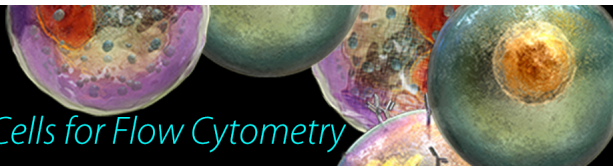


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Cyclophilin B Attenuates the Expression of TNF- α in Lipopolysaccharide-Stimulated Macrophages through the Induction of B Cell Lymphoma-3

Adeline Marcant, Agnès Denys, Aurélie Melchior,¹ Pierre Martinez, Audrey Deligny,² Mathieu Carpentier, and Fabrice Allain

Extracellular cyclophilin A (CyPA) and CyPB have been well described as chemotactic factors for various leukocyte subsets, suggesting their contribution to inflammatory responses. Unlike CyPA, CyPB accumulates in extracellular matrixes, from which it is released by inflammatory proteases. Hence, we hypothesized that it could participate in tissue inflammation by regulating the activity of macrophages. In the current study, we confirmed that CyPB initiated *in vitro* migration of macrophages, but it did not induce production of proinflammatory cytokines. In contrast, pretreatment of macrophages with CyPB attenuated the expression of inflammatory mediators induced by LPS stimulation. The expression of TNF- α mRNA was strongly reduced after exposure to CyPB, but it was not accompanied by significant modification in LPS-induced activation of MAPK and NF- κ B pathways. LPS activation of a reporter gene under the control of TNF- α gene promoter was also markedly decreased in cells treated with CyPB, suggesting a transcriptional mechanism of inhibition. Consistent with this hypothesis, we found that CyPB induced the expression of B cell lymphoma-3 (Bcl-3), which was accompanied by a decrease in the binding of NF- κ B p65 to the TNF- α promoter. As expected, interfering with the expression of Bcl-3 restored cell responsiveness to LPS, thus confirming that CyPB acted by inhibiting initiation of TNF- α gene transcription. Finally, we found that CyPA was not efficient in attenuating the production of TNF- α from LPS-stimulated macrophages, which seemed to be due to a modest induction of Bcl-3 expression. Collectively, these findings suggest an unexpected role for CyPB in attenuation of the responses of proinflammatory macrophages. *The Journal of Immunology*, 2012, 189: 2023–2032.

Macrophages are a primary source of a number of proinflammatory cytokines and chemokines, which are expressed in response to pathogen infection and other pathological stimuli. Although secretion of these proinflammatory mediators plays a beneficial role in protecting host from infection or injury, prolonged and excessive exposure is associated with a number of pathological disorders, including chronic inflammation, septic shock, or rheumatoid arthritis. Thus, the inflammatory response of macrophages must be tightly regulated, and a number of negative-feedback systems have evolved to reduce the production of proinflammatory mediators. These include the expression of B cell lymphoma-3 (Bcl-3), which has been reported to target NF-

κ B-dependent gene expression. Bcl-3 was originally identified as a proto-oncogene in B cell leukemia (1). It was later found to be expressed in various cell types, including monocytes/macrophages, lymphocytes, and keratinocytes, in which it modulates cell proliferation and TLR signaling. Bcl-3 is a member of the I κ B family of NF- κ B inhibitors. In contrast to the cytoplasmic I κ Bs, Bcl-3 associates with p50 and p52 homodimers but not with other NF- κ B dimers in the nucleus (2). Binding of NF- κ B p50 dimers to κ B sites was demonstrated to repress LPS-induced TNF- α gene transcription in macrophages, which has been related to a negative-feedback system to regulate production of the cytokine (3, 4). Bcl-3 is induced by LPS with delayed kinetic in macrophages and facilitates accumulation of NF- κ B p50 homodimers in the nucleus. Therefore, LPS presumably causes transient induction of activating NF- κ B p65/p50 heterodimers, which are then gradually replaced by p50 dimers associated to Bcl-3 (5). Bcl-3 has also been implicated in the anti-inflammatory properties of a number of regulatory cytokines. For instance, IL-10 induces the expression of Bcl-3 in LPS-stimulated macrophages, which is required to attenuate the production of TNF- α (6). Bcl-3 was also implicated in the down-regulatory effect of IL-4/IL-13 on the production of antimicrobial peptides from keratinocytes (7).

Originally described as the host cell receptors for the immunosuppressive drug cyclosporin A (8), cyclophilins are ubiquitously distributed intracellular proteins, mainly involved in the regulation of protein folding (9). However, accumulating data have implicated extracellular cyclophilins (i.e., cyclophilin A [CyPA] and B [CyPB]), as intercellular mediators in inflammation (10). High levels of extracellular cyclophilins have been observed in inflammatory disorders, such as sepsis, rheumatoid arthritis, and atherosclerosis (11–13). Although cytosolic, CyPA can be secreted by macrophages and vascular smooth muscle cells in response to LPS and oxidative stress

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Abbreviations used in this article: Bcl-3, B cell lymphoma-3; ChIP, chromatin immunoprecipitation; CRE, cAMP response element; CyPA, cyclophilin A; CyPB, cyclophilin B; MMP, matrix metalloproteinase; qPCR, quantitative PCR; siRNA, small-interfering RNA.

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(14, 15). Unlike CyPA, CyPB is constitutively secreted by tissue-resident cells (e.g., fibroblasts, chondrocytes, and keratinocytes) and accumulates within the extracellular matrix (16–18). Importantly, it is released intact by heparanase and matrix metalloproteinases (MMPs), suggesting that it can participate in tissue inflammation. Both CyPA and CyPB exhibit potent chemotactic properties *in vitro* and *in vivo*, indicating that they contribute to leukocyte infiltration during the acute phase of inflammation (14, 19–22). CD147, also known as an extracellular MMP inducer, was demonstrated to act as a signaling receptor for extracellular cyclophilins and mediate their chemotactic activity via a mechanism involving Ca^{2+} mobilization and p44/p42 MAPK activation (19, 20).

Although the role of cyclophilins in mediating leukocyte recruitment during the acute phase of inflammation is now well established, their contribution to the expression of proinflammatory mediators in monocytes/macrophages is more controversial. CyPA was reported to induce the production of proinflammatory cytokines and chemokines, such as TNF- α , IL-1 β , IL-6, CXCL8/IL-8, and CCL2/MCP-1 (23). However, these findings are in contradiction with other studies, which demonstrated that the levels of proinflammatory mediators were either undetectable or very low, but without any comparison with those obtained with TLR agonists (24–26). This discrepancy might be explained by the presence of LPS or other bacterial contaminations in recombinant CyPA preparations. This is in agreement with the study of Payeli et al. (24), who reported that some commercially available CyPA exhibits low purity and can be contaminated by endotoxin.

We have previously reported that CyPB is released from the extracellular matrix as a consequence of the activation of inflammatory MMPs and that it was more efficient than CyPA to trigger chemotaxis and integrin activation in T lymphocytes (17, 20). However, the contribution of CyPB to the inflammatory responses of macrophages has never been addressed. In the current study, we have therefore investigated the effects of CyPB on the major responses elicited by macrophages *in vitro*. Our results indicate that CyPB was efficient to initiate migration of macrophages but it did not induce the production of proinflammatory cytokines and chemokines. In contrast, pretreatment of macrophages with CyPB strongly reduced the expression of inflammatory mediators induced by subsequent stimulation with LPS. We then analyzed the inhibitory mechanisms by which CyPB inhibits LPS-induced production of TNF- α . We found that CyPB potently reduced TNF- α gene transcription in LPS-stimulated macrophages, and this was dependent on the expression of Bcl-3. Collectively, our results suggest an unexpected role for CyPB in the modulation of proinflammatory responses of macrophages.

Materials and Methods

Production of recombinant cyclophilins

Recombinant human CyPA and CyPB were produced in *Escherichia coli* essentially as described previously (20, 27). The material was further purified by heparin-sepharose chromatography and detoxified on Detoxi-Gel Endotoxin Removing Gel (Pierce Biotechnology). The purity of recombinant proteins was determined to be >98% by SDS-PAGE. LPS contamination was analyzed with the *Limulus* amoebocyte lysate assay (BioWhittaker) and found to be <0.009 endotoxin units/ μg .

