

## RESEARCH ARTICLE

## Evidence for IL-6 promoter nuclear activation in U937 cells stimulated with *Salmonella enterica* serovar Typhimurium porins

Emiliana Finamore<sup>1\*</sup>, Mariateresa Vitiello<sup>1\*</sup>, Marina D'Isanto<sup>1</sup>, Emilia Galdiero<sup>2</sup>, Annarita Falanga<sup>3</sup>, Aikaterini Kampanaraki<sup>1</sup>, Katia Raieta<sup>1</sup>, Marilena Galdiero<sup>1</sup>

<sup>1</sup> Department of Experimental Medicine, Section of Microbiology and Clinical Microbiology, Second University of Naples, Naples, Italy

<sup>2</sup> Department of General Physiology, Faculty of Science MM.FF. NN., Hygiene and Microbiology Section, University of Naples "Federico II", Naples, Italy

<sup>3</sup> Department of Biological Science, Division of Biostructures, University of Naples "Federico II", Naples, Italy

**Correspondence:** Prof. M. Galdiero, Department of Experimental Medicine, Section of Microbiology and Clinical Microbiology, Second University of Naples, Via De Crecchio, 7 - 80138, Naples, Italy  
<marilena.galdiero@unina2.it>

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**ABSTRACT.** Interleukin-6 (IL-6) is a pleiotropic cytokine and plays an active role in inflammatory and immune responses, contributing to a multitude of physiological and pathophysiological processes. In this study, we address the molecular mechanism of IL-6 transcriptional induction and propose a correlation between activated NF- $\kappa$ B localization and IL-6 expression. In particular, we detected, by ChIP assay, that occupation of the IL-6 gene promoter site is dependent on activated NF- $\kappa$ B. In fact, after porin stimulation, the NF- $\kappa$ B p65 subunit is activated, translocates to the nucleus and binds to the IL-6 promoter sequence. Elucidation of the host signaling pathways and identification of the transcription factors that contribute to IL-6 expression, may aid in the understanding of host susceptibility to gram-negative infections and in identifying new therapeutic strategies in a variety of infectious diseases.

**Keywords:** porins, IL-6, NF- $\kappa$ B, nuclear activation

During gram-negative infections, lipopolysaccharide (LPS) and several outer membrane proteins (OMPs), among these porins [1], carry out a fundamental role, and are the main initiators of inflammatory reactions that may lead to circulatory failure and organ injury. Several data regarding the structure, function and expression of bacterial OMPs have accumulated over the last three decades, and indicate that these proteins represent important factors in bacterial virulence, and play an essential role in the pathobiology of gram-negative bacterial pathogens in comparison to other known determinants of pathogenesis such as flagellin [2]. It is well established that signaling and transcriptional activities for proinflammatory cytokine synthesis are important processes induced by LPS and porins [1]. In particular, under various circumstances, a predominance of interleukin-6 (IL-6) release may induce systemic inflammation, sepsis and damage to a variety of vital organs. As reported by several authors, the plasma level of IL-6 during the first 6h of sepsis could be used as a predictor of early death [3, 4].

The specific cellular porin receptors remain unknown, but previous studies have reported that the TLR2/

MyD88 pathway plays an essential role in Hib porin-mediated cytokine production [5]. The members of the Toll-like receptor (TLRs) family recognize specific molecular patterns present on microbial pathogens, triggering inflammatory responses and activating several transcription factors such as nuclear factor (NF)- $\kappa$ B, activating protein-1 and interferon regulatory factor that induce specific immune responses. For example, TLRs activate NF- $\kappa$ B and lead to the induction of the IL-6 gene [6]. The genes for human and mouse IL-6 have been cloned and sequenced [7], and Dendorfer *et al.* [8] have shown that multiple mechanisms underlie IL-6 gene regulation and expression. Regulation of the expression of the IL-6 gene is adapted to the pivotal role of this cytokine, namely as a systemic alarm signal that recruits diverse host defence mechanisms that serve to limit tissue injury. Inflammatory cytokines, such as tumor necrosis factor (TNF), IL-1, and bacterial products such as LPS, enhance IL-6 gene expression [9]. Several bacterial components modulate many of the pathways involved in the host inflammatory response and in innate and adaptive immune functions.

