BIOCHEMICAL PHARMACOLOGY 76 (2008) 1214–1228

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Actin cytoskeleton differentially modulates NF-κB-mediated IL-8 expression in myelomonocytic cells

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ARTICLE INFO

Article history:
Received 10 July 2008
Accepted 13 August 2008

Keywords:
Actin cytoskeleton
NF-κB
Cytochalasin D
TNFα
LPS
Interleukin-8

ABSTRACT

Many physiopathological events such as phagocytosis, pathogen invasion, cellular adhesion and chemotaxis governed by actin-based cytoskeleton are often accompanied by nuclear factor κB (NF-κB) activation and expression of pro-inflammatory genes. In the present study, we demonstrated that reorganization of actin cytoskeleton induced by Cytochalasin D (CytD), an actin-polymerization inhibitor, enhanced *il-8* gene expression induced by TNFα and LPS in HL-60 monocyte-like cells. Both transcriptional and post-transcriptional mechanisms were involved. CytD potentiated NF-κB-mediated transcription induced by both TNFα and LPS but via different mechanisms. In the case of LPS, the perturbation of actin dynamics increased the TLR4 levels at the cell membrane and consequently enhanced the IKK complex activation and NF-κB nuclear translocation. However, the canonical pathway involving the IKK complex and leading to the NF-κB translocation into the nucleus was not affected by actin remodelling in the case of TNFα. Interestingly, actin disruption primed p65 phosphorylation induced by TNFα and LPS, on Ser^{276} and Ser^{536}, respectively, which suggested actin cytoskeleton could also modulate p65 transactivating activity.

1. Introduction

Actin cytoskeleton is involved in many aspects of cellular function, such as cell movement, muscle contraction, phagocytosis and mitosis [1–4]. The dynamic assembly and spatial organization of actin filaments in response to extracellular signals are at the basis of these fundamental processes [5]. Recently, several studies established a link between actin dynamics and alteration of gene expression by demonstrating direct interactions between actin and members of signal transduction pathways. Indeed, it has been demonstrated that cytoskeleton-disrupting agents can modulate post-transcriptional and transcriptional events [6–10]. In another hand, many recent studies support the idea of a role for nuclear actin in the control of gene transcription. Nuclear actin is required for chromatin remodelling and for the transcription by all three RNA polymerases [11–13].

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Abbreviations: Act D, Actinomycin D; CBP, cAMP-response element binding protein; ChIP, chromatin immunoprecipitation; CytD, cytochalasin D; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; EMSA, electromobility shift assay; ERK1/2, extracellular signal-regulated kinase 1/2; FBS, fetal bovine serum; GST, glutathione S-transferase; IκBα, inhibitor of κB alpha; HIV-1, human immunodeficiency virus type 1; IKK, IκB kinase; IL-1β, interleukin-1β; LPS, lipopolysaccharide; LUC, luciferase; MSK1, mitogen- and stress-activated protein kinase-1; NF-κB, nuclear factor κB; PMA, phorbol myristate acetate; qRT-PCR, quantitative real-time reverse transcriptase-PCR; RNA pol II, RNA polymerase II; TNFα, tumor necrosis factor α.

0006-2952/$ – see front matter
doi:10.1016/j.bcp.2008.08.017

Several pro-inflammatory stimuli, such as TNFα (tumor necrosis factor α) and IL-1β (interleukin-1β) as well as some bacterial products like LPS (lipopolysaccharide) known to activate the transcription factor nuclear factor κB (NF-κB), are also able to induce actin cytoskeleton remodelling [14–16]. Moreover, many physiopathological events such as phagocytosis, pathogen invasion, cellular adhesion and chemotaxis governed by actin-based cytoskeleton are often accompanied by NF-κB activation and the expression of pro-inflammatory genes [3,17–19]. Furthermore, we demonstrated that a strong perturbation of actin dynamics induced NF-κB activation in myelomonocytic cells [20]. For these different reasons, we decided to determine the modulating effect or the potential role of the actin cytoskeleton in the transduction pathways leading to NF-κB activation by two inducers: TNFα or LPS.

The transcription factor NF-κB regulates the expression of a large-scale of genes involved in a series of important cellular processes such as inflammatory and immune responses, cellular proliferation and differentiation as well as cell survival (for a review, see [21]). The NF-κB transcription factor binds specific DNA sequences as homo- or heterodimers composed of members of the Rel/NF-κB family [22]. The most ubiquitous complex is the heterodimer p50/p65(RelA). At the N-terminal end, the Rel proteins bear a related, but non-identical, 300-residue-long Rel homology domain (RHD), which is responsible for dimerization, nuclear translocation and specific DNA binding. In addition, some Rel proteins such as p65(RelA), contain one or two C-terminal transactivating domain (TAD).

In the resting state, NF-κB complexes are sequestered in an inactive form in the cytoplasm of the cells through its association with an inhibitory protein belonging to the inhibitory κB (IκB) family comprising notably IκBα. The classical NF-κB-activating pathway is induced by a variety of stimuli such as pro-inflammatory cytokines TNFα and IL-1β or bacterial products such as LPS [23–26]. While these numerous stimuli induce the activation of NF-κB through different receptors and adaptor proteins, they all converge to a specific complex called the IKK (IκB kinase) complex. This complex is composed of two catalytic subunits, IKKα and IKKβ, a regulatory subunit, IKKγ (NEMO, NF-κB essential modulator) and different scaffold proteins [27,28]. Upon cell stimulation, the IKK complex is activated by phosphorylation [29,30] and then phosphorylates the IκBα protein on Ser^{32} and Ser^{36}, targeting IκBα for polyubiquitination and degradation by the 26S proteasome. The released NF-κB translocates into the nucleus regulating the expression of its target genes such as those coding for cytokines, adhesion molecules and chemokines which have a crucial role in both immune and inflammatory responses [22,29–31].

Moreover, the NF-κB functions are regulated by post-translational modifications including phosphorylation and acetylation [32–34]. Recent studies have demonstrated that p65 can be phosphorylated by various cytoplasmic and nuclear kinases at multiple sites either in the N-terminal RHD or C-terminal TAD in a stimulus- and cell-type dependent manner leading either to an increase in p65 DNA binding and/or transcriptional activity [32–34]. The best characterized phosphorylable residues are Ser^{276} and Ser^{536}. The phosphorylation of these residues may change the conformation of p65 and promote its interaction with its coactivators CBP/p300 [35]. These proteins CBP/p300 are recruited to specific gene promoter by association with sequence-specific transcription factors and modulate the promoter activities by acetylation of both histone and non-histone substrates [34,36–39]. The acetylation of specific sites of histones, especially histones H3 and H4, surrounding specific genes is an important step in the chromatin remodelling which is often required to promote the access to the basal transcription machinery and can consequently favor the expression of specific genes [32].

In the present study, we showed that actin disruption by F-actin-depolymerizing compound, Cytochalasin D (CytD), up-regulated IL-8 expression in response to TNFα and LPS through transcriptional and post-transcriptional events in myelomonocytic cells. In both cases, the synergistic effect of CytD on *il-8* gene transcription resulted from an increased NF-κB-mediated transcription. However, this up-regulating effect of CytD on NF-κB-mediated transcription involved different mechanisms according to the inducer. While CytD potentiated the canonical NF-κB activation pathway induced by LPS, this compound did not absolutely interfere with this pathway in the case of TNFα. Interestingly, actin disruption primed p65 phosphorylation induced by TNFα and LPS, on Ser^{276} and Ser^{536}, respectively, which suggested that actin cytoskeleton could also modulate the p65 transactivating activity.

2. Material and methods

2.1. Chemicals

CytD was obtained from MP Biomedicals (Asse-Relegem, Belgium). LPS from *Escherichia coli* (serotype 0111: B4), LPS from *E. coli* (serotype 0111: B4) FITC conjugate and ActD were purchased from Sigma–Aldrich (Bornem, Belgium). Human recombinant TNFα was purchased from Peprotech (Tebu Bio, Boechart, Belgium).

2.2. Plasmids

The reporter construct $(κB)_5$LUC (where LUC stands for luciferase) was obtained from Stratagene (La Jolla, CA, USA). The reporter plasmid (133-IL8)LUC contained a human IL-8 promoter fragment of 133 bp. In the reporter construct (133-IL8-NF-κB-mut)LUC the NF-κB site GGAATTTCCT (−80 to −71 bp) was mutated to TAACTTTCC whereas in the plasmid (133-IL8-AP-1-mut)LUC the AP-1 site TGACTCA (−126 to −120 bp) was mutated to TATCTCA. These 3 constructions were kindly provided by Dr W. Vanden Berghe and Dr G. Haegeman (Ghent University, Ghent, Belgium).

2.3. Antibodies

Anti- IKKβ, -IKKγ, -RNA polymerase II, -TLR4 (H80) and -p65 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-NBS-1 was purchased from BD Biosciences Pharmingen (Erembodegem, Belgium). Anti-hsp60 used for western blot was from Stressgen (San Diego, USA). Anti-IκBα phosphorylated on Ser^{32} and Ser^{36}, -p65 phosphorylated on Ser^{536} and -p65 phosphorylated on Ser^{276} were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-histone H3

acetylated on Lys[9] used for immunoprecipitation was from Upstate (Charlottesville, VA, USA). Anti-unmodified histone H3 was from Abcam Limited (Cambridge, UK).

2.4. Cell lines

Human promyelocytes HL-60 (A.T.C.C., Rockville, MD, USA) were cultivated in RPMI 1640 supplemented with 2 mM glutamine and 10% (v/v) FBS (fetal bovine serum) (Biowhittaker, Petit Rechain, Belgium).