Preparation of human monocyte-derived macrophages and cell culture

Human citrated venous blood samples were obtained from the local blood transfusion center (Etablissement de Transfusion Sanguine, Lille, France). Following isolation of PBMC by density centrifugation on Lymphoprep (AbCys), monocytes were purified by selection with magnetic beads coupled to CD14, according to the instructions of the manufacturer (BD Biosciences). Macrophages were obtained by incubating freshly isolated

monocytes (10^6 cells/ml) in complete RPMI 1640 medium supplemented with 10 ng/ml M-CSF (AbCys) for 5 d. The purity of cell populations was assessed by FACS and found to be >95%. Human promonocytic leukemia THP-1 cells (88081201; European Collection of Cell Cultures) were routinely cultured in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FCS and 10 mM gentamicin. To induce responsiveness to CyPB, THP-1 cells were differentiated for 72 h with 50 nM 1-25-dihydroxy-vitamin D₃, as described (28).

In vitro chemotaxis assays

Cell chemotaxis was assayed *in vitro* essentially as described (20). In brief, macrophages were adjusted at 8×10^6 cells/ml in RPMI 1640 culture medium supplemented with 0.5% BSA. Chemotactic activity was evaluated in a microchemotaxis chamber containing 8- μm pore polycarbonate membranes (Corning Costar). The chemotactic index was calculated as the number of cells migrating toward the test sample divided by the number of cells migrating toward control medium.

Measurement of cytokine/chemokine production in culture supernatants

For cytokine and chemokine immunoassays, macrophages or THP-1 cells were plated at 1×10^5 cells/well in 96-well plates and incubated in the absence or presence of various concentrations of recombinant cyclophilins and/or 10 ng/ml LPS (*E. coli* 055:B5; Sigma-Aldrich). Supernatants were collected after 4 or 16 h of stimulation, and the production of TNF- α , IL-1 β , IL-6, CCL5/RANTES (AbCys), CXCL8/IL-8, and IL-10 was measured by sandwich ELISA, according to the instructions of the manufacturer (R&D Systems).

Western immunoblotting

Cells (1×10^6 /point) were lysed in 60 μl 1% Triton X-100 lysis buffer (20 mM HEPES, 500 mM NaCl, and 0.2 mM EDTA [pH 7.9]) containing 25% (v/v) glycerol, 1 mM sodium NaVO_3 , 10 mM NaF, and 1 \times protease inhibitor mixture (Roche) for 3 h at 4°C. The lysates were clarified by centrifugation at $10,000 \times g$ for 30 min at 4°C, mixed with Laemmli buffer, and boiled for 10 min. Proteins were separated by SDS-PAGE (10%, w/v) and transferred onto nitrocellulose membranes (Schleicher & Schuell BioScience). Immunoblotting was performed using Abs to p44/p42 MAPK (Erk1/2; Sigma-Aldrich), phospho-JNK1/2 (Thr¹⁸³/Tyr¹⁸⁵ and Thr²²¹/Tyr²²³; Upstate Biotechnology), JNK1/2, p38 MAPK, Bcl-3, GAPDH (Santa Cruz Biotechnology), NF- κB p65, phospho-NF- κB p65 (Ser⁵³⁶), phospho-p44/p42 MAPK (Thr²⁰²/Tyr²⁰⁴), I $\kappa\text{B}\alpha$, phospho-I $\kappa\text{B}\alpha$ (Ser³²/Ser³⁶), or phospho-p38 MAPK (Thr¹⁸⁰/Tyr¹⁸²; Cell Signaling Technology). In all cases, membranes were blocked for 1 h at room temperature in TBS supplemented with 0.1% (v/v) Tween-20 and 5% (w/v) nonfat dry milk and then probed for 2 h with primary Abs in TBS supplemented with 1% (w/v) nonfat dry milk. After washing, the blots were developed using HRP-conjugated secondary Abs and an ECL detection kit (Amersham Biosciences).

RNA isolation and real-time RT-PCR

Total RNA was isolated from 3×10^6 cells using a NucleoSpin RNA II kit, according to the instructions of the manufacturer (Macherey-Nagel). Reverse transcription of mRNA encoding TNF- α and Bcl-3 was performed from 2 μg total RNA with an oligo(dT) primer and Moloney murine leukemia virus reverse transcriptase (Promega). The transcript of GAPDH was used as a control to normalize for total mRNA input and confirm efficiency of cDNA synthesis. PCR reactions were performed essentially as described in Deligny et al. (29). The synthetic primers were designed by using Primer Premier 5.0 (Biosoft International), according to the published cDNA sequences of TNF- α and Bcl-3 (accession numbers: NM_000594.2 and NM_005178.3, respectively). Primer sets, purchased from Eurogentec (Seraing, Belgium) were as follows: TNF- α , 5'-CTT CTC CTT CCT GAT CGT GG-3' (sense) and 5'-TCT CAG CTC CAC GCC ATT-3' (antisense); and Bcl-3, 5'-GAA AAC AAC AGC CTT AGC ATG GT-3' (sense) and 5'-TGC CGG AGT ACA TTT GCG-3' (antisense). PCR reactions were performed using the Mx3000P QPCR System (Stratagene), and relative expression of amplified products was calculated with the MxPro QPCR software (Stratagene). The sequence of each amplified product was confirmed by sequencing (Genoscreen, Lille, France).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed using the MAGnify ChIP System from Invitrogen. According to the instructions of the manufacturers, cells (2×10^5 /sample) were suspended in lysis buffer and

sonicated to obtain chromatin fragments containing DNA sequences of 200–500 bp. Chromatin was then diluted 10 times in ChIP buffer supplemented with Abs coupled to protein A/G agarose magnetic beads. Immunoprecipitation was performed using Abs to NF- κ B p65 (Cell Signaling Technology), NF- κ B p50, Bcl-3, c-Jun (Santa Cruz Biotechnology), or appropriate isotype-matched control IgGs (all from Sigma-Aldrich). Following treatment with reverse cross-linking buffer and proteinase K, DNA fragments from ChIP complexes or input control samples were purified with DNA purification magnetic beads and analyzed by real-time PCR. Three sets of primers were designed to amplify the sequences of the TNF- α gene promoter covering the κ B1 site alone, a first cluster containing κ B3 and cAMP response element (CRE) sites (κ B3/CRE), and a second cluster containing κ B2, ξ , and κ B2a sites (κ B2-2a) (accession number: M16441). Primer sets were as follows: κ B1, 5'-CTC TGA GGA ATG GGT TAC AGG A-3' (sense) and 5'-CCA GTG TGT GGC CAT ATC TTC-3' (antisense); κ B2-2a, 5'-CCA AGA CTG AGA CCA GCA TTA TG-3' (sense) and 5'-TGA GTC CTG AGG CCT GTG TTT G-3' (antisense); and κ B3/CRE, 5'-CCC TCC AGT TCT AGT TCT ATC-3' (sense) and 5'-GGG GAA AGA ATC ATT CAA CCA G-3' (antisense).

Reporter gene assays

Induction of NF- κ B-dependent gene transcription was analyzed by using a 5 \times multimerized κ B luciferase reporter construct (pNF- κ B-luc; Agilent Technologies). Briefly, macrophages were transiently transfected with 1 μ g poly- κ B reporter gene using the nucleofection technology according to Amaxa Biosystems instructions. After transfection, complete medium was added to the cells and incubated for 16 h. Cells were then stimulated with recombinant cyclophilins (50 nM) or LPS (10 ng/ml) for 8 h. Because the efficacy of transfection was weak in primary macrophages, we decided to use adenoviral construct to study the activation of TNF- α promoter. The adenoviral vector encoding the luciferase reporter gene under the control of human TNF- α gene promoter (Advp5') has been described previously (30) and was kindly provided by B. Foxwell (Kennedy Institute of Rheumatology, London, U.K.). For macrophage infection, 1×10^5 cells/point at a density of 1×10^6 cells/ml were exposed to virus at the optimal multiplicity of infection of 40:1 for 4 h in culture medium with 2.5% FCS, followed by washing and reculturing for 16 h in complete medium. Infected cells were either directly used for analyzing the activation of the TNF- α promoter by CyPB (50 nM) or LPS (10 μ g/ml) or preincubated with CyPB (50 nM) or medium alone for 16 h and thereafter stimulated with LPS (10 ng/ml). After stimulation, cells were treated with luciferase assay kit (Promega), and luciferase activity was measured using a Tristar multimode microplate reader LB 941 (Berthold Technologies). Relative luciferase activities were normalized to protein content (micro BCA protein assay reagent kit; Pierce Biotechnology).