It has been shown that pore-forming bacterial toxins can promote inflammatory processes in the host organism

\* These authors contributed equally to this work.

through the cell surface shedding of TNF- $\alpha$  and interleukin-6 receptor (IL-6R) [10]. Previous data demonstrated that 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 [11]. Porins activate the Raf-1-MEK1/2 MAPK pathway [12]. We have also reported that porins induce significant phosphorylation of STAT1/STAT3, and IL-6 production through MAPK, but not through JAK activation [13]. Early, post-receptor events in porin- and LPS-induced signal transduction involve several phosphorylation pathways, but it remains unclear, especially as regards IL-6 release, which of these are the key events. In view of its pleiotropic nature, studies on the regulation of IL-6 gene expression may be of paramount importance. The characterization of the human IL-6 promoter revealed a highly conserved control region of 300 bp upstream of the transcriptional initiation site that contains the elements necessary for its induction by a variety of stimuli commonly associated with acute inflammatory or proliferative states. In particular, electrophoretic mobility shift assays, as well as promoter deletion and point mutation analysis, revealed the presence of an NF- $\kappa$ B-binding element. NF- $\kappa$ B plays a central role in the transcriptional activation of numerous genes encoding for immunological and pro-inflammatory cytokines. NF- $\kappa$ B is present in the cytoplasm of resting cells, bound to its inhibitor I $\kappa$ B. Only as a consequence of cellular activation and proteolytic degradation of I $\kappa$ B, is NF- $\kappa$ B released and transduced to the nucleus, where it transactivates several genes [14]. We have already reported that porins are able to activate the transcriptional factors AP-1 and NF- $\kappa$ B [12]. In the present work, we have shown that *Salmonella enterica* serovar Typhimurium porins enhance the synthesis and release of IL-6 in U937 cells, regulating the transcriptional activity of the IL-6 gene by nuclear transduction of NF- $\kappa$ B.

## METHODS AND MATERIALS

### Cell line

U937 monocytes (ATCC CRL-1593.2) were grown at 37°C in 5% CO<sub>2</sub> in RPMI 1640 with HEPES supplemented with 10%, heat-inactivated FCS, glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 U/mL), and differentiated as previously described [13]. Before experimentation, U937 cells were cultured in serum-free medium, for 24 h at 37°C to prevent any interference with nuclear activation, by serum factors.

### Preparation of porins and LPS

Porins were isolated from the lysozyme-EDTA-extracted envelopes of *S. enterica* serovar Typhimurium strain SH5014 as previously reported [12]. The protein content of the porin preparation was determined by the method of Lowry *et al.* [15], and checked by SDS-PAGE according to Laemmli [16]. 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 [12]. All possible traces of LPS were revealed on SDS-PAGE stained with silver nitrate as described by Tsai and Frasch [17] and by the *Limulus*-amoebocyte-lysate assay (*Limulus* test, pbi international, Milan, Italy), according to Yin *et al.* [18]. Purity of the porin and LPS preparations from *S. enterica* serovar Typhimurium was reported in a previous paper [12]. In some experiments, Polymyxin-B (PB) was incubated with porins to neutralize the biological activity of any possible traces of LPS. The porins were incubated with PB at room temperature for 1 h at a ratio of 1:100 [19]. LPS, porins and the PB mixture were used in pyrogen-free, phosphate-buffered saline (PBS).

### Cytokine release

For the cytokine release assay, U937 cells ( $3 \times 10^6$ /mL) were washed, resuspended in complete medium and stimulated with porins (1  $\mu$ g/mL) or LPS (1  $\mu$ g/mL) for 18 h at 37°C in 5% CO<sub>2</sub>; the optimal time for stimulation and the concentration of stimuli were determined in preliminary experiments in order to induce maximum release (data not shown). After incubation, the samples were centrifuged at 1 800 rpm, at 4°C, for 10 min; 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).

### RNA preparation

Total RNA was extracted in RLT RNA extraction buffer (Rneasy kit; Qiagen), and treated with DNase I. The integrity of the RNA was assessed by gel and RT-PCR, and the concentration was measured by absorbance at 260 nm. Reference RNA was prepared from different U937 cell line. All assays were carried out using U937 cells ( $1 \times 10^7$ /mL) stimulated with the appropriate porin concentrations (0.1-5  $\mu$ g/mL) for between 2 h to 8 h.

### Northern blot

Total RNA was electrophoresed in 1.2% agarose gel, in a formaldehyde running buffer system. Northern transfer onto Genescreen plus Nylon membrane was conducted by passive blotting. Prehybridization and hybridization were conducted in Hybrisol II. The IL-6 probe (0.6 Kbp *Xba*I-*Eco*RI fragment of the plasmid pBSF 2.5 carrying the cDNA of the IL-6 gene) was added at a concentration of  $12 \times 10^6$  cpm/mL hybridization mix and incubated with the membrane at 65°C for 2 h. The membrane was washed twice in 2 x sodium chloride 0.3 M, sodium citrate 0.3 M, pH7 buffer (SSC) at 65°C for 15 min. Autoradiography was performed at - 80°C using Kodak X-OMAT film. We accounted for possible differences in band intensities due to differences in RNA loading, by using the relative intensity of the 18S ribosomal band in each sample to normalize the respective probe value.  $\beta$ -actin was used as the internal control and as the house-keeping control gene.

