A Novel Signaling Pathway Mediates the Inhibition of CCL3/4 Expression by Prostaglandin E$_2$*

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In response to pathogen-associated molecular patterns, dendritic cells initiate an innate immune response characterized by expression and release of pro-inflammatory cytokines and chemokines. The extent of the inflammatory response is limited by various endogenous factors, including lipid mediators such as prostaglandin E$_2$ (PGE$_2$). We described previously the inhibitory effect of PGE$_2$ on the expression and release of the inflammatory chemokines CCL3 and CCL4 from activated dendritic cells. In this study we describe a novel PGE$_2$ signaling pathway that proceeds through EP-2 → cAMP → EPAC → phosphatidylinositol 3-kinase → protein kinase B → GSK-3 and results in increased DNA binding of the CCAAT displacement protein (CDP), a potent mammalian transcriptional repressor. The direct link between CDP and CCL3/4 transcription was established in knock-down experiments using CDP small interference RNA.

Dendritic cells (DCs)$^1$ are poised at the interface between innate and adaptive immunity. Following stimulation through the Toll-like receptors (TLR), DCs express and release pro-inflammatory cytokines and chemokines, up-regulate the expression of costimulatory molecules, and migrate to the nearest lymph node where they induce the activation and proliferation of naïve T cells (reviewed in Refs. 1–7). Although the inflammatory process is required to contain and eliminate pathogens, uncontrolled inflammation is detrimental to the host. Endogenous factors such as anti-inflammatory cytokines (reviewed in Ref. 8), neuroendocrine hormones and neuropeptides (reviewed in Refs. 9 and 10), and lipid mediators such as prostaglandins (reviewed in Refs. 11 and 12) maintain homeostasis by eliminating activated effector T cells and/or limiting the degree of macrophage/microglia/DC activation.

PGE$_2$, generated from arachidonic acid by cyclooxygenases and prostaglandin E synthases (13), affects the activation of macrophages and T cells, exerting a general anti-inflammatory effect (reviewed in Ref. 12). For mature TLR-activated DC, PGE$_2$ acts primarily as an anti-inflammatory agent, inhibiting the production of IL-12p70, tumor necrosis factor, and IL-6 (14–16) promoting the release of IL-10 (17), and skewing CD4+ T cell differentiation toward Th2 effectors (18, 19). In agreement with the general anti-inflammatory effect, we reported previously that PGE$_2$ inhibits the expression and release of CCL3 and CCL4, two potent pro-inflammatory chemokines, from LPS- and PGN-stimulated DCs (20).

The inhibitory effect of PGE$_2$ on CCL3/4 production is mediated through the EP-2 and possibly EP-4 receptors (20). In this report we identified the signaling pathway initiated by PGE$_2$ and leading to the suppression of CCL3/4 expression. We demonstrate that PGE$_2$ signals through the EPAC → PKB → GSK-3 mediators resulting in increased DNA binding of the CDP repressor and that siRNA knock-down of CDP reverses the inhibitory effect of PGE$_2$ on CCL3/4 expression.

**Experimental Procedures**

**Mice—**Male B10.A mice 6–8 weeks old were purchased from The Jackson Laboratory (Bar Harbor, MA) and were maintained in the Rutgers State University (Newark, NJ) animal facility under pathogen-free conditions.

**Reagents—**LPS (Escherichia coli O26:56), prostaglandin E2 (PGE$_2$), avidin peroxidase, and FITC-conjugated F(ab)$^2$ goat anti-rabbit IgG, LiCl, and SB 216763 were purchased from Sigma. TMB ELISA substrate was purchased from Pierce. Capture and biotinylated anti-mouse CCL4 antibody were purchased from R&D Systems (Minneapolis, MN). Purified and biotinylated anti-mouse CCL3 antibodies and recombinant murine CCL4 was purchased from R&D Systems (Minneapolis, MN). Purified and biotinylated anti-mouse CCL3 antibodies and recombinant murine CCL4 was purchased from PeproTech (Rochy Hill, NJ). The cAMP Biotrak EIA System was purchased from Amersham Biosciences. PD98059, H89, dibutyryl cAMP (Bt2cAMP), and the EPAC-specific activator 8-CPT-2‘-OMe-cAMP, and the GSK-3 inhibitor II were obtained from Calbiochem. Antibodies to total and phospho-PKB, ERK1/2, p38, JNK, and CREB were obtained from Cell Signaling Technology (Beverly, MA). Anti-CD11c antibody was purchased from BioLegend (San Diego, CA).