RNA interference

Synthetic small interfering RNA (siRNA) duplexes with symmetric 3' deoxythymidine overhangs were used to carry out RNA interference [essentially as described in Ref. (29)]. A set of three distinct synthetic siRNA duplexes, corresponding to the Bcl-3 mRNA sequences 5'-GAG CCU UAC UGC CUU UGU A(dTdT)-3', 5'-GAG CUC GAC AUC UAC AAC A(dTdT)-3', and 5'-CCC AAU UUC UUC CUU CCU U(dTdT)-3' was designed (Eurogentec) and tested for its efficiency to downregulate the expression of Bcl-3. Negative control siRNAs, in which two nucleotides have been changed from the target sequence, were used to demonstrate the specificity of silencing (modified nucleotides, U \rightarrow A and A \rightarrow U, are underlined in target mRNA sequences). A synthetic siRNA duplex (siGFP) targeting GFP mRNA was used as an irrelevant control. THP-1 cells were transiently transfected by nucleofection, thereafter transferred into prewarmed complete maintenance medium, and cultured as described before. To monitor the transfection efficiency, a fluorescein-tagged siRNA duplex was transfected in parallel, and the transfection rate was evaluated by FACS and found to be >85%.

Statistical analysis

Results are representative of at least three independent experiments conducted with either monocyte-derived macrophages from distinct donors or different preparations of differentiated THP-1 cells. Data are presented as means \pm SD. Statistical significance of the differences between means was performed by one-way ANOVA or two-tailed Student *t* test using GraphPad Prism software (GraphPad). The *p* values < 0.05 were considered significant.

Results

CyPB induces signaling events and chemotaxis on human primary macrophages

In previous studies, we demonstrated that CyPB is a chemoattractant factor for human peripheral blood CD4⁺ T lymphocytes

via a mechanism dependent on the activation of p44/p42 MAPK (20, 31). In the current study, we confirmed these findings with human macrophages and extended them to show that CyPB also activates the NF- κ B pathway (Fig. 1). As expected, *in vitro* migration of macrophages was achieved at 10 nM, which was similar to the concentration required to induce a migratory response in T cells. Moreover, Western blot experiments demonstrated that p44/p42 MAPK were rapidly phosphorylated following stimulation with 50 nM CyPB, peaking at 15 min. Next, we showed that I κ B α , which is complexed to NF- κ B and inhibits its nuclear translocation in resting cells, was rapidly phosphorylated and subsequently degraded following cell stimulation with CyPB. In parallel experiments, we found that NF- κ B p65 was phosphorylated, thus confirming activation of the canonical transcription factor. In contrast, CyPB failed to activate p38 MAPK and JNK at concentrations giving a full activation of p44/p42 MAPK and NF- κ B. Increasing the concentrations of CyPB to 500 nM had no more effect on the phosphorylation status of these kinases (data not shown). This is in agreement with our previous work showing that CyPB at physiological concentrations did not activate p38 MAPK and JNK in T lymphocytes (31).

CyPB does not induce the expression of TNF- α in macrophages

Previous studies had reported that CyPA induced the production of inflammatory factors, such as TNF- α , from monocytes/macrophages (23–26). Hence, we investigated whether CyPB was capable of inducing a similar response. Unlike LPS used as positive control (10 ng/ml), we found that CyPB, at concentrations varying from

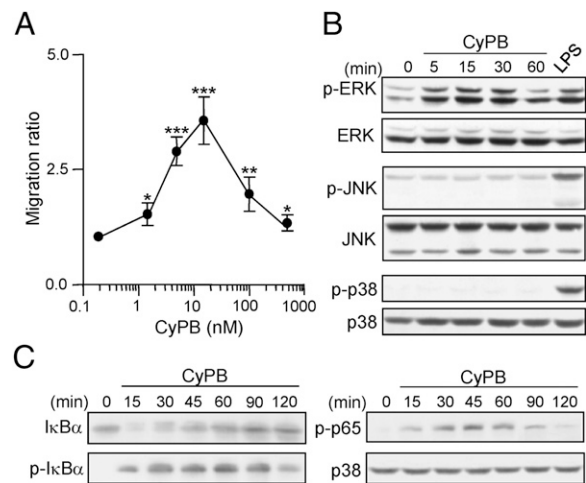


FIGURE 1. CyPB induces migration of macrophages and activation of p44/p42 MAPK and NF- κ B pathways. **(A)** The chemotactic response of monocyte-derived macrophages was expressed as the number of cells migrating toward the indicated concentrations of CyPB divided by the number of cells migrating toward culture medium. Data represent means \pm SD of triplicate obtained with macrophages from a single individual and are representative of three experiments conducted with cells of different donors. **p* < 0.05, ***p* < 0.01, ****p* < 0.001. **(B)** Macrophages were stimulated in the presence of 50 nM CyPB for the indicated times. Cell lysates were then subjected to Western blotting for phospho-p44/p42 MAPK (p-ERK), phospho-JNK (p-JNK), or phospho-p38 MAPK (p-p38). Parallel immunoblotting with anti-total ERK, JNK, and p38 confirmed equal loading of samples. **(C)** CyPB-induced activation of NF- κ B pathway was analyzed by measuring modifications in the levels of total I κ B, phospho-I κ B α (p-I κ B α), and phospho-NF- κ B p65 (p-p65). Total p38 was used as a loading control. In each case, experiments were performed with monocyte-derived macrophages from three different individuals with comparable results.

0.1 to 1000 nM, neither induced the expression of TNF- α (Fig. 2A) nor the production of TNF- α mRNA (Fig. 2B) from macrophages in culture. NF- κ B activated by CyPB was functional, because CyPB was efficient in inducing luciferase activity in a luciferase assay carried out with a poly-NF- κ B-dependent reporter gene (Fig. 2C). Importantly, there was no major difference with the activation induced by LPS (10 ng/ml), indicating that CyPB was almost as efficient as LPS in activating the NF- κ B pathway. We then analyzed the effect of CyPB on macrophages that transiently expressed the TNF- α promoter linked to the luciferase gene. To this end, we used an adenoviral construct (Advp5'), which encodes for luciferase under the control of TNF- α gene promoter (30). As expected, LPS strongly induced luciferase activity in infected cells. In contrast, CyPB was not capable of enhancing transcription of the Advp5' construct (Fig. 2D). A weak luciferase activity could be noted at 6 h, but it was not statistically different from the constitutive activation of the reporter gene. Collectively, these data seemed to indicate that the signaling events elicited by CyPB were not sufficient to activate the TNF- α promoter.

The promoter of the gene encoding TNF- α has been studied in great detail. It contains five κ B-binding elements, which can be transactivated by NF- κ B p65/p50. Two of them are denoted κ B1 and κ B3 and are distinctly located at distal (−873 to −864 nt) and proximal (−98 to −89 nt) regions of the promoter, respectively. Others, termed κ B2, ξ , and κ B2a sites, are clustered within a 39-nt segment located 598 nt upstream of the transcriptional start. The TNF- α also contains several binding elements for c-Jun. Notably, a CRE binding site, which is close to the κ B3 element (−106 to −99 nt), is critically required for the binding of c-Jun/activating transcription factor-2 complexes and consequent activation of TNF- α promoter (32–36). To investigate the effect of CyPB on the binding of transcription factors to the TNF- α promoter, we performed ChIP assays on nuclear fractions extracted from macrophages that had been stimulated for 1 h with 50 nM CyPB or 10 ng/ml LPS. We used three pairs of primers for amplification of the sequences containing κ B1, κ B3/CRE, or clustered κ B2, ξ , and κ B2a sites (termed κ B2-2a). As expected, LPS stimulation of macrophages induced the binding of NF- κ B p65 and c-Jun to the TNF- α gene promoter, as demonstrated by amplification of the sequences from DNA complexes that have been precipitated with anti-p65 and anti-c-Jun Abs. Consistent with other studies (32, 35), the strongest binding of NF- κ B p65 was observed at the sites κ B2-2a and κ B1. Following CyPB stimulation, the sequences containing κ B1 and κ B2-2a sites were also amplified, indicating that CyPB induced the binding of NF- κ B to distal κ B sites. However, CyPB did not induce the binding of c-Jun to the CRE site of TNF- α promoter, and this was accompanied by a weak binding of NF- κ B p65 to κ B3 site (Fig. 2E). In all cases, DNA amplification in negative controls, in which ChIP reaction was performed with irrelevant Abs, was either undetectable or lower than the basal levels of DNA complexes in unstimulated macrophages, thus confirming the specificity of the method. Collectively, these data indicate that CyPB was not capable of transactivating the proximal TNF- α promoter in macrophages, thus giving a possible explanation for the nonproduction of the proinflammatory cytokine.