### ***Inhibitors***

In a selected set of experiments, U937 cells were pre-treated, for 1 h, with caffeic acid phenethyl ester (CAPE) (10  $\mu$ M) (Calbiochem-Novabiochem Corp., CA, USA) a non-specific inhibitor of the NF- $\kappa$ B pathway [20], and 7-methoxy-5,11,12-trihydroxy-coumestan (IKK Inhibitor II) (10  $\mu$ M) (Calbiochem-Novabiochem Corp., CA, USA), a selective and irreversible inhibitor of IKK- $\alpha$  and  $\beta$  kinase activity [21]. The optimal concentrations of inhibitors were calculated according to 50% inhibitory concentration (IC<sub>50</sub>) values and specificities reported in the literature [20, 21].

### ***NF- $\kappa$ B activation analysis***

To detect and quantify NF- $\kappa$ B activation in U937 cells, we used ELISA-based Trans-Am transcription factor kits (Active Motif, Carlsbad, CA, USA), according to the manufacturer's recommendations. One microgram of protein was added to the wells of a 96-well plate coated with immobilized oligonucleotide containing the NF- $\kappa$ B consensus site (5'-GGGACTTTC-3') according to the transcription factors analysed [22]. After a 1 h incubation period at room temperature the wells were washed three times with the washing buffer included in the kit; 100  $\mu$ L of the anti-p65 and anti-p50 antibodies provided were added at a 1:1000 dilution. The plate was incubated at room temperature for 1 h and the wells were washed three times. HRP-conjugated anti-rabbit IgG was added at a 1:1000 dilution and incubated for 1 h at room temperature. Cells were washed four times, and developing solution was added, followed by the stop solution. The level of p65 and p50 activation was measured at 405 nm in an HTS 700 BioAssay reader (Perkin Elmer, Norwalk, CT, USA).

The specificity of the assays was checked by measuring the ability of soluble wild-type or mutated NF- $\kappa$ B oligonucleotides to inhibit binding. In preliminary assays, the Trans-Am kits showed a good correlation with an EMSA in detecting the DNA-binding capacity of NF- $\kappa$ B.

The optimal time of stimulation (1 h) and amount of porins (1  $\mu$ g/mL) or LPS (1  $\mu$ g/mL) used in the NF- $\kappa$ B ELISA were determined in preliminary experiments.

### ***Chromatin immunoprecipitation (ChIP) analysis***

To examine the association of transcription factors NF- $\kappa$ B and IL-6 promoter, U937 cells ( $2 \times 10^7$ /mL) were stimulated with porins (1  $\mu$ g/mL) or LPS (1  $\mu$ g/mL) for 0, 2, 4, and 8 h, and ChIP assays were performed using a ChIP assay kit (Upstate), according to the manufacturer's specification. Briefly, proteins/DNA were cross-linked with 1% formaldehyde for 15 min on ice; then cross-linking was blocked with 0.125 M glycine. Cells were then lysed in lysis buffer (50 mM Tris [pH 7.4]; 2 mM EDTA; 0.1% Nonidet P-40; and 10% glycerol) supplemented with protease inhibitors, and chromatin was sheared by sonication (three times for 35 seconds each time). The extracts were precleared for 1 h with salmon sperm-saturated protein A/G-agarose (Upstate Biotechnology). Immunoprecipitation was carried out overnight at 4°C using 10  $\mu$ g of NF- $\kappa$ B p65 subunit specific

antibody (Santa Cruz Biotechnology, Inc.). Immune complexes were collected with salmon sperm-saturated protein A/G-agarose for 2 h and washed twice in high-salt buffer (20 mM Tris pH 8.0; 2 mM EDTA, 500 mM NaCl, 1% Nonidet P-40, 0.1% SDS), followed by two washes with no-salt buffer (Tris-EDTA 1x), and two washes in 0.5 M LiCl washing buffer (10 mM Tris pH 8.0, 1 mM EDTA, 0.25 M LiCl, 1% Nonidet P-40, 1% Na deoxycholate). Samples were rotated for 5 min at 4°C in between every washing step. Immune complexes were extracted twice with 250  $\mu$ L of freshly prepared extraction buffer (1% SDS, 0.1 M NaHCO<sub>2</sub>). DNA cross-links were reversed overnight at 65°C and deproteinated with proteinase K (Sigma) (100  $\mu$ g for 1 h at 45°C). DNA was phenol/chloroform-extracted, ethanol-precipitated and used for PCR. DNA isolated from an aliquot of the total nuclear extract was used as a loading control for the PCR (input control).