2.5. Flow cytometry

For the measure of membranar TLR4, human adherent monocytes, extracted as described previously [20], were saturated with PBS supplemented with 5% of human AB serum (Sigma–Aldrich, Bornem, Belgium) for 30 min on ice. Cells were then stained with 8 μg/ml anti-TLR4 (Dako Cytomation, Heverlee, Belgium) for 30 min on ice, washed with PBS and then were incubated with 40 μg/ml secondary anti-rabbit Ig coupled to FITC (Dako Cytomation, Heverlee, Belgium) for 30 min on ice. For the measure of LPS-FITC fluorescence, cells were treated with LPS-FITC (10 μg/ml) for different times and then were detached with versene and washed with PBS. Finally, for all experiments, cells were analyzed by flow cytometry with DNA software (FACSCanto II, Benton Dickinson, Erembodegem, Belgium). A monocyte gate was used to analyse data from at least 10 000 events.

2.6. Confocal microscopy

HL-60 cells (10^5 cells) were rinsed and fixed with 4% (w/v) paraformaldehyde for 10 min at room temperature. After washing with PBS, the cells were incubated in PBS containing 1% (v/v) FBS and were centrifuged with a cytospin at 600 × *g* for 2 min (Staspin, Aartselaar, Belgium). Cells were then incubated in PBS containing 1% (v/v) FBS and 0.1% Triton X-100 for 10 min at room temperature and then with Alexa488-phalloïdin (1/40) (Invitrogen, Merelbeke, Belgium) 30 min at 37 °C. Cells were then washed with water and were incubated 2 min at room temperature with TO-PRO-3 (1/200) (Invitrogen, Merelbeke, Belgium). After washing with water, slides were mounted with mowiol. Samples were analyzed by confocal microscopy (Leica TCS NT, Leica Microsystems Belgium, Groot-Bjigaarden, Belgium).

2.7. IL-8 detection

The release of IL-8 in cell supernatants was quantitatively assayed by a double-antibody ELISA kit with recombinant IL-8 as a standard in accordance with the manufacturer's protocol (Immunosource, Hall-Zoersel, Belgium).

2.8. Gene reporter assays

The HL-60 cells were transfected by the DEAE-dextran method as described previously [40]. Twenty-four or 48 h after transfection with a reporter plasmid $(\kappa B)_5$LUC or with different constructions of IL8-LUC, cells were stimulated by TNFα or LPS in the presence or not of CytD. At 8 h post-treatment, cells were lysed and assayed for luciferase activity (Luciferase Reporter Gene Assay, Roche Molecular Biochemicals, Vilvoorde, Belgium) in accordance with the manufacturer's instructions. The luciferase activity of the samples was normalized with the protein concentration measured by the Bradford method (Bio-Rad, Hercules, CA, USA).

2.9. Extraction of nuclear proteins

The extraction method was described previously [41]. Briefly, after the extraction of cytoplasmic proteins in a cold hypo-osmotic buffer, the pellets of nuclei were gently washed and resuspended in a cold saline buffer during 30 min. After centrifugation at 20 000 × *g* for 15 min, the supernatant were harvested and used in EMSA or Western blotting as previously described [42].

2.10. Total protein extraction for phospho-Western blottings

Treated cells were washed with ice-cold phosphate-buffered saline and rapidly lysed in SDS-blue lysis buffer [62.5 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 0.03% bromophenol blue and 50 mM dithiothreitol]. Total lysates were sonicated for 50 s, boiled for 3 min, and used for Western blotting as previously described [42].

2.11. mRNAs detection by quantitative real-time reverse transcription-PCR

Total RNA samples were isolated with the Tripure reagent (Roche Molecular Biochemicals, Vilvoorde, Belgium). One microgram of RNA was submitted to reverse transcription with the Moloney murine leukemia virus reverse transcriptase (Invitrogen, Merelbeke, Belgium). Two microliters of cDNA obtained for each sample were submitted to a qPCR using the SYBR green Master mix method (Applied Biosystems, Foster City, USA) in the ABI Sequence Detection System. The results were normalized with the 18S transcript. PCR was performed with the following primers designed using the software Primers Express™: *il-8*: W 5′-GAAGGAACCATCTCACTGTGTGTAA-3′ and RV 5′-ATCAGGAAGGCTCCAAGAG-3′ and *18S*: FW 5′-AACTTTCGATGGTAGTCGGCCC-3′ and RV 5′-CCTTGGATGTGGTAGCCGTTT-3′.

2.12. IKK complex immunoprecipitation and in vitro IKK kinase assay

IKK complex immunoprecipitation and in vitro IKK kinase assay were performed as previously described [43] with purified GST-IκBα-(1–54) fusion protein as substrate (a gift from R. Gaynor, University of Texas Southwestern Medical Center, Dallas). This was followed by a Western blotting using an anti-IκBα phosphorylated on Ser^{32} and Ser^{36} antibody.

2.13. Electrophoretic mobility-shift assay (EMSA)

In brief, 5 μg of nuclear proteins were incubated for 30 min at room temperature in a volume of 10 μl with 0.2 ng ^{32}P-labeled oligonucleotide probe in binding buffer [20 mM HEPES-KOH

(pH 7.9), 75 mM NaCl, 1 mM EDTA, 5% (v/v) glycerol, 0.5 mM $MgCl_2$, 1 mM DTT] containing 2 μg BSA and 1.25 μg poly(dI-dC)-poly(dI-dC) (Amersham Pharmacia Biotech, Roosendael, The Netherlands). DNA-protein complexes were then resolved by electrophoresis on a non-denaturing 6% (w/v) polyacrylamide gel for 2 h at 300 V in 0.25X TBE [2.5 mM Tris, 2.5 mM H_3BO_3, 2 mM EDTA]. The gels were then dried and autoradiographed an a Fuji X-ray film. The sequence of the double strand κB probe was as follows: 5′-GGTTACAAGGGACTTTCCGCTG-3′ and 5′-TTGGCAGCGGAAAGTCCCTTGT-3′. The oligonucleotide probes were labeled by infilling with the Klenow DNA polymerase (Roche Molecular Biochemicals, Vilvoorde, Belgium).

2.14. *Chromatin immunoprecipitation assays (ChIP assays)*

This experiment was performed as described previously [44]. Briefly, immunoprecipitations of sheared chromatin were performed with 2 μg of different antibodies: anti-p65, -p65 phosphorylated on Ser^{276}, -p65 phosphorylated on Ser^{536}, -RNA polymerase II, -histone H3, -histone H3 acetyled on Lys^9 and anti-flag (aspecific antibody). After a phenol/chloroform extraction, qPCRs were done using the SYBR green Master mix method (Applied Biosystems, Foster City, CA, USA) in the ABI Sequence Detection System. All ChIP assays were performed two times in triplicate. The primers used were designed using the software Primers Express™: *il-8* FW 5′-GCCATCAGTTGCAAATCGTG-3′ and RV 5′-AGTGCTCCGTGGCTTTT-3′. These primers amplify the DNA sequence corresponding to the −101 to +2 bp region of the *il-8* promoter and thus containing the κB site (−80 to −71 bp).

3. Results

3.1. *Actin disruption potentiates the expression of various inflammatory genes in TNFα- or LPS-stimulated myelomonocytic cells*

To test the modulating effect or the potential role of actin cytoskeleton in TNFα- or LPS-induced NF-κB signalling, CytD, a naturally occurring actin-disrupting substance, was used. CytD inhibits actin polymerization by capping actin filaments and stimulating ATP hydrolysis on G-actin [45]. The staining of actin filaments (F-actin) in promyelocytic cells, especially HL-60 cells, showed a cortical cytoskeleton (Fig. 1A). The treatment with CytD disrupted actin cytoskeleton but did not induce a complete F-actin depolymerization (Fig. 1B). Indeed, F-actin aggregates accumulated on one side of the cell. This cell polarization after CytD treatment was already shown by Uematsu et al. [46].

The function of the actin cytoskeleton on the expression of various inflammatory genes was first evaluated by measuring steady state levels of the *il-8* mRNA by real-time qRT-PCR. IL-8 is an important chemokine involved in the innate immunity,