CyPB reduces LPS-induced cytokine production from human primary macrophages

Next, we have analyzed the ability of CyPB to modulate the expression of other cytokines and chemokines by measuring the secretion of IL-1 β , IL-6, IL-10, CXCL8/IL-8, and CCL5/RANTES from human macrophages in culture (Fig. 3). By comparison with LPS, CyPB induced a low but significant production of IL-6 from macrophages (0.32 ± 0.16 ng/ml with 50 nM CyPB, $p < 0.05$,

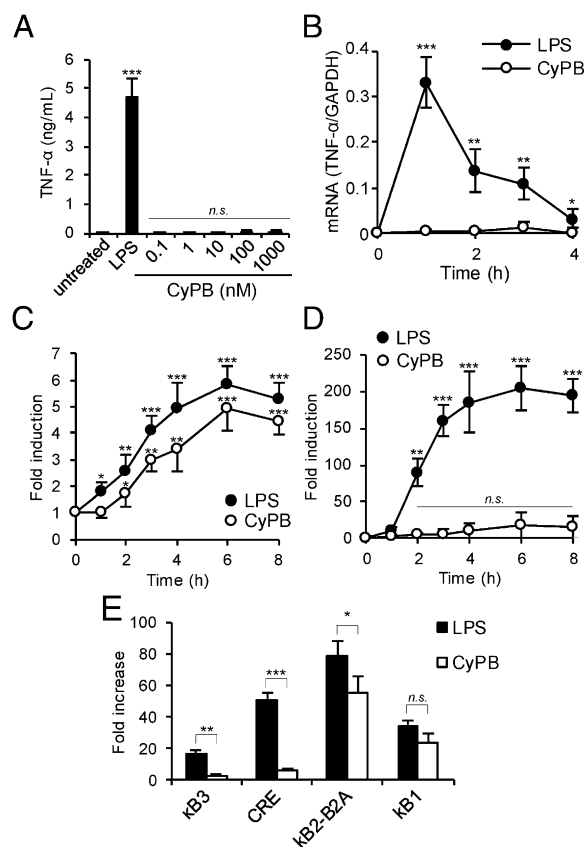


FIGURE 2. CyPB does not induce the expression of TNF- α in macrophages. (A) Monocyte-derived macrophages were treated with the indicated concentrations of CyPB or 10 ng/ml LPS. TNF- α production was assessed by ELISA in culture supernatant after 4 h of incubation. Results represent means \pm SD of triplicate obtained with cells from a single individual and are representative of three separate experiments. (B) Macrophages were treated with 10 ng/ml LPS (●) or 50 nM CyPB (○). At the indicated times, the level of TNF- α mRNA was measured by quantitative PCR (qPCR). Relative transcript abundance was normalized to endogenous control GAPDH mRNA. Data are means \pm SD from triplicates and representative of at least three experiments performed independently. Macrophages were transfected with the pNF- κ B-luc construct (C) or infected with the Advp5' vector (D) and thereafter stimulated with 10 ng/ml LPS (●) or 50 nM CyPB (○). At the indicated times, cells were harvested and prepared for analysis of luciferase activity. The luciferase data, normalized to protein content, are expressed as means \pm SD of triplicate and represent fold changes in test samples by comparison with unstimulated cells. Results are representative of three separate experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus untreated cells. (E) Macrophages were stimulated with LPS (10 ng/ml) or CyPB (50 nM) for 1 h, and the binding of NF- κ B and c-Jun to the TNF- α promoter was assessed by ChIP. Following precipitation with Abs to p65 or c-Jun, the relative binding of the transcription factors to *cis*-binding elements (κ B1, κ B2-2a, κ B3, and CRE) was assessed by qPCR of DNA fragments using TNF- α promoter-specific primers. Data were normalized with the levels of *cis* elements present in the samples (input). Results are expressed as fold changes by comparison with unstimulated cells and representative of three experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, significant difference between CyPB- and LPS-stimulated cells.

versus 4.56 ± 0.42 ng/ml with 10 ng/ml LPS, $p < 0.001$; $n = 6$). However, CyPB was not capable of inducing the production of other LPS-inducible inflammatory factors.

We then addressed the possibility that CyPB could modulate the inflammatory responses in activated macrophages. In the first experiments, the production of cytokines and chemokines in cells stimulated with CyPB alone or costimulated with LPS was com-

pared. The levels of inflammatory proteins secreted in culture supernatants were not significantly modified, indicating that CyPB did not synergize with LPS to increase the expression of these cytokines (Fig. 3). Surprisingly, we found that a 16-h pretreatment with CyPB greatly decreased LPS-induced production of TNF- α , IL-1 β , CXCL8/IL-8, and CCL5/RANTES. The inhibitory effect of CyPB was not general because LPS-induced production of IL-6 and IL-10 was not significantly reduced in CyPB-treated cells (Fig. 3). Collectively, these data support the hypothesis that CyPB downregulates a proinflammatory pathway that is commonly involved in the expression of TNF- α , IL-1 β , CXCL8/IL-8, and CCL5/RANTES, but not IL-6 and IL-10.

CyPB attenuates LPS-induced expression of TNF- α by a transcriptional mechanism

To investigate the mechanisms by which CyPB downregulates the expression of proinflammatory cytokines, we next focused our studies on the expression of TNF- α . In a time-course experiment, a significant decrease in TNF- α production was observed after 4-h pretreatment with CyPB ($n = 3$; $p < 0.05$). Longer exposure of macrophages to CyPB (16 h) resulted in an ~75% decrease. As expected, CyPB-mediated attenuation of TNF- α production was concentration dependent. A concentration of 15 nM was efficient in obtaining a 50% decrease in LPS-induced TNF- α production ($n = 3$; $p < 0.01$), and maximal inhibition was reached at 50 nM of CyPB. Moreover, CyPB did not reduce cell viability at concentrations ranging from 0.5–500 nM, indicating that the dramatic loss in cytokine production was not due to cytotoxic effect (data not shown).

CyPB may inhibit TNF- α production by inhibiting LPS-induced signaling events or by interfering with one of the three following steps: mRNA transcription, protein translation, or even secretion. To discriminate between these possibilities, we first analyzed the effect of CyPB on signaling pathways elicited by LPS in macrophages. In our hands, LPS-induced activation of p38 MAPK, JNK, or p44/p42 MAPK was not significantly altered by a 16-h pretreatment with CyPB. A weak difference in the extent of phosphorylation of p38 was observed. However, this difference was trivial compared with the dramatic loss in the cytokine production from the same cells. CyPB was also unable to inhibit I κ B α phosphorylation, degradation, and/or resynthesis, and neither was LPS-dependent