**Generation and Purification of DC from Bone Marrow—**DCs were generated from B10.A bone marrow as described previously (20). At day 7, CD11c$^+$ DCs were harvested and purified from the nonadherent cells by immunomagnetic sorting using anti-CD11c-coated magnetic beads and the autoMACS system (Miltenyi Biotec, Bergish-Gladbach, Germany). The purity of the sorted cells was determined by FACS analysis ($>96\%$ CD11c$^+$).

**Culture of the DC2.4 Cell Line—**DC2.4 cell line was derived from C57BL/6 bone marrow cells and was generously provided by Dr. Kenneth L. Rock (University of Massachusetts Medical Center, Worcester, MA). The cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM l-glutamine, 50 μM 2-mercaptoethanol, and 1% minimum Eagle’s medium nonessential amino acids.

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$^1$ The abbreviations used are: DC, dendritic cells; BM-DC, bone marrow-derived dendritic cells; CDP, CCAAT displacement proteins; Bt2cAMP, dibutyryl cAMP; LPS, lipopolysaccharide; PGE$_2$, prostaglandin E$_2$; PGN, proteoglycan; PKA, protein kinase A; PKB, protein kinase B; siRNA, small interference RNA; TF, transcription factors; TLR, Toll-like receptors; PSEK, phosphatidylinositol 3-kinase; ELISA, enzyme-linked immunosorbent assay; CREB, cAMP-response element-binding protein; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; EMISA, electrophoretic mobility shift assay; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; RT, reverse transcription; Ab, antibody; IL, interleukin; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EPAC, exchange protein directly activated by cAMP; MOPS, 4-morpholinepropanesulfonic acid.
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CCL3 and CCL4 Measurement by ELISA—Purified DCs (or DC2.4) were cultured in 24-well culture plates at 1 × 10⁶ cells per well in a final volume of 1 ml and stimulated with LPS (1 μg/ml) in the presence or absence of PGE₂ (1 μM). Cell-free supernatants were harvested and subjected to CCL3/4 ELISAs. In some inhibitors, MAPKs or PKA were added to cultures 1 h before LPS and PGE₂. For the detection of CCL3, we used 0.5 μg/ml purified polyclonal antibody as capture Ab, followed by detection with biotinylated anti-mouse CCL3 Ab (0.2 μg/ml). CCL4 was assayed with AG5–2 as capture Ab (4 μg/ml) followed by detection with the biotinylated anti-mouse CCL4 Ab (2 μg/ml).

Real Time PCR—The SYBR green-based real time PCR technique was used to detect the expression of chemokines, prostanoid receptors (Eps), and the CDF gene. Total RNA was prepared from BM-DCs or DC2.4 cells, and 1 μg of RNA was reverse-transcribed into cDNA in the presence of 200 units of Moloney murine leukemia virus-RT, 40 units of RNase-free MMLV reverse transcriptase (Biolabs, Beverly, MA), and equal amounts of total protein were separated by 12% SDS-PAGE. To determine the amount of total and phosphorylated intracellular PKB, we used intracellular flow cytometry.