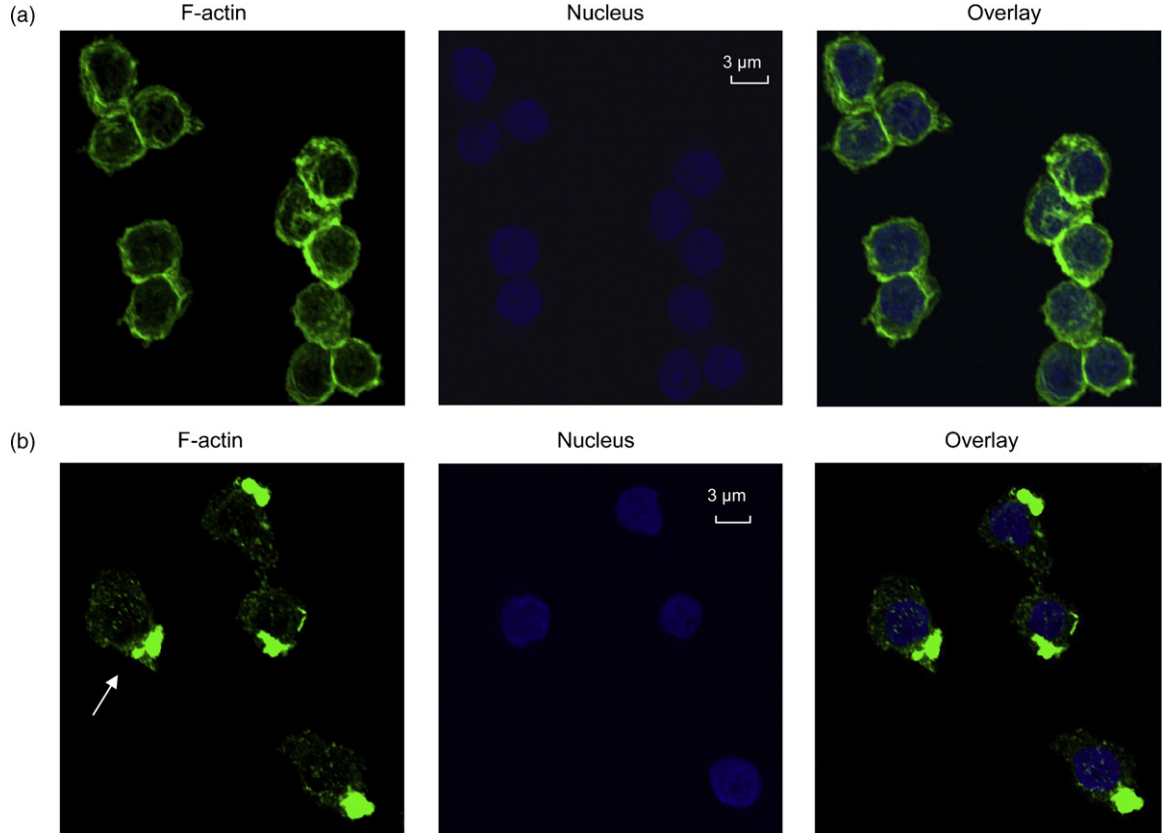

Fig. 1 – Actin disruption by CytD in HL-60 cells. Actin filaments and DNA were stained with Alexa488-phalloidin and TO-PRO-3, respectively. Confocal microscopy images show untreated HL-60 cells (A) or HL-60 cells treated with CytD (5 μM) during 2 h (B). Arrows indicate polarized cells.

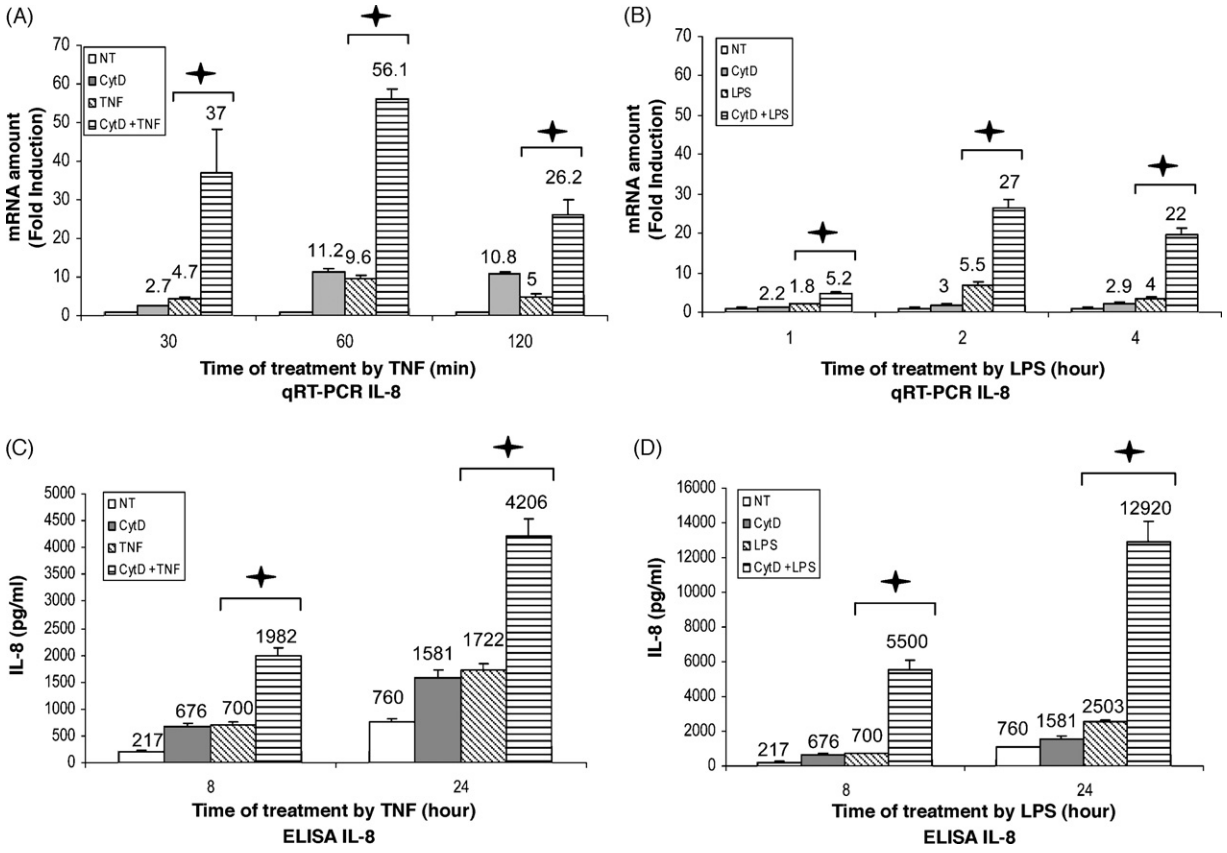

Fig. 2 – Effect of CytD on the expression of *il-8* gene in response to TNFα or LPS in HL-60 cells. Influence of CytD on *il-8* mRNAs levels (A and B) and on IL-8 secretion (C and D) induced by TNFα or LPS in HL-60 cells. After a pre-treatment (1 h) or not with CytD (5 μM), cells were stimulated or not with TNFα (100 U/ml) (A and C) or with LPS (10 μg/ml) (B and D) during various indicated times. (A and B) Total RNAs were isolated and real-time qRT-PCRs with specific primers were performed to analyze the *il-8* mRNAs expression. The results were normalized with the *18S* ribosomial transcript. (C and D) The supernatants were collected and the release of IL-8 was determined by specific ELISA. Shown were means ± S.D. from three independent experiments performed in duplicate. ✦, significantly different (*p* value <0.05).

notably through its effect on the recruitment of innate immune cells at infection sites [47–49]. HL-60 cells were pre-treated with CytD for 1 h before addition of TNFα or LPS. As shown in Fig. 2A and B, both inducers as well as the CytD alone led to an increase of *il-8* mRNA level. Interestingly, mRNAs levels induced by TNFα or LPS were much higher when cells were pre-treated with CytD. For TNFα, the most pronounced effect of CytD was observed after 30 min of stimulation (approximately 37-fold vs. 4.7-fold). This effect was sustained for up to 1 h but decreased at longer time-points. For LPS, a synergistic effect of CytD appeared only after stimulation for 2 h and further increased after 4 h (approximately 22-fold vs. 4-fold). CytD also synergistically increased *tnfα* and *iκbα* mRNA levels induced by TNFα and LPS (data not shown). In conclusion, these experiments clearly showed that actin disruption by CytD in HL-60 myelomonocytes up-regulated the mRNA levels of various inflammatory genes induced by both TNFα and LPS.

To further investigate the influence of actin cytoskeleton on TNFα- and LPS-induced gene expression, we assessed IL-8 secretion by ELISA. As shown in Fig. 2C and D, both inducers as well as CytD alone stimulated the IL-8 secretion at 8 h and 24 h. As expected, the pre-treatment with CytD increased IL-8 release in response to both inducers but the induction was higher for LPS (approximately 5500 pg vs. 700 pg of IL-8 released after 8 h for LPS and 1980 pg vs. 700 pg of IL-8 released after 8 h for TNFα). The pre-treatment with CytD also significantly increased the production of TNFα in response to both inducers (data not shown). These results suggest that actin disruption by CytD increased the release of pro-inflammatory cytokines and chemokines by myelomonocytes and could prime an inflammatory response.

3.2. *Actin disruption increases il-8 expression in response to TNFα and LPS by up-regulating NF-κB-mediated transcription and by stabilizing il-8 mRNA*

We next wanted to explore the molecular mechanism involved in CytD-mediated up-regulation of inflammatory genes and more specially the *il-8* gene.

Expression of cytokines and chemokines, such as TNFα and IL-8, is known to be regulated at the transcriptional level but also at the post-transcriptional level. Therefore, the effect of

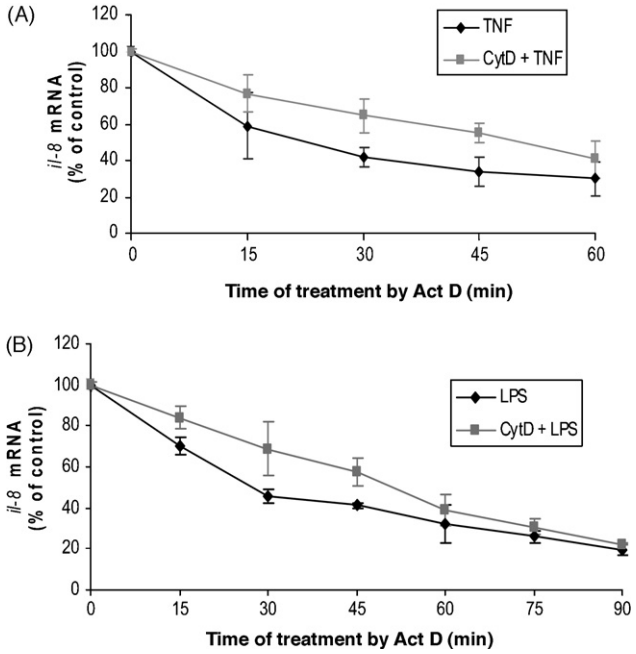

Fig. 3 – CytD increases the stability of *il-8* mRNA induced by TNFα and LPS. HL-60 cells were incubated or not with CytD (5 μM) for 1 h and then were stimulated or not with TNFα (100 U/ml) during 1 h (A) or with LPS (10 μg/ml) during 2 h (B) before the addition of Act D (5 μg/ml) to block further transcription (time 0). Total RNAs were harvested after the indicated times and *il-8* mRNAs levels analyzed by real-time qRT-PCR with specific primers. The results were normalized with the *18S* ribosomial transcript. Values are presented as means ± S.D. (*n* = 3).