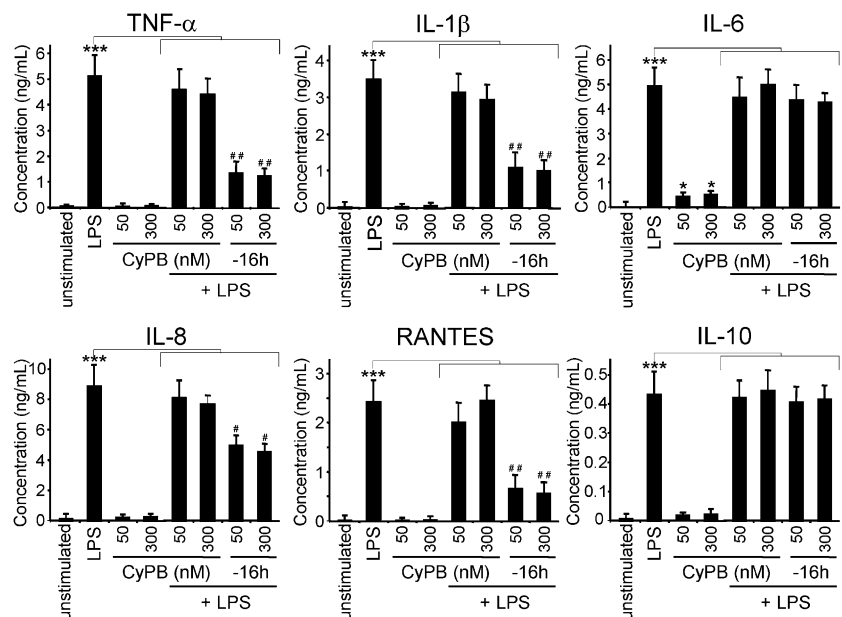
phosphorylation of NF- κ B p65 (Fig. 4). This strongly suggests that CyPB does not attenuate TNF- α production by inhibiting one of the signaling pathways that is activated by exposure of macrophages to LPS.

Using real-time RT-PCR, we next analyzed the expression of TNF- α at the mRNA level in primary macrophages and differentiated THP-1 cells. In the absence of CyPB, LPS rapidly and transiently induced the expression of TNF- α mRNA in both cell types (Fig. 5A). Pretreatment of cells with CyPB strongly reduced the transcription of TNF- α induced by LPS. After 1 h of LPS stimulation, the levels of transcription were reduced by ~75 and ~90% in THP-1 cells and primary macrophages, respectively (Fig. 5A). This was highly correlated with the inhibition of cytokine production (~75% in both cell types), thus supporting the idea that CyPB likely acts at the transcriptional level (Fig. 5B). To test this hypothesis, macrophages and THP-1 cells were infected with adenoviral construct Advp5' at the optimal multiplicity of infection of 40:1, as previously described (30). As expected, LPS activation of the reporter gene resulted in a potent stimulation of luciferase activity in both cell types. The kinetics of luciferase activity and TNF- α production followed similar profiles, with maximal expression reached after 4 h of stimulation with LPS. Advp5' virus-infected cells were then pretreated with CyPB for 16 h, and luciferase activity was assayed. In both cell types, CyPB inhibited luciferase activity by ~75% in comparison with controls (Fig. 5C), which was identical to the extent of inhibition of TNF- α production. This indicates that the inhibitory activity of CyPB on the reporter gene and the endogenous gene are similar, thus supporting the hypothesis that transcriptional control of TNF- α gene promoter is the target of CyPB. These results also validate the use of the immortalized cell line THP-1 for further mechanistic studies.

CyPB induces the expression of Bcl-3

Because CyPB downregulates the activation of TNF- α gene promoter without interfering with LPS-induced signaling pathways, we focused our investigation on the expression of inducible factors that act downstream by inhibiting TNF- α synthesis at the transcriptional level. Among them, Bcl-3 was reported to negatively regulate TNF- α expression in macrophages by enhancing the binding of NF- κ B p50 homodimer to κ B elements on the TNF- α gene promoter, thus inhibiting the transcriptional activity of ca-

FIGURE 3. CyPB inhibits LPS-induced cytokine production from macrophages. Monocyte-derived macrophages were treated with the indicated concentrations of CyPB and subsequently stimulated or not with 10 ng/ml LPS. Cytokine/chemokine production was assessed by ELISA in culture supernatant after 4 h of incubation for TNF- α and IL-1 β and after 16 h of incubation for IL-6, CCL5/RANTES, CXCL8/IL-8, and IL-10. Each bar of the histograms represents mean \pm SD of triplicate obtained with cells from a single individual, and results are representative from at least three separate experiments. * $p < 0.05$, *** $p < 0.001$ versus unstimulated cells, # $p < 0.05$, ## $p < 0.01$, significant decrease compared with LPS alone.



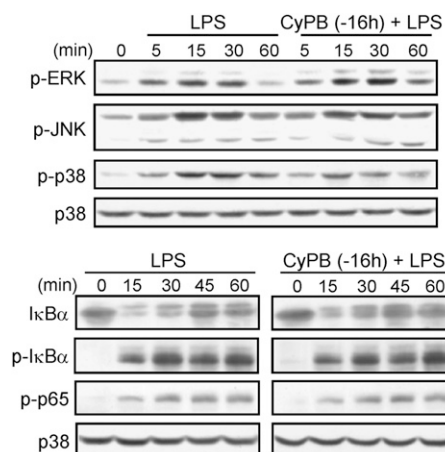


FIGURE 4. CyPB does not affect LPS-induced activation of signaling pathways. Monocyte-derived macrophages were either untreated or treated with 50 nM CyPB for 16 h and subsequently stimulated with 10 ng/ml LPS. At the indicated times, cell lysates were subjected to Western blotting for the phosphorylated forms of MAPK (p-ERK, p-JNK, and p-p38), phosphorylated and total forms of I κ B, and phospho-NF- κ B p65. Total p38 was used as a loading control. In each case, experiments were performed with macrophages from three different individuals with comparable results.

nonical NF- κ B p65/p50 heterodimer (3–5). Using real-time RT-PCR and Western blot, we showed that CyPB effectively induced the expression of Bcl-3 in primary macrophages and THP-1 cells. In both cell types, the expression of Bcl-3 mRNA peaked at 4–6 h and decreased thereafter to return to basal levels by 16 h. As expected, Bcl-3 was immunostained in samples from cells stimulated with CyPB, but contrary to its mRNA, the high level of the protein persisted at later time points (8–16 h) (Fig. 6A). Therefore, it seems that CyPB induced signaling events that not only induced the expression of Bcl-3 but also stabilized the protein.

To investigate whether CyPB-induced accumulation of Bcl-3 could be related to the downregulation of TNF- α gene expression, we performed ChIP assays on nuclear fractions extracted from cells pretreated with CyPB and thereafter stimulated with LPS (Fig. 6B). In absence of CyPB, LPS stimulation induced the binding of NF- κ B p65 and p50 to κ B sites of TNF- α gene promoter. As expected, Ab to Bcl-3 did not allow immunoprecipitation of any DNA complexes. In contrast to cells treated with LPS only, CyPB pretreatment critically suppressed LPS-induced binding of NF- κ B p65 to κ B sites, as shown by the strong decrease in amplification of sequences targeted by anti-p65 Ab. Conversely, the three sequences were amplified following immunoprecipitation with anti-p50 and anti-Bcl-3 Abs. Moreover, the relative patterns of amplification of DNA sequences were similar to those obtained in absence of CyPB, thus indicating that access of NF- κ B p65 to the κ B sites was similarly repressed by Bcl-3 (Fig. 6B).

Silencing the expression of Bcl-3 reduced the inhibitory effect of CyPB

To gain evidence into the relationships between expression of Bcl-3 and inhibition of LPS-induced production of TNF- α , we used a model based on RNA interference in THP-1 cells. First, we checked that treatment of cells with either irrelevant siRNA (siGFP) or negative control siRNA, in which two nucleotides have been changed from the target sequence, did not induce any decrease in the expression of Bcl-3 induced by CyPB (data not shown). In contrast, cell treatment with specific siRNA (termed siBcl-3) resulted in a significant decrease in Bcl-3 expression at the mRNA and protein levels (~80% of inhibition by comparison with negative control siRNA) (Fig. 7A). We next analyzed whether silencing