To detect the presence of IL-6 promoter in the processed sample, PCR reactions were carried out with total DNA (2  $\mu$ L, input control) and immunoprecipitated DNA (2  $\mu$ L) using forward primer: 5'- CCTAGTTGTGTCTTGCGATG - 3'; reverse primer: 5'- GGAGGGGAGATAGAGCTTCT - 3', which are specific for human IL-6 promoter (a.n.: AF048692). The cycling parameters for one-step PCR were: denaturation 95°C for 30 s, annealing 57°C for 30 s and extension 72°C for 75 s (repeated 40 times). The PCR products were size-separated on a 2% agarose gel and visualized with ethidium bromide.

### ***Lactate dehydrogenase (LDH) assay***

An LDH assay was carried out according to the manufacturer's instructions, using a cytotoxicity detection kit (Roche Diagnostic GmbH, Roche Molecular Biochemicals). LDH is a stable, cytoplasmic enzyme present in all cells and is rapidly released into cell culture supernatant when the plasma membrane is damaged. LDH activity was determined by a coupled enzymatic reaction whereby the tetrazolium salt was reduced to formazan. An increase in the number of dead or damaged cells resulted in an increase in LDH activity in the culture supernatant. The amount of LDH showed that treated and untreated cells were healthy.

### ***Reproducibility***

The results were expressed as mean values  $\pm$  standard errors of three independent observations. Statistically significant values were compared using Student's *t*-test and  $p \leq 0.01$  and were considered statistically significant. Gels were scanned for densitometry analysis using Sigma Gel software, and the results shown below are averages of the values from three different experiments.

## **RESULTS**

### ***IL-6 release in porin-activated U937 cells***

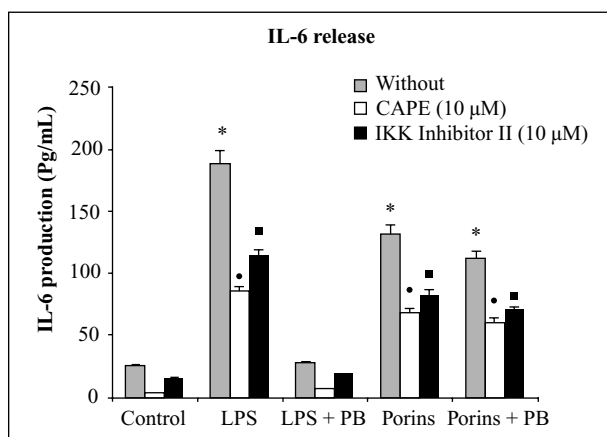
The supernatant of U937 cell cultures was tested using an ELISA for the presence of secreted IL-6 in response to porins and in comparison with that induced by LPS. We



found that highly purified porins (1  $\mu\text{g/mL}$ ) induced release of IL-6 (figure 1), 24 h after stimulation, representing at least 70% of the induction obtained by 1  $\mu\text{g/mL}$  of LPS. The time points and the concentrations chosen for analyses of IL-6 were previously optimized and were not toxic to the cells; in fact, the treatment did not induce any significant release of LDH in the cell supernatants (data not shown). The effects caused by porins were not attributable to any possible contaminating traces of LPS. The porin preparation used contained a biologically insufficient amount of LPS (about 50 pg/ $\mu\text{g}$  of porins), which did not induce any cytokine release (data not shown). Moreover, in order to show that the stimulatory capacity of the porin preparation was not due to contamination with LPS, we performed some control experiments by adding polymyxin B (PB) (a specific, LPS-inhibitory oligopeptide) to the porin and LPS, prior to stimulation, within a concentration range that is non-toxic to monocytes. LPS stimulation was abolished in the presence of PB, whereas porin stimulation was not significantly affected (figure 1).

### IL-6 transcripts induced by porins

To analyse the effect of porins on IL-6 genes, U937 cells were treated with porins (0.1 up to 5  $\mu\text{g/mL}$ ), and total RNA was extracted 2 h later and analysed by Northern blotting for the presence of IL-6 transcript. IL-6 mRNA was increased by treatment with porins (figure 2A). The highest increase was at a porin concentration of 1  $\mu\text{g/mL}$ . The smallest amount of porins able to stimulate mRNA synthesis was 0.5  $\mu\text{g/mL}$ . To examine the expression kinetics of the IL-6 transcripts, U937 cells were treated for different lengths of time with 1  $\mu\text{g/mL}$  of porins (figure 2B). A shorter stimulation time did not increase mRNA levels. IL-6 transcripts began to increase 2 h after porin treatment; there was a peak at 4 h and a return to



