 60 min) and stimulated with porins (a) or LPS (b) for 15 min. Western blot analysis on total cell extracts was performed using anti-phosphotyrosine antibodies after immunoprecipitation with specific antibody anti-STAT1 or anti-STAT3. Western blot signals were quantitated by Sigma Gel Software and reported as the ratio of the value for each stimulation time to the value for an non-stimulated control. The results shown are the average of three independent experiments, and the error bars indicate the standard error of the means. Band intensity was quantified densitometrically. $*P \leqslant 0.05$ indicates statistically significant difference compared with those from cells receiving stimuli alone.

challenged with either porins or LPS. The optimal concentrations of inhibitors were calculated according to 50% inhibitory concentration (IC_{50}) values and specificities reported in literature [23–27]. As shown in Fig. 7, the pretreatment with PD-098059 or SB203580 prior to stimulation with either porins or LPS induced a modest effect on IL-6 release. The pretreatment with both inhibitors simultaneously caused a clear decrease in IL-6 release after 24 h. The inhibitor AG490 modified slightly IL-6 release, confirming that porins did not activate the JAKs pathway unlike LPS. The serine/threonine kinase inhibitor (H7) or the broad-range tyrosine kinase inhibitor (genistein) reduced markedly IL-6 release suggesting that tyrosine phosphorylation of STATs in this system involves a tyrosine kinase other than JAK family members [34], and that the action of the serine/threonine kinase inhibitor is directed to a kinase required for incrementing activation of other tyrosine kinases.

Moreover, treatments with these concentrations of inhibitors did not significantly affect the basal level of IL-6 secretion, strongly indicating that pharmacological results in this study were not contaminated by non-specific cell damage, as confirmed by LDH assay (data not shown).