siRNA Transfection—The 21-nucleotide small interfering RNA duplexes with two overhang GT nucleotides at 3′-ends were designed according to the method of Elbashir et al. (21) and synthesized by Dharmaco Inc. (Lafayette, CO). The sequence of the sense strand of CDF siRNA is AAGAGCUAAUUGAUGUUCUA. The control siRNA was an irrelevant siRNA with random nucleotide and similar GC ratio to CDF siRNA (Dharmacon). Transfections were conducted by using the GeneSilencer siRNA Transfection reagent as recommended by the manufacturer (Gene Therapy System, San Diego). Briefly, DC2.4 cells (2 × 10⁶ cells) were plated in 6-well plates overnight and changed into 1 ml of serum-free RPMI 1640 medium before transfection. Five μl of 40 μM CDF siRNA or control siRNA duplexes were incubated with 5 μl of Lipofectamine 2000 (Invitrogen) for 5 min and these mixtures were added to the serum-free DC2.4 cells. Mock controls were transfected with 5 μl of siRNA transfection reagent alone. Four hours later, 1 ml of RPMI 1640 containing 20% FBS was added to each well. RNA was prepared from DCs 24–72 h after transfection and subjected to real time PCR for CDF expression. In some experiments, DC2.4 cells were transfected with siRNA for 72 h and then treated with LPS plus PGE₂, followed by RNA isolation and gene expression analysis.

Western Blots—Purified DC11c⁺ DCs were cultured at a concentration of 1 × 10⁶ cells/ml with LPS (1 μg/ml) in the presence or absence of PGE₂ (1 μM), and harvested at the designed time points. Total cell lysates were prepared by using cell lysis buffer (New England Biolabs, Beverly, MA), and equal amounts of total protein were separated on 12% SDS-PAGE. After transfer to nitrocellulose membranes and blocking with 5% nonfat dry milk, the membranes were probed with antibodies, followed by incubation with appropriate secondary Abs conjugated to horseradish peroxidase. Peroxidase activity was detected by using enhanced chemiluminescence reagents (Pierce) as recommended by the manufacturer.

Measurement of Intracellular cAMP—Intracellular cAMP production following PGE₂ challenge was measured by using the cAMP Biotrak Measurements kit (Amersham Biosciences). Purified DC or DC2.4 cells (1 × 10⁶ cells/200 μl) were cultured in the presence of the cAMP phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (Sigma). Cells were treated with or without PGE₂ (1 μM) or LPS (1 μg/ml) for the designed time points (as indicated in text). The reaction was stopped by brief centrifugation, followed by addition of prewarmed 25% trichloroacetic acid. The cell lysates were washed with ethanol, followed by incubation with appropriate secondary Abs conjugated to horseradish peroxidase. Peroxidase activity was detected by using enhanced chemiluminescence reagents (Pierce) as recommended by the manufacturer.

PKB Assay by FACS—To determine the amount of total and phosphorylated intracellular PKB, we used intracellular flow cytometry. Briefly, the cells were fixed and permeabilized using Cytofix/Cytoperm kit (Pharmingen) according to the manufacturer's instructions. Cells were stained with rabbit polyclonal anti-phospho-PKB (Cell Signaling Technology) or mouse monoclonal anti-PKB (Cell Signaling Technology) antibodies for 45 min, followed by the appropriate FITC-conjugated secondary antibodies for 30 min. Control rabbit and mouse IgG were used to confirm specificity. After extensive washing, cells were analyzed by FACS analysis.

GSK-3 Kinase Assay—Purified BM-DCs were treated with 1 μM PGE₂ or with different GSK-3 inhibitors (10 mM LiCl, 20 μM SB216753, or 20 μM GSK-3 Inhibitor II) for 1 h followed by total cell lystate preparation. Untreated cells were used as control. The GSK-3 kinase activity was assayed by using a kit from Upstate Biotechnology, Inc. (Lake Placid, NY), as recommended by the manufacturer. In brief, 10 μg of total protein was incubated for 30 min at 30 °C with 1 μl of [γ-³²P]ATP (10 μCi/μl) and 62.5 μM GSK (GSK-3 substrate peptide) in a 40-μl total volume containing 2 mM MOPS (pH 7.2), 0.05 mM EDTA, 2.5 mM magnesium acetate, 15 mM MgCl₂, and 100 μM unlabeled ATP. 25 μl of the reaction mixture was spotted onto P81 phosphocellulose paper followed by extensive washing with 0.75% phosphoric acid. Then the assay papers were transferred into scintillation mixture, and we