**Figure 1**

Effects of the NF- $\kappa$ B inhibitors on IL-6 secretion by U937 cells stimulated with *S. enterica* serovar Typhimurium porins and LPS. U937 cells ( $3 \times 10^6/\text{mL}$ ) were pretreated for 1 h with CAPE (10  $\mu\text{M}$ ) or IKK Inhibitor II (10  $\mu\text{M}$ ) before stimulation with LPS (1  $\mu\text{g/mL}$ ) or LPS plus PB or porins (1  $\mu\text{g/mL}$ ) or porins plus PB. The cytokine level was measured by ELISA 24 h after stimulation. The results are representative of three independent experiments, and the error bars indicate the standard error of the mean ( $p \leq 0.01$ ; Student's *t*-test). (\*)  $p \leq 0.01$  versus untreated cells; (•)  $p \leq 0.01$  versus CAPE pretreated cells; (■)  $p \leq 0.01$  versus IKK Inhibitor II pretreated cells.

the basal level 8 h post-treatment. Similar amounts of IL-6 mRNA were obtained when U937 cells were stimulated with porins plus PB.

As a control, we measured the levels of  $\beta$ -actin mRNA, which is a cell-cycle-independent mRNA. Actin mRNA levels remained unchanged, indicating that the values for IL-6 mRNA levels were not caused by a general increased in all poly-(A)<sup>+</sup>RNA species (data not shown).

### NF- $\kappa$ B activation by porin stimulation

NF- $\kappa$ B regulates gene activity in response to a plethora of stimuli, including cytokines, growth factors, bacterial and viral products. Therefore, in order to demonstrate the activation of NF- $\kappa$ B, we investigated porin induction of NF- $\kappa$ B p50 and p65 subunits in whole-cell extracts using antibodies specific for epitopes that are accessible only when the nuclear factors are phosphorylated and bound to their target DNA. Following treatment of U937 cells with porins at 1  $\mu\text{g/mL}$ , NF- $\kappa$ B-binding increased significantly, by 30 min (data not shown), was maintained at the same level by 1 h as shown in figure 3, and returned to background levels by 120 min (data not shown). NF- $\kappa$ B induction by porins occurred at similar levels of LPS. We have previously reported that the lowest concentration of LPS that activates NF- $\kappa$ B was 100 ng/mL [12]. In our preparation, 50 pg of LPS/ $\mu\text{g}$  of porins were present. Using this concentration, we were not able to observe any NF- $\kappa$ B activation (data not shown). In this *in vitro* model, the porin-PB complex induced activation of NF- $\kappa$ B, while the LPS-PB complex did not induce NF- $\kappa$ B-binding, indicating that it was the porins and not traces of LPS that activated NF- $\kappa$ B in our assays.