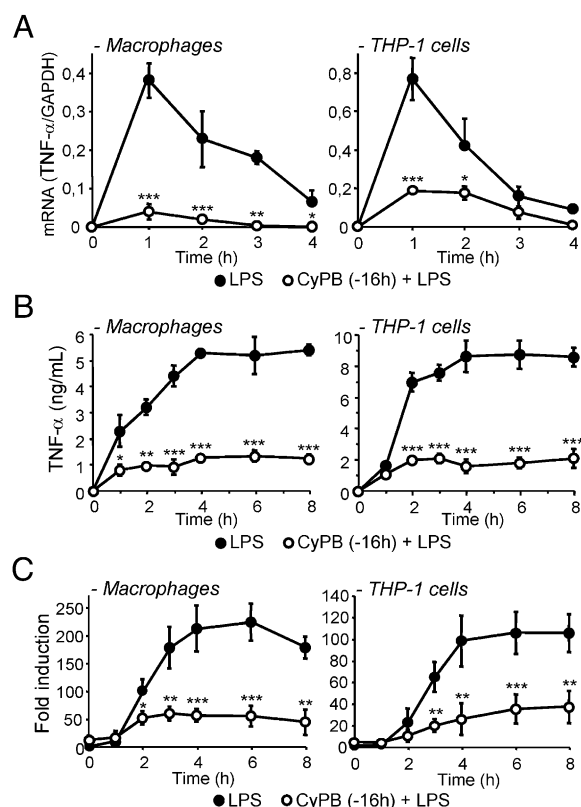


FIGURE 5. CyPB attenuates LPS-induced expression of TNF- α through inhibition of gene transcription. **(A)** Monocyte-derived macrophages and THP-1 cells were either untreated (●) or treated with 50 nM CyPB (○) for 16 h and subsequently stimulated with 10 ng/ml LPS. At the indicated times, the level of TNF- α mRNA was measured by RT-qPCR. Relative transcript abundance was normalized to endogenous control GAPDH mRNA. Data are means \pm SD from triplicates and representative of at least three experiments performed independently. **(B)** Supernatants from the same experiments were subjected to ELISA to determine TNF- α production. **(C)** Macrophages and THP-1 cells were infected with the Advp5' vector. Following a subsequent 16-h incubation without (●) or with (○) 50 nM CyPB, infected cells were stimulated with 10 ng/ml LPS. At the indicated times, cells were prepared for analysis of luciferase activity. The luciferase data, normalized to protein content, are expressed as means \pm SD of triplicate and represent fold changes in test samples by comparison with unstimulated cell samples. Results are representative of three separate experiments. * p < 0.05, ** p < 0.01, *** p < 0.001, significant decrease compared with LPS alone.

the expression of Bcl-3 led to a consequent decrease in the inhibitory activity of CyPB. In cells treated with negative control siRNA, CyPB potently reduced LPS-induced expression of TNF- α mRNA, indicating that the transfection procedure had not modified cell responsiveness. The same experiment was then reproduced with specific siRNA. As expected, cell treatment with CyPB had a poor inhibitory effect on TNF- α expression in LPS-stimulated cells. After 1 h of LPS stimulation, the expression of TNF- α mRNA was only reduced by 25% in siBcl-3 transfected cells compared with 75% inhibition obtained in samples from cells transfected with negative control siRNA (Fig. 7B). Finally, we analyzed the effect of silencing Bcl-3 in THP-1 cells infected with the adenoviral vector Advp5'. As expected, treatment with negative control siRNA did not modify cell responsiveness, because LPS-induced luciferase activity was strongly reduced following a 16-h incubation with CyPB. In contrast, siBcl-3 treatment resulted in a dramatic loss in the activity of CyPB. Indeed, a modest inhibition of LPS-induced luciferase activity was observed in treated cells, thus confirming

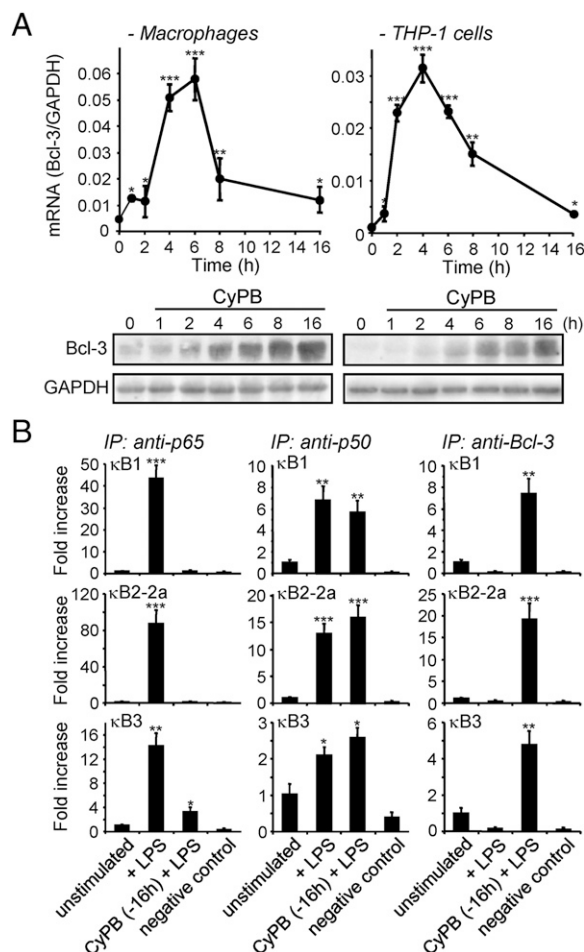


FIGURE 6. CyPB induces the expression of Bcl-3. **(A)** Monocyte-derived macrophages and THP-1 cells were stimulated with 50 nM CyPB. At the indicated times, cells were harvested, and the relative abundance of mRNA for Bcl-3, normalized to endogenous control GAPDH, was measured by qPCR. In parallel experiments, whole-cell lysates were separated by SDS-PAGE and immunostained with Ab to Bcl-3. Western blot for GAPDH served as loading control. In each case, results are representative of at least three experiments performed independently. **(B)** Untreated and CyPB-treated macrophages were stimulated with LPS (10 ng/ml) for 1 h, and changes in the binding of NF- κ B to specific sites on the TNF- α promoter were assessed by ChIP. Following precipitation (IP) with Abs to p65, p50, or Bcl-3, the relative binding of transcription factors to κ B elements (κ B1, κ B2-2a, κ B3) was assessed by qPCR. Negative control samples incubated with irrelevant Abs were represented. Results were normalized with the levels of κ B elements present in the samples (input). Data are expressed as fold changes by comparison with unstimulated cells and representative from three experiments conducted with macrophages of different individuals. * p < 0.05, ** p < 0.01, *** p < 0.001 versus untreated cells.

that knockdown of Bcl-3 has restored the capability of LPS to activate TNF- α gene promoter in the presence of CyPB (Fig. 7C). Altogether, these results clearly demonstrate that CyPB reduced TNF- α expression in LPS-stimulated macrophages via a transcriptional mechanism dependent on the expression of Bcl-3.

CyPA is not efficient in reducing LPS-induced TNF- α production from macrophages

It has been reported that CyPA induces chemotaxis and activation of p44/p42 MAPK and NF- κ B pathways in monocytes/macrophages (26). Consequently, our results suggest that both CyPA and CyPB may induce the same responses in macrophages. To