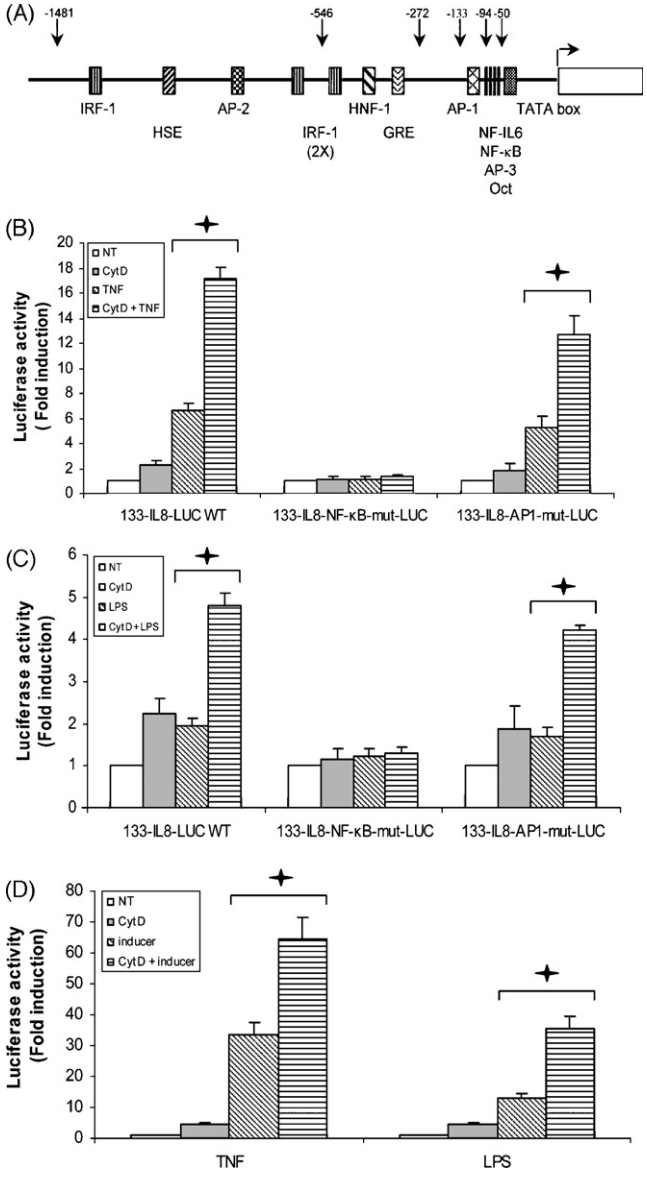

Fig. 4 – CytD has a synergistic effect on the NF-κB-mediated transcription induced by TNFα or by LPS. (A) Human *il-8* promoter. (B–D) HL-60 cells were transfected with different variants of IL-8-LUC reporter plasmid (B and C) or with a $(\kappa B)_5$ LUC reporter construct (D). After 24 h, cells were pre-treated with Cyt D (5 μM) during 1 h and then stimulated or not with TNFα (100 U/ml) or with LPS (10 μg/ml) for 6 h. Cells were harvested and the luciferase activity was measured. The luciferase activity of the samples was normalized with the protein concentration measured by the Bradford method. Shown were means ± S.D. from two independent experiments performed in duplicate. +, significantly different (*p* value <0.05).

CytD on the *il-8* mRNA stability after TNFα or LPS induction in HL-60 cells was investigated. HL-60 cells were pre-treated or not with CytD during 1 h and then stimulated with TNFα during 1 h or with LPS for 2 h before the addition of Actinomycin D (Act D), a transcription inhibitor. The levels of *il-8* mRNA were followed at various times after Act D addition by real-time qRT-PCR. It is important to note that MTT assays were performed in order to control the eventual cytotoxic effect of these different treatments. These experiments showed that neither the combination of CytD with TNFα or LPS nor Act D in association with the different substances induced cytotoxic effect (data not shown). Fig. 3A shows that the half-life of *il-8* mRNA induced by TNFα was increased in CytD-pre-treated cells (approximately 23 min vs. 50 min). A similar effect was observed in the case of LPS (approximately 27 min vs. 52 min, Fig. 3B). These results showed that CytD induced a significant stabilization of *il-8* mRNA.

The *il-8* gene promoter contains binding sites for several transcription factors such as NF-κB, AP-1 and NF-IL6 (Fig. 4A). The NF-κB site is often required for *il-8* gene transcription activation in many cell types [50]. To confirm the up-regulating effect of CytD on *il-8* promoter-mediated transcription in response to TNFα and LPS, a reporter construct (133-IL8-LUC WT) containing the minimal *il-8* promoter with the binding sites for essential transcription factors (AP-1, NF-κB) upstream of the luciferase gene was used. After transient transfection of HL-60 cells with the 133-IL8-LUC WT construct, cells were pre-treated or not with CytD for 1 h, then stimulated by TNFα or LPS for 6 h. Fig. 4B shows that CytD up-regulated *il-8* promoter-

mediated transcription induced by TNFα, resulting in a 2 to 3-fold increase in reporter gene activity. The mutation of the NF-κB site (133-IL8 NF-κB mut-LUC) abrogated the *il-8* promoter inducibility by CytD or TNFα alone and by combined treatment while the mutation of the AP-1 site (133-IL8-AP-1 mut-LUC) only induced a slight decrease of *il-8* promoter activation by all three treatments (Fig. 4B). These experiments indicated that the NF-κB site, unlike the AP-1 site, was required for CytD-mediated up-regulation of the *il-8* promoter. Similar results were obtained with LPS although the effect of CytD on *il-8* promoter stimulation by LPS was less marked (Fig. 4C). Fig. 4D shows that CytD also up-regulated NF-κB-mediated transcription in HL-60 cells after transfection by a $(\kappa B)_5$LUC reporter construct and stimulation by TNFα or LPS. In these experiments, other CytD concentrations (1, 10, 25 and 50 μM) were used and similar results were observed (data not shown).

Altogether, these results suggest actin disruption by CytD increased *il-8* expression in response to TNFα and LPS not only by stabilizing *il-8* mRNA but also by up-regulating NF-κB-mediated transcription.

3.3. *Actin disruption up-regulates the canonical NF-κB activation pathway induced by LPS but not by TNFα*

To further investigate the mechanism by which the actin cytoskeleton disruption up-regulated the NF-κB-mediated transcription in response to TNFα and LPS, the effect of CytD was evaluated on different steps of the canonical NF-κB activation pathway.

First, the influence of CytD on p65 nuclear translocation in response to both inducers was assessed by carrying out an anti-p65 immunoblotting on nuclear extracts. As observed in Fig. 5A (top panel), TNFα-stimulation strongly induced nuclear translocation of p65, which appeared after 15 min and lasted for 1 h. Pre-treatment with CytD neither increased the levels of nuclear p65 nor affected the kinetics of p65 translocation in response to TNFα (Fig. 5A, top panel). LPS induced p65 translocation into the nucleus with a slower kinetic, as compared with TNFα (Fig. 5B, top panel). However, pre-treatment with CytD significantly increased p65 nuclear translocation induced by LPS, as demonstrated by the higher levels of nuclear p65 at various times (approximatively 14-fold vs. 7-fold, for 60 min of treatment by LPS) (Fig. 5B, top panel). The lack of cytoplasmic protein contaminations in nuclear extracts was checked by immunoblot against a nuclear (NBS-1) and a cytoplasmic protein (hsp60) (Fig. 5A and B, bottom panels).

To be more convincing, we also performed this experiment with other LPS and TNFα concentrations. Whatever the LPS concentration, a very strong stimulating effect of CytD on LPS-induced p65 translocation could be observed (Fig. 5D), while no effect of CytD was seen in the case of TNFα, even at lower concentrations leading to a partial p65 translocation (Fig. 5C). Therefore, these dose response experiments allowed us to exclude the possibility that the lack of CytD effect resulted from too high TNFα concentrations.

CytD had also a very efficient up-regulating effect on p65 translocation when cells were stimulated by Ultrapure LPS (data not shown), which rules out the hypothesis that CytD could exert its synergistic effects on another NF-κB activation pathway induced by another bacterial component than LPS.

In order to reinforce the results obtained in p65 nuclear translocation experiments, electromobility shift assays (EMSA) with a probe containing a consensus NF-κB site were carried out. As expected, CytD strongly increased DNA binding activity in nuclear extracts of LPS-treated HL-60 cells while it had no effect on TNFα-induced binding (Fig. 5E and F).