Proteins/DNA Assays—Protein/DNA assays were used to screen simultaneously a large number of transcription factors for DNA binding activity. Nuclear extracts were prepared as described above. The protein/DNA assays were carried out with the TranSignal™ Protein/DNA Array I (Promega, Redwood City, CA). In brief, 15 μg of total nuclear proteins were incubated with biotin-labeled DNA-binding probes (TranSignal Probe Mix) to allow the formation of protein-DNA (or TF-DNA) complexes. The protein-DNA complexes were separated from free probes by electrophoresis in agarose gels. The probes present in the complexes were eluted and hybridized to the TranSignal membrane dotted with corresponding nonlabeled probes, and the signals were detected by Chemiluminescence.
The analysis of data was performed using the Student’s t test with the minimum significant level.

Phase-contracted cells were cultured at a concentration of 1 x 10⁶ cells/ml and stimulated with LPS (1 μg/ml) in the presence or absence of PGE₂ (1 μM). Supernatants were harvested 12 h later and subjected to ELISA for CCL3 and CCL4. * indicates statistically significant differences compared with the LPS group (p < 0.05).

**RESULTS**

**PGE₂ Inhibits LPS-induced CCL3/4 Expression in BM-DCs and DC2.4 Cells**—Purified CD11c⁺ DCs (BM-DC) and DC2.4 cells were stimulated with LPS in the presence or absence of exogenous PGE₂. LPS induces high levels of CCL3 and CCL4, and PGE₂ inhibits LPS-induced chemokine release in both BM-DCs and DC2.4 cells (Fig. 1A). The PGE₂ inhibitory effect on CCL3/4 release is paralleled by a decrease in CCL3/4 transcription (Fig. 1B).

Screening for Transcription Factors Affected by PGE₂—Because PGE₂ inhibits CCL3/4 expression at the mRNA level, we investigated the transcription factors involved in this event. We used first a DNA/protein array to screen for TFs that might be positively or negatively regulated by PGE₂. The DNA/protein arrays were prepared with nuclear extracts from BM-DCs stimulated with LPS or LPS plus PGE₂. Although LPS induces strong DNA binding activity for AP1, AP2, CREB, NF-κB, GATA, Egr, Smad 3/4, and USF-1, PGE₂ does not affect these TFs (Table I). PGE₂ decreased the DNA binding activity of NFAT, Sp1, SRE, TFIID, and Pbx1 and increased the DNA binding activity of CDP/CBF, Est-1/PEA3, and P53 (Table I).

Previous reports indicated that AP1, NF-κB, and CREB/ATF are crucial for CCL3/4 transcription. In agreement with our DNA/protein array data, PGE₂ does not affect the DNA binding activity of AP1, NF-κB, and CREB/ATF, as determined by EMSA (Fig. 2, A–C).

**PGE₂ Induces CDP Binding**—Since with the exception of members of the CCAAT displacement protein family (CDP), none of the factors whose binding is affected by PGE₂ have been reported to be involved in CCL3/4 expression, we focused on CDP. The DNA/protein array indicated that PGE₂ increases the DNA binding of CDP, a CCAAT displacement protein previously reported to act as a transcriptional repressor. EMSA experiments with nuclear extracts from both BM-DCs and DC2.4 cells show a significant increase in CDP binding in cells treated with PGE₂, both in the presence and absence of LPS (Fig. 2D). PGE₂ concentrations from 10⁻⁶ to 10⁻⁸ M increased CDP binding to a similar degree, and even PGE₂ 10⁻⁷ M induced CDP binding above control levels (Fig. 2E). The CDP binding is specific, as shown by successful competition with a cold specific probe and by supershift with a specific anti-CDP antibody (Fig. 2F).