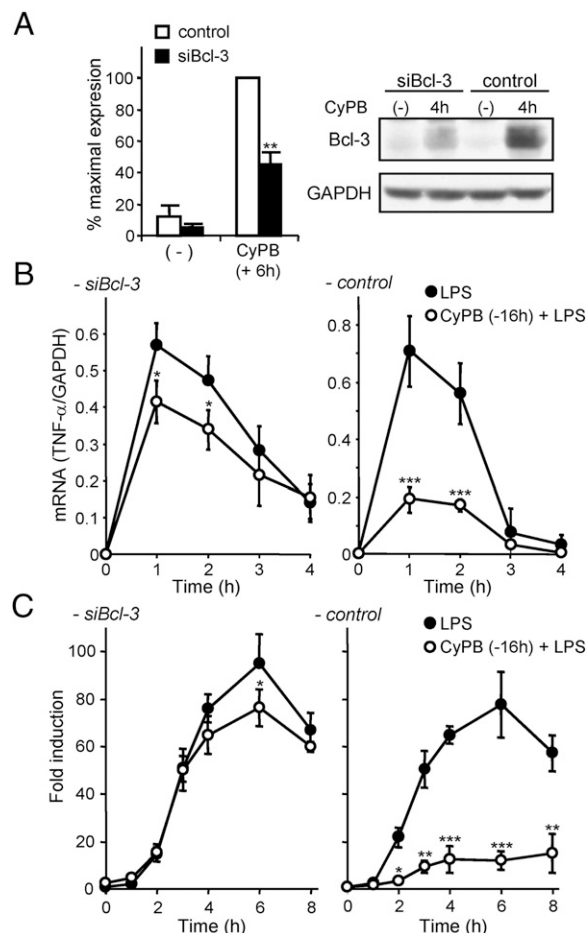


FIGURE 7. Bcl-3 deficiency affects CyPB-mediated attenuation of TNF- α . THP-1 cells were transfected with siRNA targeting mRNA for Bcl-3 (siBcl-3) or GFP (negative control), as described in *Materials and Methods*. **(A)** Transfected cells were stimulated or not with 50 nM CyPB, and the downregulation of the expression of Bcl-3 was assessed by RT-qPCR and Western blot. Data are representative of at least three separate experiments. ** p < 0.01 versus negative control. **(B)** siRNA-treated cells were either untreated (●) or treated with 50 nM CyPB (○) for 16 h and subsequently stimulated with 10 ng/ml LPS. At the indicated times, the level of TNF- α mRNA was measured by RT-qPCR. Relative transcript abundance was normalized to endogenous control GAPDH mRNA. Data are means \pm SD from triplicates from a single experiment and representative of three performed independently. **(C)** siRNA-treated cells were infected with the Adp5' vector and subsequently incubated without (●) or with (○) 50 nM CyPB for 16 h. Thereafter, infected cells were stimulated with 10 ng/ml LPS, and luciferase activity was measured at the indicated times. The luciferase data, normalized to protein content, are expressed as means \pm SD of triplicate and represent fold changes in test samples by comparison with unstimulated cell samples. Results are representative of three separate experiments. * p < 0.05, ** p < 0.01, *** p < 0.001, significant decrease compared with LPS alone.

test this hypothesis, we analyzed the effect of CyPA on the expression of TNF- α by macrophages. In our hands, CyPA was not more efficient than CyPB to induce the expression of the proinflammatory cytokine (data not shown). Similarly to what we described for CyPB, we then investigated whether CyPA could modulate the production of TNF- α from LPS-activated macrophages. To this end, macrophages from the same individuals were pretreated for 16 h with various concentrations of CyPA or CyPB before LPS stimulation. As described above, maximal inhibition of LPS-induced secretion of TNF- α was reached at 50 nM of CyPB. In contrast, a 10-fold higher concentration of CyPA was

required to induce only a modest reduction in TNF- α production (~15%) compared with cells treated with LPS alone (Fig. 8A).

Next, we analyzed the effect of CyPA on the expression of Bcl-3 at the mRNA and protein levels. As shown in Fig. 8B, CyPA induced a transient expression of Bcl-3 mRNA, although less than CyPB. Moreover, Bcl-3 was immunostained in samples from cells stimulated with CyPA, but the protein level peaked at 4 h and rapidly returned to basal level by 8 h of treatment, suggesting that it was rapidly degraded following its expression (Fig. 8C). To confirm these results, we performed ChIP assays with Bcl-3 and NF- κ B in LPS-stimulated macrophages that have been pretreated with either CyPA or CyPB (Fig. 8D). A 4-h pretreatment resulted in ~50% reduction in the binding of NF- κ B p65 compared with LPS alone, and this was accompanied by an enhanced binding of Bcl-3 to κ B sites, thus suggesting a competition for the same DNA sequences. But unlike CyPB, a 16-h pretreatment with CyPA had no inhibitory effect on the binding of NF- κ B p65 to κ B sites, and the three sequences were not amplified any more following immunoprecipitation with anti-Bcl-3 Abs. Collectively, these results indicate that CyPA was inefficient in sustaining a high expression of Bcl-3 in macrophages, which is a possible explanation for the inability to reduce the production of TNF- α to a similar extent than CyPB.

Discussion

Previous studies have reported that CyPA acts as a potent chemoattractant to monocytes/macrophages and induces the activation of p44/p42 MAPK and NF- κ B pathways (14, 24–26). Data concerning the effect of CyPA on cytokine production are, however, more contradictory. A first study reported that it could induce the production of high levels of TNF- α , IL-1 β , or RANTES from monocytes/macrophages (23). Otherwise, more recent works have casted doubt on these findings, because CyPA preparations substantially free of endotoxin failed to induce the expression of TNF- α and other proinflammatory factors. A low production of IL-6 and/or IL-8 was, however, reported, but the levels of secreted cytokines were not comparable to those obtained after stimulation by TLR agonists (24–26). Although CyPB is also secreted by inflammatory stimuli (16, 17), its contribution to the responses of macrophages has not been addressed. In the current work, we confirmed that CyPB induces a migratory response in macrophages and activates p44/p42 MAPK and NF- κ B pathways. We also found that CyPB does not induce the production of TNF- α , IL-1 β , IL-10, CXCL8/IL-8, and CCL5/RANTES from human macrophages in culture. We observed a low production of IL-6 from CyPB-stimulated macrophages, which was not, however, comparable to that obtained with LPS.

It is important to note that CyPB is constitutively secreted in the extracellular matrix, from which it can be released by the action of activated MMPs (17). This observation suggested that CyPB could participate in the regulation of tissue inflammation. Therefore, we investigated whether macrophage exposure to CyPB could modulate the production of inflammatory factors. Although CyPB did not modify the response of LPS-stimulated macrophages when added simultaneously, a 16-h pretreatment with CyPB prior the addition of LPS efficiently decreased the production of TNF- α , IL-1 β , CXCL8/IL-8, and CCL5/RANTES from the same cells. Moreover, the inhibitory effect appeared to target the expression of specific proinflammatory mediators, because the production of IL-6 and IL-10 was not affected by CyPB. These findings raised the intriguing possibility that, in addition to inducing a migratory response in macrophages, CyPB may also act as an anti-inflammatory factor and mediate tolerance to cells that were exposed to proinflammatory stimuli.