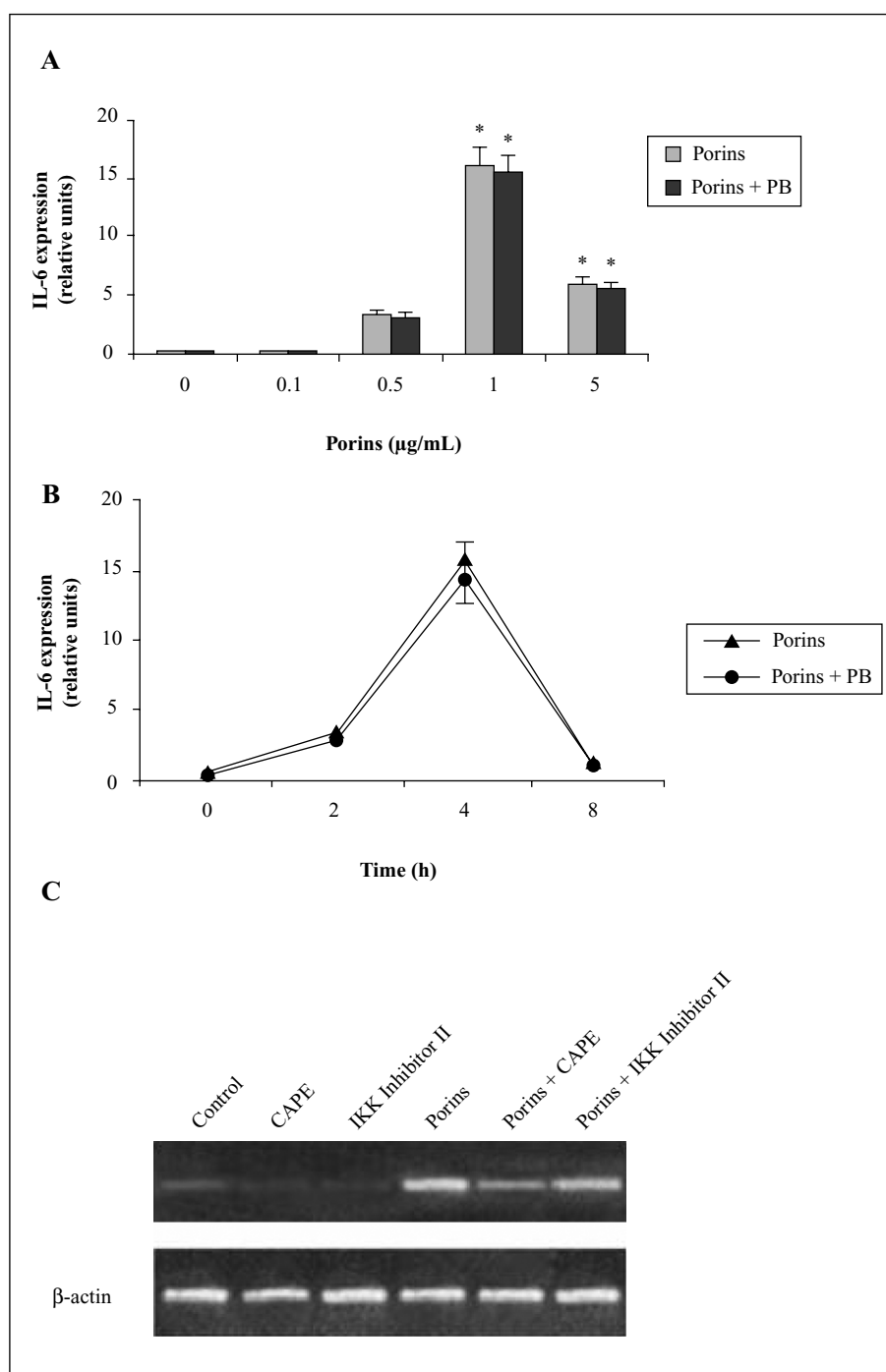
### The NF- $\kappa$ B pathway plays a pivotal role in inducing IL-6 production by U937 cells in response to porins

To evaluate the role of NF- $\kappa$ B signaling, U937 cells were treated with CAPE, a broad spectrum NF- $\kappa$ B inhibitor [20]. Pretreatment of cells with 10  $\mu\text{M}$  of CAPE for 1 h before porin or LPS exposure led to a significant inhibition of IL-6 expression (figures 1, 2C). Since CAPE is a rather non-specific inhibitor of NF- $\kappa$ B, the importance of this signaling cascade was confirmed with the use of additional, more specific blocker. Similar to what was observed with CAPE, the IKK Inhibitor II, which affects NF- $\kappa$ B signaling downstream by blocking the phosphorylation and degradation of I $\kappa$ B [21], was also capable of attenuating IL-6 expression (figures 1, 2C). Cell viability assays revealed that neither CAPE nor IKK Inhibitor II were cytotoxic at the concentration examined, indicating that the inhibitory actions of these compounds were not responsible for cell death (data not shown).

Collectively, these findings indicate that NF- $\kappa$ B signaling is a primary pathway for inducing IL-6 production in response to *S. enterica* serovar Typhimurium porins.

### NF- $\kappa$ B binds to the IL-6 promoter after porin stimulation

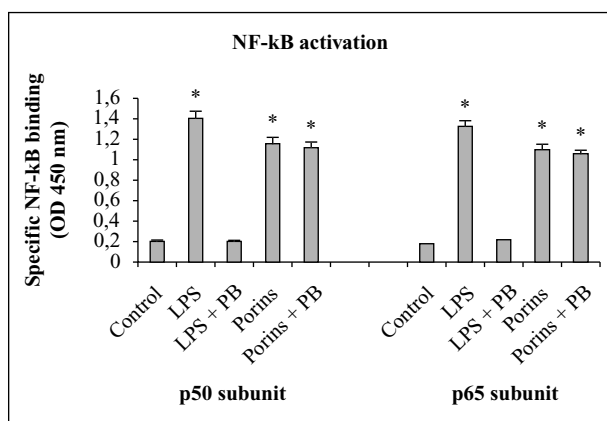
The nuclear regulation of NF- $\kappa$ B activity requires multiple co-activators [23]. One of the major questions in eukaryotic transcriptional regulation concerns the way