Knock-down of CDP Reverses the Inhibitory Effect of PGE₂ on CCL3/4 Production—To address the question whether CDP acts as a CCL3/4 repressor, we knocked down CDP expression with siRNA. DC2.4 cells were transfected with CDP siRNA, an siRNA control for 72 h, followed by treatment with LPS in the presence or absence of PGE₂. The expression of CCL3/4 was determined by real time RT-PCR. Compared with untreated cells, the cells transfected with mock or siRNA control express comparable levels of CCL3 and CCL4, whereas cells transfected with CDP siRNA exhibit significantly lower levels of CCL3 and CCL4 expression. This inhibition is partially reversed in cells transfected with CDP siRNA (Fig. 3A). The CDP levels were low even 72 h after transfection (data not shown). Next, DC2.4 cells were transfected with CDP siRNA or control for 72 h, followed by treatment with LPS in the presence or absence of PGE₂. The expression of CCL3/4 was determined 3 h later by real time RT-PCR. In nontransfected or mock-transfected cells, PGE₂ inhibits LPS-induced CCL3/4 expression. This inhibition is partially reversed in cells transfected with CDP siRNA (Fig. 3B). The reversal was also observed at protein level in cells transfected with CDP siRNA (Fig. 3C). Taken together, these results indicate that CDP mediates at least partially the inhibitory effect of PGE₂ on LPS-induced CCL3/4 expression.
cAMP Mediates the Inhibitory Effect of PGE2—Previously, we showed that the inhibitory effect of PGE2 on CCL3/4 production in BM-DCs is mediated through EP-2 and possibly EP-4 receptors. Real time RT-PCR experiments indicate similar patterns for EP-2 and EP-4 expression in BM-DCs and DC2.4 cells (Fig. 4A). To assess the contribution of the EP receptors to the increase in CDP binding observed with PGE2, we used three EP receptor agonists, i.e. butaprost, an EP-2-specific agonist, misoprostol, a preferential EP-3/EP-2 agonist that also binds EP-4 at high concentrations, and sulprostone, an EP-1/EP-3 agonist. Because butaprost has a 10-fold lower affinity for EP-2 than PGE2, we used butaprost concentrations corresponding from $10^{-6}$ to $10^{-8}$ M PGE2. The two higher concentrations of butaprost induced CDP binding, which indicates the involvement of the EP-2 receptor (Fig. 4B, lower panel). Misoprostol induced strong CDP binding at high concentrations, where it acts as an EP-3/EP-2 receptor (Fig. 4B, upper panel). The possible involvement of the EP-3 receptor in the induction of CDP binding was eliminated by the fact that sulprostone, an EP-1/EP-3 agonist, did not induce CDP binding, even at $10^{-6}$ M (Fig. 4B, lower panel). These results indicate that the effects of PGE2 on CDP binding are mediated through the EP-2 receptor.

Because EP-2 receptors are coupled to Gαs resulting in the activation of adenylate cyclase, we investigated whether PGE2 induces cAMP in BM-DCs and DC2.4 cells. PGE2 induced a rapid increase in cAMP as early as 5 min following stimulation, reaching a maximum around 10 min, followed by a gradual decrease (results not shown). The effect on cAMP levels is dose-dependent, with significant increases for $10^{-6}$–$10^{-7}$ M PGE2 (Fig. 4C). PGE2 induced comparable amounts of cAMP in the presence and absence of LPS (Fig. 4C). The involvement of

### Table I

| Effect on DNA binding activity | TFs |
|-------------------------------|-----|
| ↑                             | NF-AT, Sp1, SRE, TFIID, Pbx1 |
| ↓                             | NF-AT, Sp1, SRE, TFIID, Pbx1 |
| ↔                             | CREB, NF-KB, AP1, AP2, GATA, EGR, smad3/4, USF-1 |