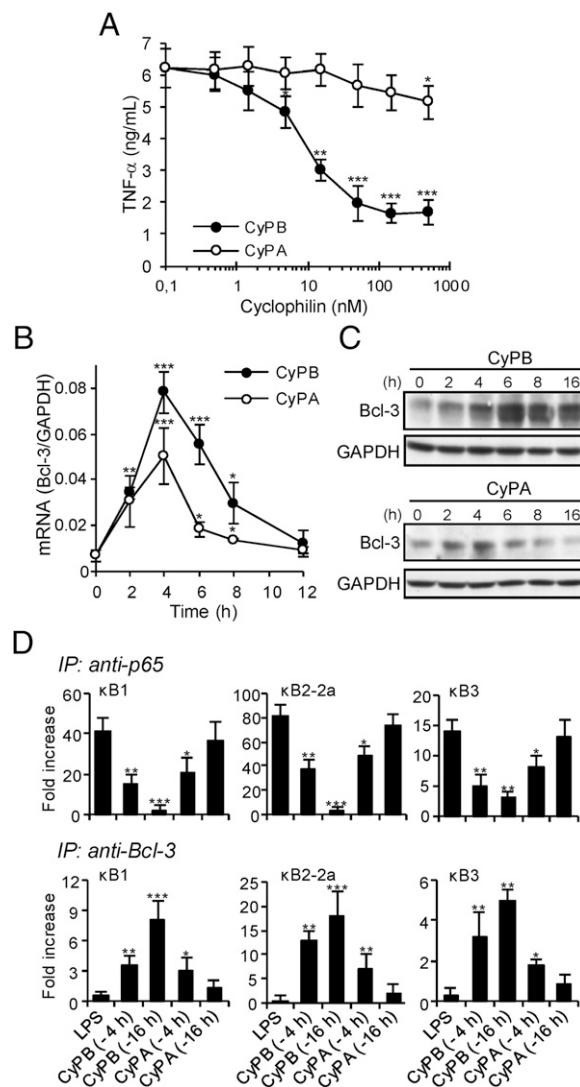


FIGURE 8. Comparison of the effects of CyPA and CyPB on LPS-induced expression of TNF- α . (A) Monocyte-derived macrophages were treated with the indicated concentrations of CyPA (○) or CyPB (●) for 16 h and subsequently stimulated with 10 ng/ml LPS. Supernatants were collected after 4 h of stimulation, and the production of TNF- α was measured by ELISA. Data are expressed as mean \pm SD of triplicate obtained with cells from the same individual, and results are representative from three separate experiments. * p < 0.05, ** p < 0.01, *** p < 0.001, significant decrease compared with LPS alone. (B) Macrophages were treated with 50 nM CyPB (●) or CyPA (○). At the indicated times, the level of Bcl-3 mRNA was measured by RT-qPCR. Relative transcript abundance was normalized to GAPDH mRNA. Data are means \pm SD from triplicates and representative of three experiments performed independently. * p < 0.05, ** p < 0.01, *** p < 0.001 versus untreated cells. (C) Macrophages were stimulated with either CyPA or CyPB (both at 50 nM) for the indicated times. Whole-cell lysates were separated by SDS-PAGE and immunostained with Ab to Bcl-3. Western blot for GAPDH served as loading control. The experiment was performed with cells from three different individuals with comparable results. (D) Macrophages were treated with CyPA or CyPB for 4 or 16 h and subsequently stimulated with LPS (10 ng/ml) for 1 h. Change in the binding of NF- κ B to the TNF- α promoter was assessed by ChIP with Abs to p65 and Bcl-3. The relative binding of transcription factors to κ B elements was assessed by qPCR. Results were normalized by the levels of κ B elements present in the samples (input), expressed as fold changes by comparison with unstimulated cells, and are representative from three distinct experiments. * p < 0.05, ** p < 0.01, *** p < 0.001 versus LPS alone.

The expression of TNF- α requires phosphorylation and degradation of I κ B as well as activation of p44/42 MAPK, JNK, and p38 MAPK, which ultimately leads to the activation of transcription factors such as NF- κ B, activating transcription factor-2, and c-Jun. These factors have been shown to functionally cooperate with each other for the recruitment of coactivators and efficient gene transcription (32–34, 36). We demonstrated in this study that NF- κ B activated by CyPB was functional. However, CyPB was unable to activate JNK and p38 MAPK in macrophages. Consistent with these results, our ChIP experiments showed that c-Jun did not bind to the TNF- α promoter in CyPB-treated macrophages. This may explain why CyPB was unable to induce the expression of TNF- α and possibly other proinflammatory cytokines and chemokines that have NF- κ B and c-Jun binding sites in their promoters in a similar configuration to the TNF- α promoter. Importantly, other studies also reported that CyPA did not induce the activation of p38 MAPK and JNK pathways in responsive cells (15, 31, 37). Hence, our current work extends previous studies to demonstrate that, besides stimulating monocytes/macrophages chemotaxis, extracellular CyPA and CyPB may induce a low production of specific cytokines (e.g., IL-6) without leading to a generalized production of proinflammatory mediators.

TNF- α produced by TLR-dependent activation of monocytes/macrophages is usually considered to be a key driver of the subsequent inflammation. Although beneficial in protecting the host from infection or injury secretion, the production of this proinflammatory mediator must be tightly regulated to avoid deleterious effects. This regulation may be achieved by inhibition of TLR-dependent signaling events, mRNA transcription, protein translation, or secretion. In this study, we have studied in depth the mechanisms by which CyPB attenuated the production of TNF- α from LPS-stimulated macrophages, and our findings strongly suggest a transcriptional mechanism of inhibition. First, 16-h pretreatment of macrophages with CyPB did not significantly affect the LPS-induced activation of MAPK and NF- κ B pathways in macrophages. Second, LPS-induced expression of TNF- α mRNA was strongly reduced in cells that were exposed to CyPB, which correlated with the inhibition of the cytokine production. Finally, LPS activation of a reporter gene under the control of TNF- α gene promoter was strongly inhibited in cells that have been treated with CyPB. A number of cellular processes could inhibit transcriptional induction of proinflammatory protein gene expression. However, the demonstration that efficient inhibition of TNF- α production required a long-term exposure to CyPB prior to LPS stimulation was suggestive of a mechanism that requires the expression of an inducible inhibitory factor. Previous studies have reported that the action of many regulatory mediators, including IL-4, IL-9, IL-10, or adiponectin, was dependent, at least in part, on the induction of Bcl-3 (6, 7, 38, 39). As expected, we found that Bcl-3 was highly expressed in macrophages that were stimulated with CyPB and remained present at high levels even after 16 h of stimulation. Although Bcl-3 is a member of the I κ B family of NF- κ B inhibitors, it does not interact with canonical NF- κ B heterodimers in the cytosol, but rather accumulates in association with p50 or p52 homodimers in the nucleus. Notably, Bcl-3 was reported to negatively regulate the expression of proinflammatory cytokines by enhancing the binding of inhibitory NF- κ B p50 dimers to κ B sites on target gene promoters (1–6, 38). Consistent with this, we found that expression of Bcl-3 was related to an inhibition of NF- κ B p65 binding to the TNF- α gene promoter. These data strongly support the hypothesis that CyPB inhibits LPS-induced TNF- α expression by a transcriptional mechanism, in which Bcl-3 associates with NF- κ B p50 homodimers and forms

inhibitory complexes with high affinity to κ B sites. Interestingly, other studies have reported on the absence of repressive effect of Bcl-3 on the expression of IL-6 and IL-10 (5, 6). This may explain our finding that CyPB also reduced the expression of IL-1 β , CXCL8/IL-8, and CCL5/RANTES, whereas it had no significant effect on the production of IL-6 and IL-10 from LPS-activated macrophages.

Both CyPA and CyPB interact with the same signaling receptor (i.e., CD147), which leads to the activation of common signaling pathways in responsive cells (10, 19, 20). Although most, if not all, responses triggered by CyPA can also be induced by CyPB, we demonstrated in this study that CyPA was not efficient in attenuating the production of TNF- α from LPS-stimulated macrophages. These findings were not surprising, because we previously reported that, in contrast to CyPA, CyPB is capable of inducing adhesion of T lymphocytes and monocytes/macrophages to fibronectin, a mechanism dependent on the activation of β_1 integrins. We also demonstrated that CyPB is a high-affinity ligand for cell-surface proteoglycans and that this interaction is required to induce sustained signaling and consequent activation of integrins (20, 31). In the current work, we showed that both cyclophilins induced the expression of Bcl-3. However, CyPA was not capable of sustaining a high level of expression of Bcl-3. It has been reported that the activity of Bcl-3 is tightly regulated by phosphorylation and polyubiquitination (40). Consistent with these observations, our results support the hypothesis that CyPB likely induced sustained and/or complementary signaling events that posttranslationally regulate Bcl-3, in addition to inducing its expression. Further investigations are currently in progress to validate this model and identify the underlying mechanisms responsible for the induction and subsequent accumulation of Bcl-3 in CyPB-treated macrophages.

Our findings on a potential anti-inflammatory effect of CyPB are intriguing because extracellular cyclophilins are commonly described as proinflammatory factors (10). However, other studies have already reported that numerous mediators, primarily identified for their proinflammatory properties, may also display anti-inflammatory action toward LPS-stimulated macrophages. As an example, chemerin is a potent chemoattractant factor for immature dendritic cells, macrophages, and NK cells (41). It was also reported to strongly reduce inflammatory responses of macrophages induced by LPS stimulation, thus indicating that CyPB and chemerin share common properties. Cyclophilins have also been reported to induce the expression of MMPs (23–26). These enzymes are commonly associated with acute inflammation, in which they are required for the degradation of extracellular matrix and establishment of a chemotactic gradient for infiltrating leukocytes. However, several lines of evidence have illustrated their involvement in remodeling of collagens and cell proliferation, which is a key part of the resolution of inflammation and tissue repair (42). Besides its role in MMP regulation, our study suggests another participation of CyPB to all the phases of inflammation. Indeed, it can act as a potent chemoattractant to monocytes/macrophages during the acute phase of inflammation, and participate later to resolution, by attenuating the production of proinflammatory factors from activated macrophages.

In conclusion, our findings demonstrate that CyPB may control macrophage activation by attenuating the production of inflammatory factors, thus highlighting an unexpected anti-inflammatory activity. Complete comprehension of the mechanisms by which CyPB regulates the responses of macrophages will allow for understanding its contribution to the different phases of inflammation.

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Disclosures

The authors have no financial conflicts of interest.

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