**Figure 2**

Densitometric analysis of IL-6 mRNA expression from U937 cells treated with *S. enterica* serovar Typhimurium porins. U937 cells ( $1 \times 10^7$ /mL) were stimulated for 2 h with 0.1 up to 5 µg/mL of porins (A) or for designated times (B) with 1 µg/mL of porins. Northern blotting was performed as described in Methods and materials. Gels were scanned for densitometry analysis with the Sigma Gel software, and the values for each stimulation are expressed as arbitrary units. The results shown are the average of three independent experiments, and the error bars indicate the standard error of the means ( $p \leq 0.01$ ; Student's *t*-test). C) Representative experiments of the effect of CAPE (10 µM) and IKK Inhibitor II (10 µM) on IL-6 expression by U937 cells stimulated with optimal porin concentration (1 µg/mL) and time (4 h). β-actin was used as the housekeeping control gene.

in which the transcription machinery gains access to promoter DNA. To examine further which NF-κB factor is involved in the activation of IL-6 expression in porin-stimulated cells, association of IL-6 promoter with individual NF-κB factors was examined using the ChIP assay. For this analysis, the antibody to the NF-κB p65 subunit that holds a characteristic transcriptional activation domain was chosen. U937 cell lysates, after stimula-

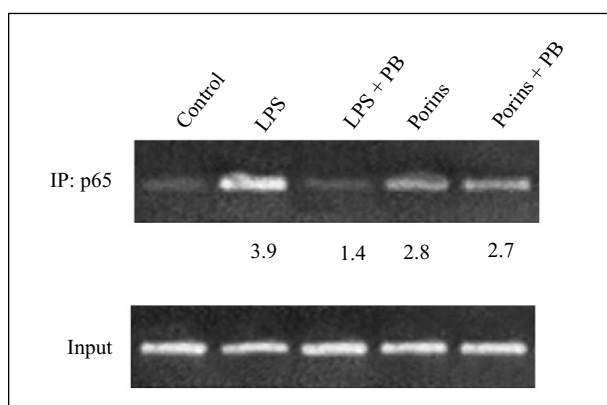
tion with 1 µg/mL of porins or LPS, were immunoprecipitated with NF-κB p65 mouse monoclonal antibodies. PCR amplification was performed using primers designed to amplify a fragment containing the NF-κB binding consensus sequence of human IL-6 promoter (see "Methods and materials"). The predicted 240 bp PCR product was amplified from samples derived from mouse monoclonal NF-κB p65 antibody immunoprecipi-



**Figure 3**

Effect of *S. enterica* serovar Typhimurium porins on binding of NF- $\kappa$ B p50 and p65 subunits to an NF- $\kappa$ B-binding consensus sequence. U937 cells were stimulated with porins (1  $\mu$ g/mL) or LPS (1  $\mu$ g/mL) for 1 h. Cell lysates (10  $\mu$ g/mL) were tested for binding of the activated p50 and p65 NF- $\kappa$ B subunits to an NF- $\kappa$ B consensus sequence using the Trans-Am NF- $\kappa$ B 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  $\pm$  standard errors of triplicate determinations ( $p \leq 0.01$ ; Student's *t*-test).

tates. No PCR amplification was observed in unstimulated U937 cells (control). The ChIP assays showed that NF- $\kappa$ B proteins could be found at the IL-6 promoter 2 h after porin treatment (figure 4), and the binding level increased up to 4 h after stimulation (data not shown). Quantitative analysis by Sigma-Gel demonstrated that at 8 h post-stimulation, the binding of NF- $\kappa$ B to IL-6 promoter decreased to a basal level (data not shown). The porins/PB complex was still active in determining the binding of NF- $\kappa$ B to IL-6 promoter, while the LPS/PB complex was ineffective.



**Figure 4**

Porin-mediated IL-6 gene induction is dependent on activated NF- $\kappa$ B. U937 cells were stimulated with LPS (1  $\mu$ g/mL) or porin (1  $\mu$ g/mL) for 2 h. ChIP analysis was performed as described in Methods and materials. Briefly, DNA was immunoprecipitated (IP) with p65 NF- $\kappa$ B antibody, and PCR was performed with primers specific for the IL-6 gene promoter. Input samples show equal loading. Fold-increases in p65 NF- $\kappa$ B binding, compared to unstimulated cells (control), are shown below each lane.

Our experimental data demonstrate that anti-p65 antibody precipitated the IL-6 promoter, a finding that is consistent with the possibility that NF- $\kappa$ B p65 plays a role in the activation of IL-6 promoter by porins.