To extend these results in a more physiological setting, chromatin immunoprecipitation (ChIP) assays were carried out. After 15 min, TNFα very efficiently induced the recruitment of p65 on endogenous *il-8* promoter and this step was not significantly affected by CytD pre-treatment (Fig. 5G). Conversely, CytD significantly increased p65 recruitment on the *il-8* promoter at 60 min of treatment by LPS (13.2-fold vs. 4.2-fold) (Fig. 5H).

In the canonical pathway, NF-κB translocation into the nucleus results from the activation of the IKK complex which phosphorylates IκBα, leading to its polyubiquitination and degradation by the 26S proteasome. Accordingly, we examined whether the effect of CytD on LPS-induced NF-κB translocation could be explained by a modulating effect on the IKK complex activation. The IKK complex was then immunoprecipitated from cytoplasmic extracts and an in vitro kinase assay with a purified GST-IκBα_{1-54} fusion protein as substrate was carried out. The phosphorylated GST-IκBα was detected by western blotting with an antibody directed against IκBα phosphorylated on Ser^{32} and Ser^{36} (Fig. 6A and B, top panels). An anti-IKKβ immunoblot was also performed to check the efficiency of the immunoprecipitation in each sample (Fig. 6A and B, bottom panels). As illustrated in Fig. 6A, TNFα induced a strong and transient IKK complex activation which was not significantly modified by the presence of CytD. LPS induced an IKK complex activation delayed compared to TNFα but which was increased by CytD pre-treatment (3.15-fold vs. 1.5-fold for 1 h of treatment by LPS) (Fig. 6B). This up-regulating effect of CytD on IKK complex activation by LPS could at least partly explain the stronger nuclear translocation of p65 (Fig. 5B) and its more efficient recruitment to *il-8* promoter (Fig. 5H).

To induce NF-κB activation, LPS must be delivered onto its membrane receptor complex composed of dimerized TLR4 receptors (Toll-like receptor 4) and two molecules of the extracellular adapter MD-2 [25]. Then, TLR4 initiates signalling cascades through different adapters which eventually lead to the activation of the NF-κB [25]. The TLR4 resides in the endoplasmic reticulum (ER)/Golgi and on the plasma membrane and its sub-cellular distribution is regulated by different proteins [51]. Indeed, it has been demonstrated that the TLR4-MD2 complex can be endocytosed. In this way, this complex can be recycled between the plasma membrane and the Golgi apparatus in the absence and in the presence of the ligand [52]. Since receptor-mediated endocytosis can be dependent on actin cytoskeleton [53], CytD could slow down TLR4 endocytosis. Thus, the effect of CytD on the surface TLR4 levels in human monocytes was analyzed by flow cytometry. CytD alone had not a significant effect on surface TLR4 levels (Fig. 6C). Consistent with published reports [54,55], LPS treatment at 37 °C induced a decrease of cell surface TLR4 staining (a loss of 20% after 60 min), which means that TLR4 is internalized after ligand binding (Fig. 6C). When endocytosis was inhibited by incubating the cells at 4 °C or when human

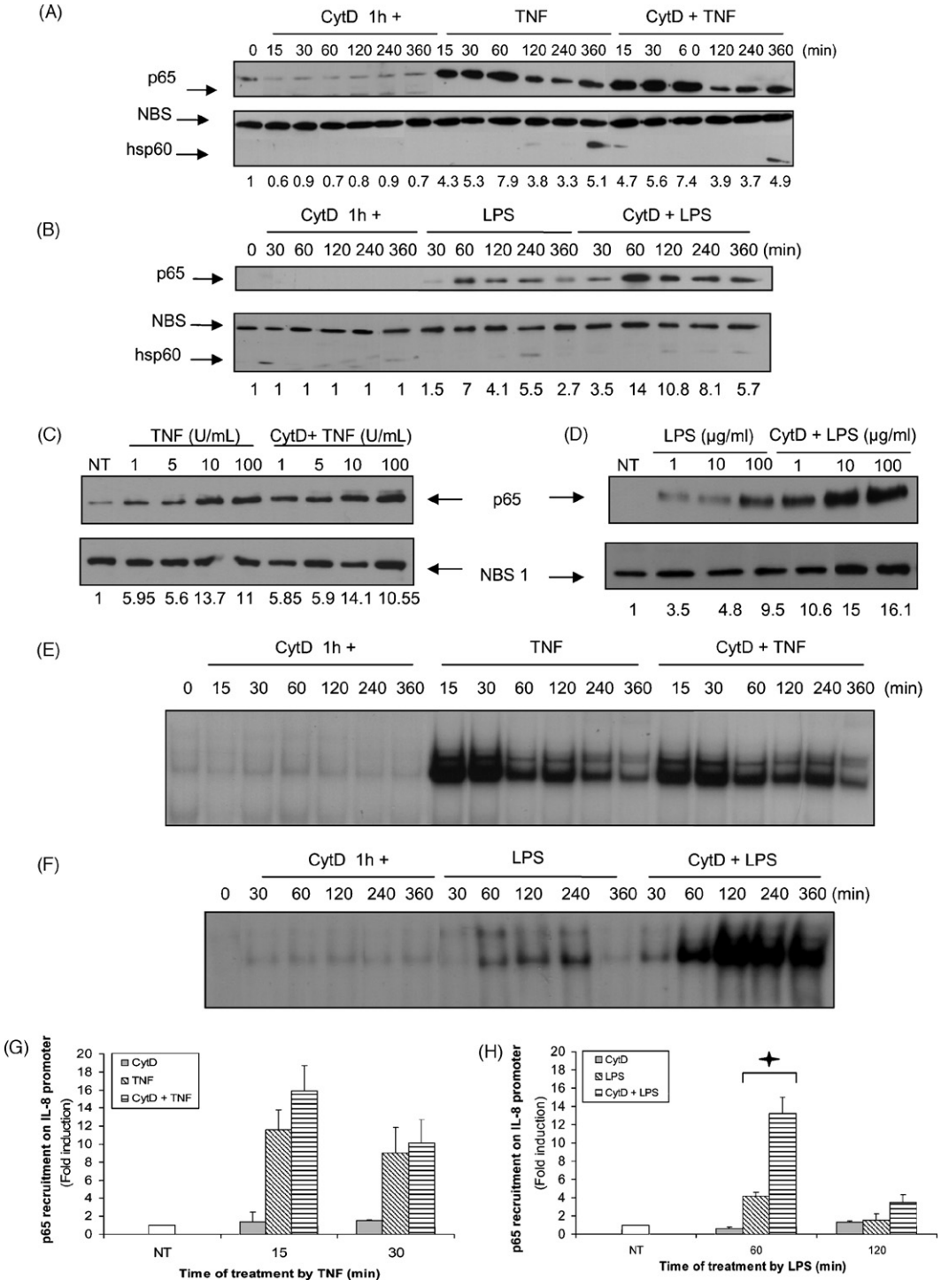

Fig. 5 – Differential impact of CytD on the p65 nuclear translocation and its recruitment on *il*-8 promoter induced by TNFα or LPS. HL-60 cells were incubated or not with CytD (5 μM) for 1 h and then treated or not with TNFα (100 U/ml) (A, E and G) or LPS (10 μg/ml) (B, F and H) for indicated times. (C and D) HL-60 cells were incubated or not with CytD (5 μM) for 1 h and then treated or not with TNFα (C) or LPS (D) at different concentrations for 30 min or 1 h, respectively. (A–D) Nuclear translocation of NF-κB was estimated by performing an anti-p65 immunoblot on nuclear extracts (top panels). The lack of contamination of nuclear extracts by cytoplasmic proteins was checked by western blotting against a nuclear protein (NBS-1) and a cytoplasmic protein (hsp60) (bottom panels). Results correspond to the ratios between NBS-1 and p65 levels which were measured with "Quantity one" (Biorad, Nazareth Eke, Belgium). (E and F) DNA-binding activities in nuclear extracts were analyzed by EMSA with a consensus NF-κB probe. (G and H) ChIPs assays were performed using anti-p65 antibody for the immunoprecipitation. Immunoprecipitated DNA was submitted to a real-time qPCR with specific primers targeting the promoter region of *il*-8. +, significantly different (*p* value <0.05).