**Fig. 2.** Effect of PGE2 on the DNA binding activity of CREB, AP-1, NF-κB, and CDP. BM-DCs were treated with PGE2 (1 μM) except for E, LPS (1 μg/ml), or LPS + PGE2 for 1 h. Nuclear extracts (10 μg) were subjected to EMSA analysis with DNA probes containing consensus motifs specific for CREB (A), AP-1 (B), NF-κB (C), and CDP (D–F). Specificity controls for NF-κB include 50-fold excess of cold NF-κB probe or a nonspecific cold probe added prior to the labeled NF-κB probe. Specificity controls for CDP include 50-fold excess of cold CDP or nonspecific probe, or 1 μl of anti-CDP Ab added to the nuclear extracts 10 min before incubation with the labeled CDP probe. One representative experiment out of three is presented.

**TABLE I**

Effects of PGE2 on the DNA binding activity of various TFs

| BM-DCs were cultured at a concentration of $1 \times 10^6$ cells/ml and stimulated with LPS (1 μg/ml) in the presence or absence of PGE2 (1 μM) for 1 h. Nuclear extracts were prepared and subjected to protein/DNA array as described under “Experimental Procedures.” ↑ indicates an increase in DNA binding (higher than 3-fold) by PGE2; ↓ indicates a decrease in DNA binding (higher than 3-fold) by PGE2; ↔ indicates the TFs whose DNA binding activity was not significant affected by PGE2. | TFs |
|---|-----|
| ↑ | NF-AT, Sp1, SRE, TFIID, Pbx1 |
| ↓ | NF-AT, Sp1, SRE, TFIID, Pbx1 |
| ↔ | CREB, NF-KB, AP1, AP2, GATA, EGR, smad3/4, USF-1 |
cAMP as a mediator in the inhibition of CCL3/4 by PGE₂ is supported by the inhibitory effect of Bt₂cAMP, a stable cAMP analog (Fig. 4D).

The CCL3/4 Inhibition by PGE₂ Is Not Mediated by the PKA/CREB Pathway—Because the classical downstream cAMP target is PKA, we pretreated BM-DCs and DC2.4 cells with the PKA inhibitor H89 (10⁻⁶–10⁻⁹ M), followed by LPS and PGE₂. No reversal of the PGE₂-induced inhibition was observed (Fig. 5A), suggesting that PKA does not participate in the PGE₂ signaling pathway. To further eliminate the possible involvement of PKA, we examined the phosphorylation of CREB (pCREB) by Western blotting. LPS induced strong CREB phosphorylation, whereas PGE₂ did not induce pCREB and did not affect LPS-induced CREB phosphorylation (Fig. 5B). These results are consistent with the fact that PGE₂ does not affect CREB DNA binding in the EMSA experiments (Fig. 2A).

PGE₂ Does Not Affect the TLR Common Core Response—LPS induces proinflammatory cytokines and chemokines in DCs through the TLR common core response, mediated through the activation of MAPKs and NF-κB. PGE₂ did not affect LPS-induced NF-κB binding as determined by both DNA/protein arrays and EMSA (Table I and Fig. 2C). To assess the involvement of the ERK1/2 and p38 MAPK pathways in the LPS-induced CCL3/4 expression, we treated BM-DCs and DC2.4 cells with LPS in the presence and absence of MAPK inhibitors. PD98059, an ERK1/2 specific inhibitor, reduced CCL3/4 production in a dose-dependent manner (Fig. 6A, and data not shown), whereas the p38 MAPK inhibitor SB 203580 did not affect chemokine production (data not shown).