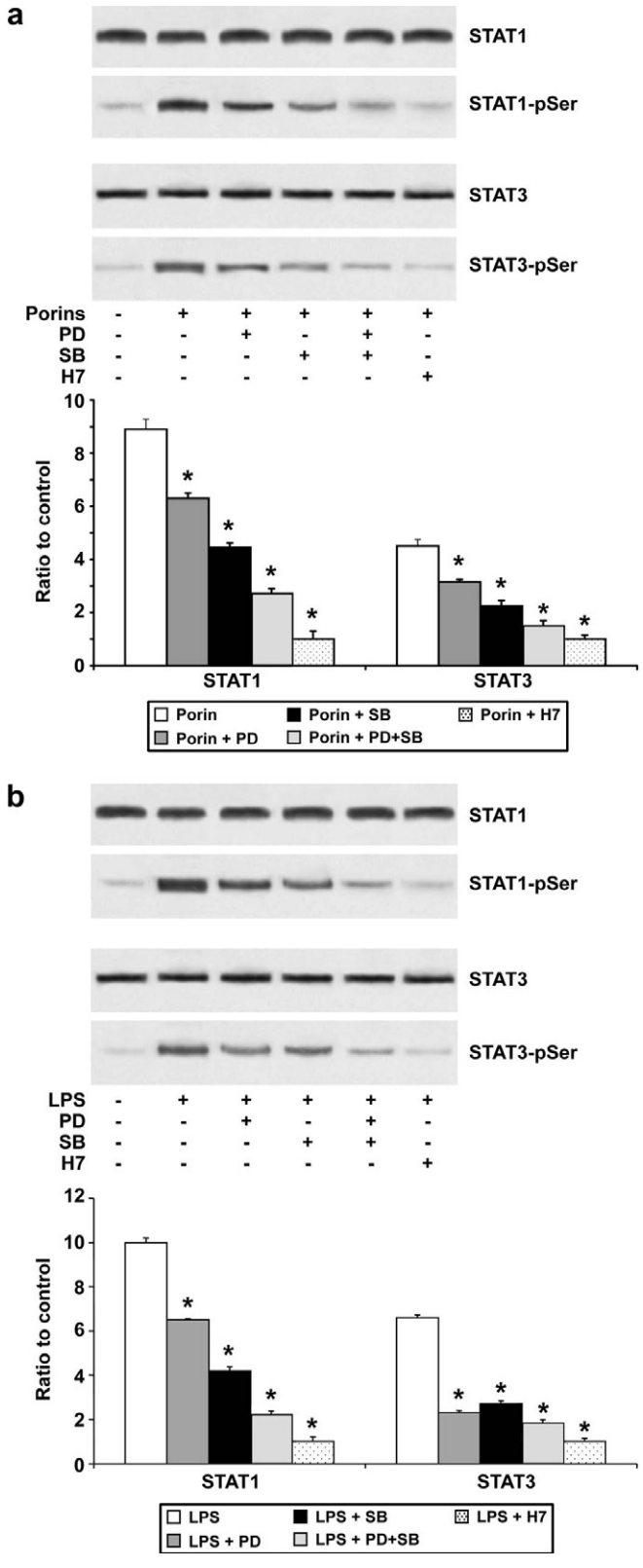

Fig. 5. Effect of PD-098059, SB203580 and H7 inhibitors on STAT1 and STAT3 serine phosphorylation in response to *S. enterica* serovar Typhimurium porins or LPS. U937 cells (3×10^6/ml) were incubated with PD-098059 (25 μM for 60 min, MEK1 inhibitor) (PD), SB203580 (10 μM for 60 min, p38 inhibitor) (SB) or H7 (a serine/threonine kinase inhibitor, 25 μM for 60 min) and stimulated with porins (a) or LPS (b) for 15 min. Western blot analysis on total cell extracts was performed using anti-phosphoserine antibodies after immunoprecipitation with specific antibody anti-STAT1 or anti-STAT3. Western blot signals were quantitated by Sigma Gel Software and reported as the ratio of the value for each stimulation time to the value for an non-stimulated control. The results shown are the average of three independent experiments, and the error bars indicate the standard error of the means. Band intensity was quantified densitometrically. $*P \leqslant 0.05$ indicates statistically significant difference compared with those from cells receiving stimuli alone.

4. Discussion

Many different models of sepsis have been used to investigate the role of STATs and JAKs in response to infection. The results obtained using these models of infection have all contributed to the current knowledge on the mechanism and function of JAK/STAT signaling in sepsis [11]. LPS and porins of Gram-negative bacteria are key factors implicated in septic shock [31]. Our results from *in vitro* experiments on U937 cells demonstrate a complex indirect mechanism of STAT1 and STAT3 activation after stimulation of cells with either porins or LPS. The treatment with porins did not results in increase of JAK phosphorylation although a significant increase in precipitation bands for phosphotyrosine STAT1 and STAT3 was observed. Using several inhibitors, we demonstrated that the activation of STAT1 and STAT3 by porins can occur through the activation of MAPK and possibly other PTKs [34] but not through JAK activation. In our experiments, both the ERKs and p38 MAPK are involved in the activation of STAT1 and STAT3 by porins or LPS, while STAT2, STAT4, STAT5 and STAT6 did not demonstrate any activation under our experimental conditions. The amount of