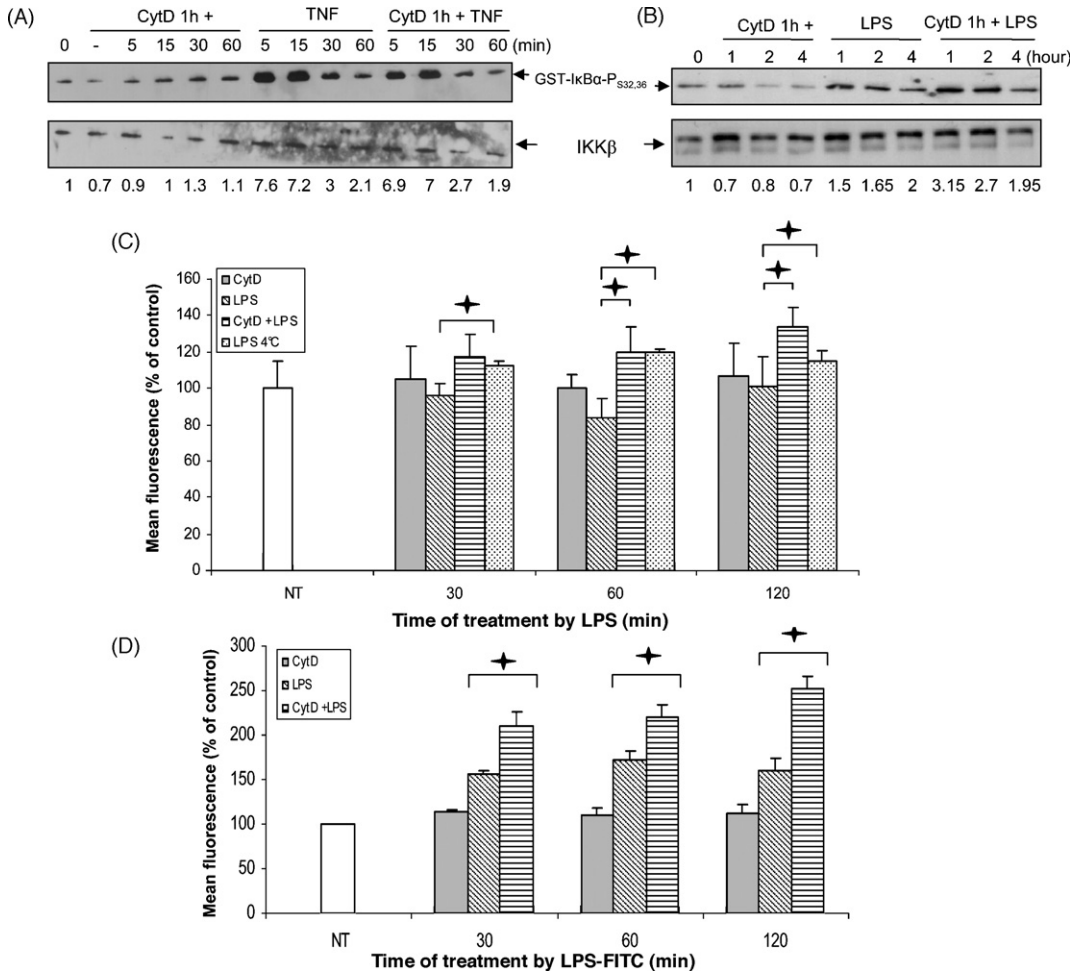

Fig. 6 – CytD up-regulates the LPS-induced IKK complex activation, prevents LPS-induced surface TLR4 down-regulation and leads to an higher LPS uptake. (A and B) HL-60 cells were incubated or not with CytD (5 μM) for 1 h and then stimulated or not with TNFα (250 U/ml) (A) or with LPS (10 μg/ml) (B) during indicated times. The IKK complex was immunoprecipitated with an anti-IKKγ antibody and a kinase assay in vitro was performed by using a purified GST-IκBα$_{1-54}$ fusion protein as substrate. Western blottings were carried out with antibodies anti-phospho-Ser^{32} and Ser^{36} IκBα and anti-IKKβ. Results correspond to the ratios between IKKβ and phospho-IκBα levels which were measured with "Quantity one software" (Biorad, Nazareth Eke, Belgium). (C) Human monocytes were incubated or not at 4 °C or with CytD (5 μM) for 1 h and then stimulated with LPS (100 ng/ml) during indicated times. The level of TLR4 at the plasma membrane was determined by using an anti-TLR4 antibody and an anti-rabbit-FITC secondary antibody before analysis by flow cytometry. +, significantly different (p value <0.05). Values on the graphs are presented as means ± S.D. (n = 3). (D) Human monocytes were pre-incubated or not with CytD (5 μM) for 1 h and then stimulated with LPS-FITC (10 μg/ml) during indicated times. The uptake of LPS-FITC after the different treatments was analysed by flow cytometry. +, significantly different (p value <0.05). Shown were means ± S.D. from three independent experiments.

monocytes were pre-treated by CytD, surface TLR4 levels did no longer decrease after LPS treatment, even they increased compared to untreated cells (Fig. 6C). The downregulation of surface CD14 after LPS stimulation of human monocytes was also prevented by CytD pre-treatment (data not shown).

To show that the increased levels of surface TLR4 upon CytD treatment correlate with a higher ability of the cell to bind LPS, we measured the LPS-FITC uptake by flow cytometry. As shown in Fig. 6D, CytD significantly up-regulated the LPS uptake by human monocytes at the different times of treatment (for example, 220% vs. 172% at 60 min of treatment).

Thus, it seems that CytD potentiated the LPS-induced NF-κB activation at least partially by increasing the membrane TLR4 levels, allowing more interactions between TLR4 and its ligand at the surface of the cells.

3.4. *Actin disruption up-regulates LPS- and TNFα-induced Ser^{536} and Ser^{276} p65 phosphorylations, respectively, and potentiates il-8 promoter activity*

A second level of NF-κB control apart from the well-studied nuclear translocation mechanism involves post-translational modifications. The best characterized process is the p65

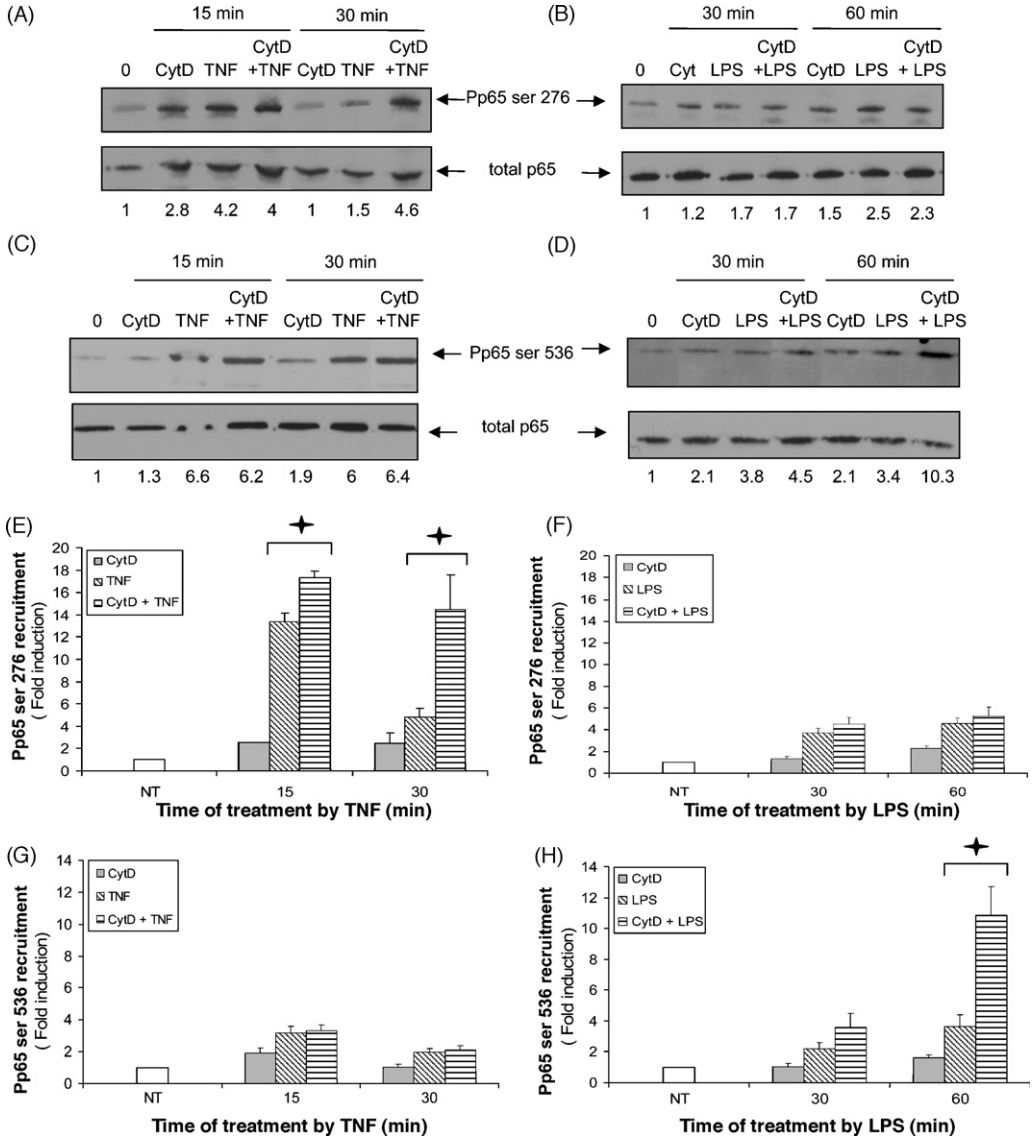

Fig. 7 – CytD treatment up-regulates TNFα-induced p65 Ser^{276} phosphorylation and LPS-induced p65 Ser^{536} phosphorylation leading to an increased recruitment of the respective p65 phosphorylated forms on il-8 promoter. (A–D) HL-60 cells were pre-treated or not with CytD (5 μM) during 1 h and then incubated or not with TNFα (100 U/ml) (A and C) or with LPS (10 μg/ml) (B and D) during indicated times. Western blots using different antibodies directed against: p65 phosphorylated on Ser^{276} (A and B, top panels) or on Ser^{536} (C and D, top panels) or total p65 (A–D, bottom panels) were performed on total extracts. Results correspond to the ratios between p65 and phospho-p65 levels which were measured with "Quantity one software" (Biorad, Nazareth Eke, Belgium). (E–H) ChIPs assays were performed on HL-60 cells pre-treated or not with CytD (5 μM) during 1 h and then incubated or not with TNFα (100 U/ml) (E and G) or with LPS (10 μg/ml) (F and H) using different antibodies directed against: p65 phosphorylated on Ser^{276} (E and F) and p65 phosphorylated on Ser^{536} (G and H). A real-time qPCR was performed with specific primers targeting the promoter region of il-8. ✦, significantly different (p value <0.05). The results presented on the graphs are an average of two independent experiments.