To determine whether the inhibitory effect of PGE₂ is mediated through the inhibition of ERK1/2, we treated BM-DCs with LPS in the presence or absence of PGE₂, followed by Western blotting for phosphorylated ERK1/2. In contrast to PD98059, PGE₂ did not affect LPS-induced ERK phosphorylation (Fig. 6B). The effect of PGE₂ on the phosphorylation of JNK and p38 was also analyzed by Western blots. Similar to ERK1/2, PGE₂ did not affect the LPS-induced phosphorylation of JNK or p38 (Fig. 6C).

Activation of EPAC Mediates the Inhibitory Effect of PGE₂ on CCL3/4—Our previous results showed that the inhibition of chemokines by PGE₂ was mediated in a cAMP-dependent but PKA-independent manner. Recently, other cAMP-dependent pathways have been reported, including cAMP-gated ion channels and EPAC. To determine whether EPAC is involved in the inhibition of chemokine production, BM-DCs were treated with LPS in the presence or absence of different concentrations of the EPAC-specific activator, 8-CPT-2'-OMe-cAMP. Similar to Bt₂cAMP and PGE₂, 8-CPT-2'-OMe-cAMP inhibits chemokine production at both the protein and mRNA level (Fig. 7, A and B).

Next, we investigated if activation of EPAC by 8-CPT-2'-OMe-cAMP induces CDP binding. BM-DCs were treated with PGE₂, Bt₂cAMP, or 8-CPT-2'-OMe-cAMP and CDP binding was analyzed by EMSA. PGE₂, Bt₂cAMP, and 8-CPT-2'-OMe-cAMP induced comparable levels of CDP binding (Fig. 7C).
These results suggest that activation of EPAC mediates the inhibitory effect of PGE₂.

PGE₂ Induces PKB Phosphorylation—Next, we investigated the downstream events regulated by EPAC. There is evidence that activation of EPAC by cAMP signals through the PI3K-PKB pathway. Because the PI3K inhibitors wortmannin and LY294002 were cytotoxic for DC except at low concentrations that proved to be inefficient in terms of PI3K inhibition, we examined whether PGE₂ activates PKB in untreated or LPS-treated BM-DCs. BM-DCs and DC2.4 cells were treated with PGE₂ in the presence or absence of LPS, and the levels of phosphorylated PKB were determined by flow cytometry. We did not detect phosphorylated PKB in cells cultured with medium or stimulated with LPS. However, phosphorylated PKB was present in the majority (>94%) of the medium- or LPS-stimulated BM-DCs treated with PGE₂ (Fig. 8).

Involvement of GSK-3 in Chemokine Production—Because PGE₂ activates PKB, which in turn phosphorylates and inactivates GSK-3, we considered the possibility that GSK-3 might be involved in CCL3/4 expression. BM-DCs were treated with LPS in the presence of different concentrations of the GSK-3 inhibitors SB216763 and GSK-3 inhibitor II. Both inhibitors decrease LPS-induced CCL3/CCL4 production in a dose-dependent manner (Fig. 9). The inhibitory effect on CCL3/4 release is paralleled by a decrease in CCL3/4 transcription (data not shown). Similar to SB216763 and GSK-3 inhibitor II, a third GSK-3 inhibitor, LiCl, inhibits chemokine production in a dose-dependent manner at both the protein and mRNA level (Fig. 10, A and B).

We propose that upon LPS stimulation, GSK-3 phosphorylates CDP, which results in reduced DNA binding and loss of repressor activity. In contrast, when DCs are treated with PGE₂, the activation of PKB results in GSK-3 phosphorylation and inactivation. Subsequently, nonphosphorylated CDP binds to the CCL3/4 promoters and represses transcription. The link between GSK-3 activity and CDP DNA binding was confirmed by the fact that nuclear extracts from LiCl-treated DCs showed increased CDP binding activity (Fig. 10C).