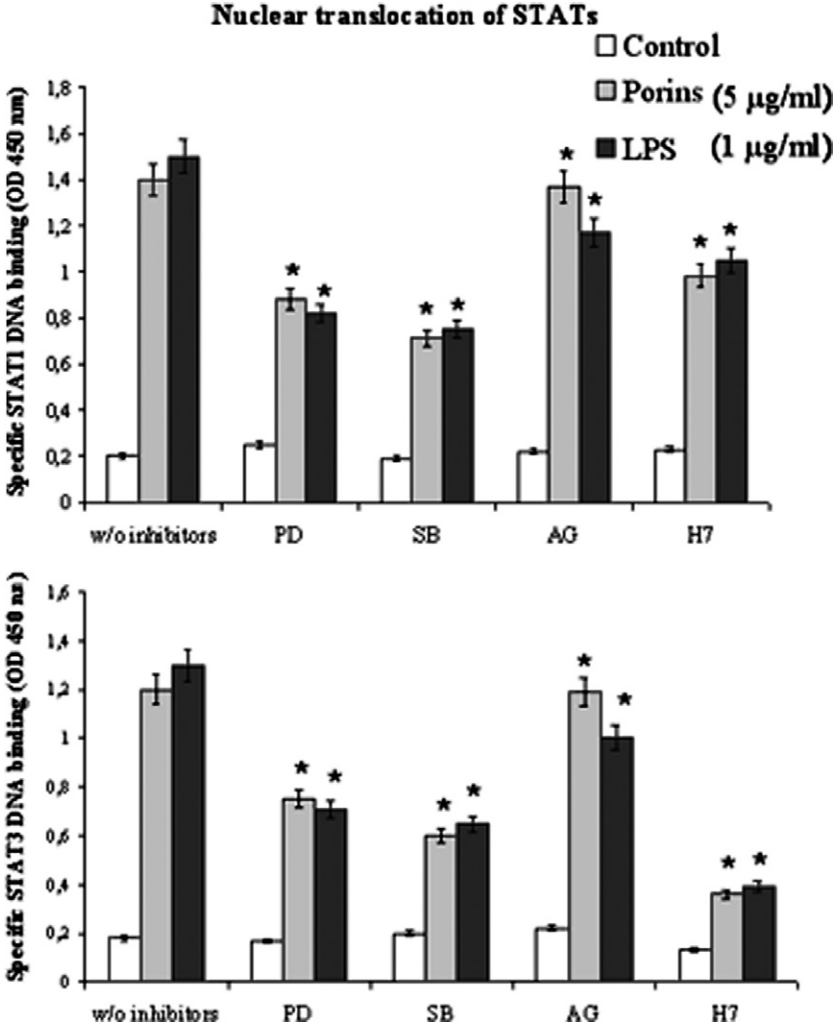

Fig. 6. Analysis of the effects of different inhibitors on binding of STAT1 and STAT3 to an STATs-binding consensus sequence. U937 cells were stimulated with *S. enterica* serovar Typhimurium porins or LPS in the absence or presence of an ERK 1/2 inhibitor (PD-098059, 100 μM) (PD), or a p38 inhibitor (SB203580, 10 μM) (SB), or a JAK/STAT pathway inhibitor (AG490, 10 μM for 60 min) (AG) or a serine/threonine kinase inhibitor (H7, 25 μM for 60 min). Cell lysates (10 μg/ml) were tested for binding of the activated STAT1 or STAT3 to an STATs consensus sequence using the Trans-Am STATs family ELISA kit. The experiment was performed in the presence of soluble wild-type or mutated consensus oligonucleotides. The results are expressed as specific binding (absorbance measured in the presence of the mutated oligonucleotide minus that measured in the presence of the wild-type oligonucleotide). The results are shown as means ± standard errors of triplicate determinations (*$P \leqslant 0.05$; Student's *t*-test).

phosphorylation of STAT1 and STAT3 induced by porins or LPS resulted in only a minor difference. Whereas LPS appeared to be superior to porins with respect to induction of STAT1/STAT3 activation by ERK1/ERK2 mediation, porins induced significantly more phosphorylation of STAT1/STAT3 and IL-6 release through p38. Moreover, the pretreatment with H7 or genistein decreased IL-6 secretion mediated by porins or LPS, indicating that both tyrosine kinases and serine/threonine kinases are essential for IL-6 production. Although porins and LPS signal through different surface receptors [35,36], our data show that there are also differences between the tyrosine kinase signaling pathways by which porins and LPS are able to activate cells. Our results suggest that JAK2 and Tyk2 phosphorylation is only required for IL6 production LPS-induced, but they are not required following porin-induction. This lends weight to the hypothesis of a different cellular mechanism exerted by porins in contrast to LPS, as also described in previous works [9,30,35,36].

Previous studies have demonstrated that bacterial infection of macrophages, or treatment with LPS resulted in rapid STAT1 phosphorylation in Serine727 independently of concomitant tyrosine phosphorylation of STAT1 in Tyr701 [10]. Serine and tyrosine phosphorylation of STAT1 occur independently of each other, but the serine kinase may recognize tyrosine-phosphorylated STAT1 preferentially in the course of an IFN-γ response [37]. Maximal activation of transcription by STAT1 and STAT3 require both tyrosine and serine phosphorylation [33]. For STAT dimer formation and DNA binding is necessary