phosphorylation on the Ser^{276} and Ser^{536} residues. Furthermore, these two serines were shown to be phosphorylated in response to TNFα and/or LPS in various cell types [35,56–59]. Therefore, we assessed CytD influence on phosphorylation of both residues after LPS- or TNFα-stimulation of HL-60 monocyte-like cells. As shown by western blottings (Fig. 7A–D), CytD alone had a slight effect on the phosphorylation of both serines, which was more pronounced in the case of Ser^{276} at short times (2.8-fold at 15 min). TNFα induced a more important increase of the cellular level of phospho-Ser^{276} p65 at 15 min (4.2-fold) which declined after 30 min (1.5-fold) (Fig. 7A). CytD significantly extended the lifetime of phospho-Ser^{276} p65 induced by TNFα until 30 min (4.6-fold vs. 1.5-fold). Oppositely, LPS slightly induced the phosphorylation of Ser^{276} and CytD did not modify the phosphorylation of this residue induced by LPS (Fig. 7B). As shown in Fig. 7C and D, the pre-

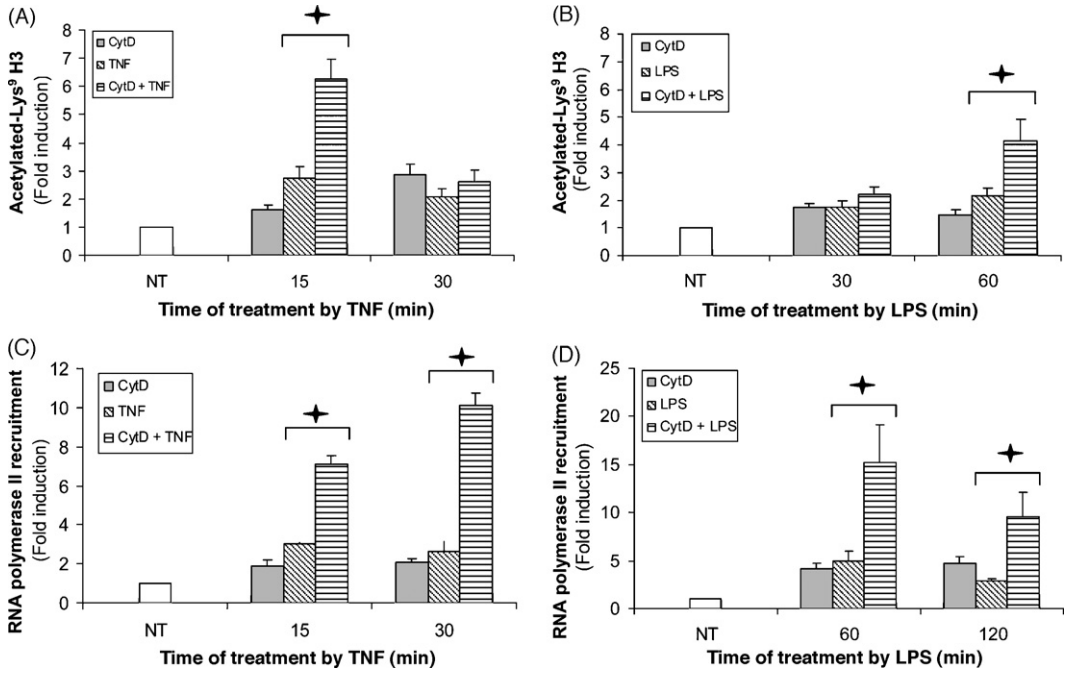

Fig. 8 – Impact of CytD on acetylated-Lys^9 H3 and RNA polymerase II recruitment on *il-8* promoter after TNFα or LPS treatment. ChIPs assays were performed on HL-60 cells pre-treated or not with CytD (5 μM) during 1 h and then incubated or not with TNFα (100 U/ml) (A and C) or with LPS (10 μg/ml) (B and D) using different antibodies directed against: H3 acetylated on Lys^9 (A and B) and RNA polymerase II (C and D). ChIPs assays analyzing acetylated Lys^9 H3 were normalized with unmodified H3. Immunoprecipitated chromatin was submitted to a real-time qPCR analysis using specific primers targeting the promoter region of *il-8*. +, significantly different (p value <0.05). Values on the graphs are presented as means ± S.D. (n = 3).

treatment with CytD seems to have the opposite effect on Ser^{536} phosphorylation. Indeed, CytD did not modify the levels of phospho-Ser^{536} p65 induced by TNFα (approximatively 6-fold with or without CytD, Fig. 7C) while it up-regulated the phosphorylation of this serine when cells were stimulated with LPS, especially at 60 min of treatment (10.3-fold vs. 3.4-fold, Fig. 7D). Thus, these experiments show that CytD modulates p65 phosphorylation differentially according to the inducer.

In order to confirm the relevance of these phosphorylation events in CytD-mediated up-regulation of *il-8* gene expression, the effects of CytD on the recruitment of phospho-Ser^{276} or phospho-Ser^{536} p65 to the native *il-8* promoter were tested. As shown by ChIP assays (Fig. 7E–H), CytD alone induced a slight recruitment of both p65 phosphorylated forms on *il-8* promoter. TNFα induced a strong increase in binding of phospho-Ser^{276} p65 at 15 min (13-fold) which declined after 30 min (5-fold) (Fig. 7E). CytD potentiated and significantly extended the TNFα-induced recruitment of phospho-Ser^{276} p65 until at least 30 min, which corroborated the results obtained by western blotting (Fig. 7A and E). LPS induced a slight recruitment of phospho-Ser^{276} p65 on endogenous *il-8* promoter in HL-60 cells which was not significantly increased with CytD pre-treatment (Fig. 7F).

In another hand, as shown in Fig. 7G and H, both inducers slightly induced the recruitment of phospho-Ser^{536} p65 at the indicated times of treatment. The pre-treatment with CytD did not modify the TNFα-induced recruitment of phospho-Ser^{536} p65 while it significantly increased the LPS-induced binding of this p65 phosphorylated form, with a strong effect at 60 min (11-fold vs. 4-fold). These ChIP assays corroborated the previous results obtained by western blotting anti- phospho-Ser^{536} p65.

Interestingly, this differential effect of CytD on the recruitment of phospho-Ser^{276} and -Ser^{536} p65 according to the inducer was also observed on the *iκbα* promoter (data not shown).

Previous works have shown that p65 phosphorylations on Ser^{276} and Ser^{536} may promote its interaction with its coactivator CBP/p300 which promotes the access of basal transcription machinery through the acetylation of specific sites of histones H3 and H4 [34–39]. Among the amino acid residues of H3, acetylation of Lys^9 is well known to be transcription-permissive [60]. So, we examined whether the up-regulating effect of CytD on the recruitment of phospho-Ser^{536} and -Ser^{276} p65 on *il-8* promoter could result in a stronger acetylation of H3 on Lys^9 and a more efficient recruitment of the RNA polymerase II.

Therefore, H3 Lys^9 acetylation was determined by ChIP assays on *il-8* promoter and was normalized with unmodified H3. Fig. 8A and B show a slight increase in Lys^9-acetylated H3 recruitment on *il-8* promoter after stimulation by both LPS and TNFα and CytD alone. The pre-treatment with CytD had an up-regulating effect on Lys^9-acetylated H3 binding on *il-8* promoter after LPS- and TNFα-stimulation (6-fold vs. 2.5-fold after 15 min for TNFα and 4-fold vs. 2-fold after 60 min for LPS).

These kinetics coincided with those of the ChIP assays analysing the recruitment of phospho-Ser^{536} and -Ser^{276} p65. Similar results were obtained with the *iκbα* promoter (data not shown).

Finally, to confirm that CytD potentiates *il-8* promoter activation by TNFα and LPS in monocyte-like cells, ChIP assays with an antibody recognizing RNA polymerase II (RNA pol II) were performed. As demonstrated in Fig. 8C and D, both inducers as well as CytD alone induced a small association of the RNA pol II on native *il-8* promoter. When cells were pre-incubated with CytD, the TNFα-induced recruitment of the RNA pol II was increased and, more importantly, extented until at least 30 min similarly to the TNFα-induced binding of phospho-Ser^{276} p65 (Fig. 8C). A significant up-regulating effect of CytD was also observed on LPS-stimulated RNA pol II association (Fig. 8D). Comparable results were observed on the *iκbα* promoter (data not shown).

4. Discussion

A growing body of evidences demonstrated that actin cytoskeleton, in addition to its well-known roles in cell morphology and motility, could modulate various signalling pathways [7–10,20]. Several classical NF-κB inducers, such as TNFα and LPS, can also lead to actin cytoskeleton reorganizations [14,16]. For these different reasons, we decided to elucidate the role of actin cytoskeleton in the NF-κB activation pathways induced by TNFα and LPS in monocyte-like cells.

We have demonstrated that actin disruption by CytD strongly potentiated NF-κB target genes expression, especially *il-8* gene, in LPS- and TNFα- stimulated monocyte-like HL-60 cells, by both transcriptional and post-transcriptional mechanisms. Transient transfection assays with reporter constructs containing the wild type or mutated *il-8* promoter upstream of the luciferase gene or with the $(\kappa B)_5$LUC reporter plasmid showed that the up-regulating effect of CytD on *il-8* gene transcription was mediated by NF-κB. By using Act D in real-time qRT-PCRs, we demonstrated that the post-transcriptional effect involved the stabilization of *il-8* mRNAs. Interestingly, the up-regulating effect of CytD on NF-κB-mediated transcription resulted from different mechanisms according to the inducer.