## DISCUSSION

Interleukin-6 is a multifunctional cytokine that regulates B and T cell function, haematopoiesis and acute inflammatory response. In addition, circulating IL-6 levels are usually high in cancer patients [24]. During infections by gram-negative microorganisms, LPS and porins or other OMPs are the main initiators of inflammatory reactions that may lead to circulatory failure and organ injury. Cytokines synthesis is dependent upon diverse signaling pathways that involve several transcription factors such as NF- $\kappa$ B. On the basis of evolutionary considerations, the original function of the NF- $\kappa$ B pathway is the initiation of inflammatory and innate immune responses via production of inflammatory mediators [25, 26]. One of the most interesting aspects of NF- $\kappa$ B is the variety and the nature of the stimuli that lead to its activation. Consequently, NF- $\kappa$ B is an important protein in the regulation of the acute phase response of inflammation. Autocrine production of IL-6 and paracrine activation by IL-6 from infiltrating inflammatory cells, amplify immune response and activate several other genes encoding proteins that regulate cell response [27]. The link between the activation of NF- $\kappa$ B has been shown in a variety of human diseases and constitutes an attractive target for therapeutic, anti-inflammatory interventions. A good deal of recent research suggests that inhibition of NF- $\kappa$ B blocks inflammation, cancer development and progression, diabetes, stroke, muscle wasting and other diseases [28]. In the past few years, it has become evident that inhibition of IKK-driven NF- $\kappa$ B activation may be considered to be a promising treatment strategy for acute illnesses such as sepsis, chronic inflammation and autoimmune diseases [29, 30]. Research into NF- $\kappa$ B has identified the molecular mechanisms of this pathway, but the details of why this pathway works differently in different cellular environments, and how it interacts with other signalling pathways remain unclear. A thorough understanding of these processes is needed in order to design better and more efficient therapeutic approaches to treat complex diseases such as acute and chronic inflammatory diseases. There are now excellent reasons to promote proteins of the NF- $\kappa$ B signalling family as potential therapeutic targets for inflammation, regardless of the stimulus [31].

Our results, in a model of U937 cells stimulated with *S. enterica* serovar Typhimurium porins, show that IL-6 synthesis and release is mediated by NF- $\kappa$ B activation. We used two different inhibitors that block distinct steps in the NF- $\kappa$ B pathway, namely CAPE and IKK Inhibitor II, to monitor NF- $\kappa$ B activation. Both inhibitors significantly inhibited the porin-induced production of IL-6 in U937 cells. These data are consistent with the interpretation that porin induction of IL-6 expression is critically dependent on IKK activity and the NF- $\kappa$ B pathway. Our data demonstrated that in response to porins, an

order of events may be assigned for the transcriptional activation of the IL-6 gene starting from its promoter. In fact, after porin stimulation, NF- $\kappa$ B is activated, translocates to the nucleus and binds to the IL-6 promoter. The ChIP assay detects that activation, translocation and binding of the NF- $\kappa$ B p65 subunit to the IL-6 promoter sequence are evident 2 h after the porin treatment.

Activation of IL-6 promoter by NF- $\kappa$ B is obviously not the only gene activation pathway able to lead to IL-6 synthesis. In fact, porin-NF- $\kappa$ B activation is also mediated through other signal transmission pathways. As previously reported, porins induce phosphorylation of different signal transmission pathways, not only the Raf-MEK1/2-MAPKs pathway, but also the pathways regulated by NT-PTKs [32]. In different cell types, in addition to NF- $\kappa$ B, porins are also able to activate other transcription factors such as AP-1 [12] and STAT1/STAT3 [13]. There are several interconnections between the different signaling pathways that use either activated NF- $\kappa$ B or activated STAT1/STAT3. STAT1/STAT3 has been shown to drive gene expression, even in the absence of tyrosine phosphorylation [33, 34]. These reports suggest that porin-induced transcriptional activation of the IL-6 gene may involve the activation of different panels of signal pathways and nuclear transcription factors.

Here we have found that, in U937 cells, NF- $\kappa$ B performs a role of paramount importance in IL-6 promoter activation. As NF- $\kappa$ B is able to enhance IL-6 gene promoter activity, activation of this nuclear factor may be responsible for porin-induced expression and release of IL-6. Elucidation of the host signaling pathways and transcription factors that are involved in IL-6 expression may help in the understanding of host susceptibility to gram-negative infections during various disease states. A recent hypothesis postulates that sepsis moves through different phases, with periods of enhanced inflammation alternating with periods of immune suppression. It has been shown in mice that die in the early phase, that there is uniformly increased inflammation, with elevated IL-6 levels in the plasma; during the late phase of sepsis, some mice die with evidence of immunosuppression (increased bacterial growth and low IL-6 levels), while other mice die with immunostimulation (high IL-6 levels and bacterial growth) [35]. The cells implicated in the inflammatory and immunological response contain a large repertoire of receptors, which are responsible for the surface interaction with microorganisms and their components. These cell surface interactions lead to the activation of several signal transmission pathways, which induce cytokine synthesis and release; of these IL-6, has an essential role in sepsis.

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