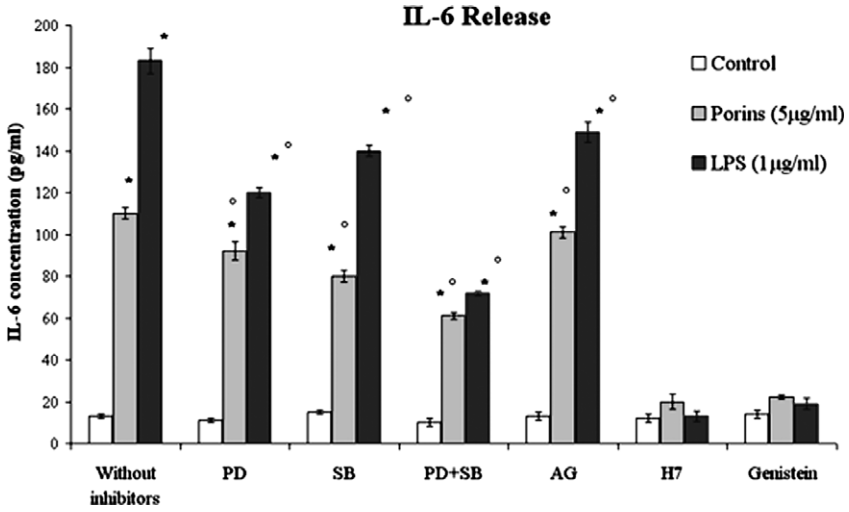

Fig. 7. IL-6 secretion induced by *S. enterica* serovar Typhimurium porin or LPS stimulation. U937 cells were pretreated with PD-098059 (25 μM for 60 min, MEK1 inhibitor) (PD), SB203580 (10 μM for 60 min, p38 inhibitor) (SB), AG490 (10 μM for 60 min, JAK/STAT pathway inhibitor) (AG), H7 (a serine/threonine kinase inhibitor, 25 μM for 60 min), genistein (a total PTKs inhibitor, 50 μM for 60 min). IL-6 level was measured by capture ELISA (see Section 2). The results are the mean of three different experiments and error bars indicate the standard errors of the mean. Symbol denoting significance: $P \leqslant 0.01$ versus unstimulated (∗) or inhibitor pretreated cells (°) (Student's *t*-test).

tyrosine phosphorylation [28]. Cytokine- and growth factor-induced STAT activation is mainly dependent of JAKs phosphorylation, while porins do not use these tyrosine kinases. Our results show that STAT1 and STAT3, after porin stimulation, are phosphorylated at tyrosine residues (Tyr701 and Tyr705, respectively) probably through other PTKs [34]; in fact treating cells with genistein, a wide spectrum inhibitor of tyrosine kinases, IL-6 release becomes strongly reduced. Early post-receptor events in porins and LPS induced signal transduction involve tyrosine phosphorylation [7,38], but it is unclear which tyrosine kinases are critically involved in the down-stream signal transduction pathways. In addition to the tyrosine kinases, a number of serine kinases was previously shown to be activated by porins and LPS, including c-Raf, the MAPKs ERK1 and ERK2, the p38 kinase, c-Jun kinase [9]. Our experiments also show serine (Ser727) phosphorylation of STAT1 and STAT3. Moreover, MAPKs inhibitors reduce, with a different extent, IL-6 release, pointing out a key role of this pathway in phosphorylation of Ser727 in STAT1 and STAT3.

These results are confirmed by nuclear translocation of STAT1 and in porin or LPS-stimulated U937 cells. Several published data show that MAPKs have specific roles in phosphorylation of Ser727 of STATs [39]. Porins and LPS while not directly influencing the out-put of the JAK/STAT signal transduction pathway, are able to play an important role in the activation of macrophages as a result of the stimulation with early cytokines release and a previous stimulus such as a microbial product (e.g. porins, LPS). Porins, as also LPS, activates also others transcriptional factors such as AP-1 and NF-κB [9], that plays a central role in the transcriptional activation of numerous genes encoding immunologically and pro-inflammatory important cytokines or other mediators. In our *in vitro* experiments we have not found evidence of phosphorylation of STATs 2, 4, 5 or 6, although they are dramatically modified in infection and especially during sepsis [11]. The *in vivo* conditions which occur during sepsis can probably not be reproduced *in vitro*, where we are able to separate an immune response following a porin stimulation from the whole response which is also influenced by the released cytokines. In conclusion the results obtained in this work demonstrate that signaling in cellular activation can be directly influenced by bacterial components and that also early cytokines, released by the effect of the same bacterial components, can interfere with signaling pathways.

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