Indeed, in the case of LPS, we demonstrated by western blot experiments and EMSAs that the CytD increased the nuclear translocation of p65. Moreover, we also showed an increased recruitment of p65 on endogenous *il-8* promoter by ChIP assays. An up-regulating effect of CytD on IKK complex activity was also observed. Finally, we showed that CytD induced an increase of the membrane TLR4 levels as well as a stronger LPS uptake by human monocytes. It is currently well-known that TLR4 is endocytosed after LPS binding by a dynamin-dependent process. This TLR4 internalization is required to induce TRAM-TRIF-dependent signaling and production of type I interferon [55]. In contrast, a specific inhibitor of dynamin does not prevent TLR4-dependent NF-κB activation, consistent with the idea that TLR4 induces this signaling pathway from the plasma membrane through TIRAP-MyD88. Furthermore, another group demonstrated that the reduced endocytic activity caused by the expression of a dominant negative mutant for the dynamin even increased LPS-induced NF-κB activation [54]. Several studies demonstrated that actin cytoskeleton is involved in clathrin- and dynamin-dependent endocytosis processes [61–63]. All these published data and our results taken together allow us to claim that Cyt D, by interfering with LPS binding-induced TLR4 endocytosis, increases the LPS uptake by human monocytes and potentiates LPS-induced canonical NF-κB activation pathway involving IKK complex.

In addition to phosphorylate the inhibitory protein IκBα, IKK-α and -β are the best characterized p65 Ser^{536} kinases and are known to phosphorylate this residue in macrophages stimulated by LPS [58]. Accordingly, we can suggest that, in the case of LPS, the up-regulating effect of CytD on IKK complex could lead to an increase of p65 translocation and Ser^{536} phosphorylation. Both events would contribute to a more efficient NF-κB-mediated transcription.

At the opposite, CytD had no effect on the NF-κB activation classical pathway induced by TNFα. However, actin disruption significantly interfered with post-traductional modifications of p65 induced by TNFα. In this way, we demonstrated by western blotting that CytD up-regulated the phosphorylation of p65 on Ser^{276} in TNFα-stimulated cells. Moreover, we observed by ChIP assays that CytD potentiated the recruitment of phospho-Ser^{276} p65, acetylated-Lys^{9} H3 and RNA polymerase II on endogenous promoters in response to TNFα. This increased binding of phospho-Ser^{276} p65 on endogenous *il-8* promoter could favor the recruitment of the major coactivators of p65, CBP/p300 [39,56]. Indeed, it has been demonstrated that the phosphorylation of p65 on Ser^{276} induces conformational changes allowing the formation of a stable complex with p300 [60]. Consequently, CBP/p300 could acetylate H3 on Lys^{9}, which would favor the recruitment of RNA polymerase II on the endogenous promoter [32]. This hypothesis is reinforced by the work of Jamaluddin et al. which has demonstrated the important role of phospho-Ser^{276} p65 and p300 in the *il-8* gene expression in TNFα-stimulated promonocytic cells [56].

As described in the literature, two kinases could potentially phosphorylate the Ser^{276} of p65. These kinases are MSK-1 (mitogen- and stress-activated protein kinase-1) and PKAc (protein kinase A catalytic subunit). The nuclear kinase MSK-1 is activated by both ERK and p38 [57,64]. However, we observed that CytD alone induced the phosphorylation of p38 and ERK but did not further increase the phosphorylation of both MAPKs in response to TNFα in HL-60 cells (data not shown). In another hand, Adachi et al. [65,66] have reported that in resting cells, non-phosphorylated ERK was complexed with MEK in the cytoplasm, and upon phosphorylation, ERK dissociated from MEK and translocated into nucleus by different mechanisms. Moreover, Smith et al. suggested that the restriction of ERK nuclear translocation required intact cytoskeleton [67]. Indeed, they have observed that actin disruption induced by CytD allowed a better nuclear entry of activated ERK [67]. So, in our experiments, CytD could potentiate the nuclear translocation of MAP kinase(s), allowing an increase of MSK-1 phosphorylation followed by up-regulated phosphorylation of p65 on Ser^{276}.

In another hand, Jamaluddin et al. have recently demonstrated that the phosphorylation of p65 on Ser^{276} in TNFα-

stimulated promonocytes U937 was mediated by the PKAc and favored the recruitment of coactivators on the *il-8* promoter [56]. They also showed that the activation of the PKAc and, consequently, the p65 phosphorylation on Ser^{276} in TNFα-stimulated monocytes required the production of ROS (reactive oxygen species). In a previous work, we have demonstrated that the perturbations of actin dynamics by CytD in monocyte-like cells induced the ROS production through their NADPH oxidase [20]. Accordingly, we can assume that CytD could up-regulate the TNFα-induced PKAc activation through ROS production, leading to an increased p65 ser^{276} phosphorylation and to a more efficient *il-8* gene transcription.

The up-regulating effect of CytD on both TNFα- and LPS-induced *il-8* gene transcription cannot explain by itself the strong synergistic effect on the *il-8* mRNAs levels. Indeed, we showed that CytD also plays an important role at the post-transcriptional level by up-regulating the TNFα- and LPS-induced *il-8* mRNA stabilization. The regulation of mRNA stability is often mediated by AUUUA sequences (ARE) within the 3′-untranslated region (UTR) [68–70]. Four of these AU-rich elements have been identified in the 3′-UTR of the *il-8* mRNA [71]. Several factors, like TTP (tristetraprolin) and HuR, which specifically interact with these AU-rich elements, have been implicated in the negative or positive regulation of the mRNA stability, respectively [72,73]. Many groups have demonstrated that p38 and ERK pathways could be involved in the stabilization of various mRNAs [71,74–76]. As described above, we did not observe any up-regulating effect of CytD on TNFα- and LPS-induced p38 and ERK activation in HL-60 cells. Alternatively, another group demonstrated that the factor HuR exerted its stabilizing effect independently on the p38 pathway when over-expressed in a macrophage cell line [77].

Moreover, Henics et al. [78] observed a stabilization of IL-2 mRNA after the remodeling of actin architecture. They showed that unknown AU rich-repeats binding proteins (AUBPs) were able to interact with actin and that actin disruption allowed an increased binding of theses AUBPs to the AUUUA sequences. Accordingly, the disruption of actin network by CytD in HL-60 cells could induce the release of an AUBP and promote its binding to the 3′-UTR of the *il-8* mRNA, leading to *il-8* mRNA stabilization.

In ChIP assays, we have observed that CytD up-regulated the recruitment of acetylated-lys^9 H3 on *il-8* gene promoter. We interpretated this result as a consequence of the more important binding of phospho-Ser^{276} or ^{536}p65 on *il-8* promoter, leading to a more efficient p300 recruitment. However, our results cannot exclude that the up-regulation of H3 acetylation resulted from another mechanism than the recruitment of CBP/p300. Indeed, acetylation of histones in response to alterations of actin cytoskeleton has been already described in other contexts involving neither CBP/p300 nor NF-κB target promoters [79].

The major aim of this work was to study the modulating effect of CytD on TNFα- and LPS-mediated IL-8 expression. However, an effect of CytD alone was observed in some experiments. Indeed, CytD alone significantly increased *il-8* mRNA levels and IL-8 production. CytD, at the concentration used in this work (5 μM), did not significantly induce the canonical NF-κB activation pathway as demonstrated by the failure of CytD alone to activate the IKK complex and to induce the p65 nuclear translocation, the binding to a probe containing a consensus NF-κB site and the p65 recruitment on endogenous *il-8* promoter. However, CytD alone had a slight effect on the binding of p65 phosphorylated forms, acetylated-Lys^9 H3 and RNA polymerase II to endogenous *il-8* promoter. These events partially explain the stimulating effect of CytD alone on *il-8* promoter-mediated transcription, *il-8* mRNA expression and IL-8 production. As already reported in the literature [78], CytD alone could also exert a post-transcriptional effect leading to a stabilization of constitutive *il-8* mRNA.

Monocytes/macrophages are important TNFα-targets and represent the major source of IL-8 in blood. TNFα inhibits the migratory response of the monocytes/macrophages and neutrophils in response to chemotactic stimuli. This phenomenon probably serves to retain monocytes/macrophages and neutrophils at infection sites [14,80]. This inhibitory effect of TNFα is explained by modulations of actin cytoskeleton. Indeed, TNFα is able to prevent the formation of Cdc42-dependent actin structures, such as filopodia, and induces a decrease of global F-actin in macrophages [14]. It seems that actin disruption could prime the pro-inflammatory response of monocytes/macrophages by different ways. First, actin disruption could reinforce the TNFα-induced inhibiting effect on monocytes/macrophages migration. Second, actin disruption could up-regulate the production of ROS and finally increase the expression of NF-κB-dependent genes.

Acknowledgements

We thank the platform Imaging and Flow cytometry, a CRCE/GIGA platform, and more especially we are grateful to S. Ormenese which is logistic collaborator FRS-FNRS of this platform for her technical assistance. This work was supported in part by the Interuniversity Attraction Poles (IAP)5/12 and IAP6/18 programs (Brussels, Belgium), Televie (Brussels, Belgium) and the National Fund for Scientific Research (FNRS Brussels, Belgium). GK and JH were supported by the IAP program and the Télévie, NEM is research assistant at the University of Liège, SLP and NJ are Research Associate and JP is Research Director of the FNRS.

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