In addition, we compared GSK-3 activity following treatment with PGE₂, LiCl, SB216763, or GSK-3 inhibitor II. BM-DCs were treated with PGE₂ or GSK-3 inhibitors for 1 h, followed by kinase assays with GSM as substrate. PGE₂ and LiCl decreased GSK-3 activity by 25%, and the two specific GSK-3 inhibitors resulted in 40% inhibition. This indicates that PGE₂ acts as a GSK-3 inhibitor, similar to LiCl, SB216763, and the GSK-3 inhibitor II.

DISCUSSION

Activated DC are major producers of proinflammatory chemokines, such as CCL3 and CCL4. Prostaglandins of the E series inhibit the expression of CCL3 and CCL4 in LPS-stimulated macrophages and LPS or PGN-stimulated DC (20, 22). Although we have shown previously that PGE₂ affects CCL3/4 expression through the EP-2 and possibly EP-4 receptors (20), there is no information on the intracellular signaling. In this study we identified the CCAAT displacement protein CDP as the major player in the effect of PGE₂ on CCL3/4 gene expression. In addition, we established that the PGE₂-induced DNA binding of the CDP repressor is mediated through the EP-2 → cAMP → EPAC → PKB → GSK-3 pathway.
Several transcription factors, i.e., NF-κB, CREB, c/EBP, c-Ets, members of the CCAAT displacement protein family, and the negative regulator ICK-1, are involved in the expression of CCL3 and CCL4 genes in murine and human macrophages and monocytes (23–27). When we tested nuclear extracts from LPS- and LPS + PGE₂-treated BM-DC for DNA binding factors, we found that PGE₂ does not modify the binding of CREB, NF-κB, and AP-1. This was confirmed by using electromobility shift assays. PGE₂ reduces the DNA binding of the transcriptional factors NFAT, Sp1, and SRE, which are not involved in CCL3/4.
expression. However, PGE₂ increases the binding of the CCAAT displacement protein CDP, a negative regulator that represses the transcription of a variety of genes, including the pro-inflammatory chemokine CXCL1 (28). The increase in CDP binding is apparent for PGE₂ concentrations that are within the range observed in inflammatory conditions (10⁻⁷–10⁻⁸ M).

The CDP proteins exert their repressor activity by competing with transcriptional activators for occupancy of DNA-binding sites and by direct recruitment of deacetylases (reviewed in Ref. 29). The DNA binding capacity of CDP is modulated through phosphorylation and/or proteolytic processing. In fibroblasts, the CDP DNA binding is increased during the G₁ →
S transition through dephosphorylation and proteolytic cleavage and decreased during the S → G2 transition through phosphorylation (30).

The direct involvement of CDP in CCL3/4 expression was demonstrated by the reversal of CCL3/4 inhibition following CDP siRNA transfection of DC2.4 cells, a dendritic cell line developed by Shen et al. (31). We used the DC2.4 cell line because primary BM-DC are quite difficult to transfect. The DC2.4 cells have been reported to up-regulate major histocompatibility complex class II and costimulatory molecules following LPS/interferon-γ stimulation and to secrete IL-12p40 (32). However, there is no information regarding their capacity to produce chemokines or to respond to PGE2. Our results show that the DC2.4 cells behave similar to BM-DC in terms of EP-2 and EP-4 receptor expression, induction of cAMP by PGE2, and effects of PGE2 on CCL3/4 expression. In addition, PGE2 treat-

Fig. 9. Active GSK-3 kinase is required for the induction of CCL3/4 by LPS. BM-DCs were treated with different concentrations of SB216763 (A) or GSK-3 inhibitor II (B) for 1 h, followed by stimulation with LPS for an additional 12 h. The levels of CCL3 and CCL4 were determined by ELISA. * indicates statistically significant differences compared with the LPS-treated group (p < 0.01). One representative experiment out of three is shown.

Fig. 10. LiCl inhibits CCL3/4 induction by LPS and increases CDP DNA binding. A. BM-DCs were treated with LPS in the presence of different concentrations of LiCl for 12 h, and the levels of CCL3 and CCL4 released in the supernatants were determined by ELISA. B. BM-DCs were treated with LPS together with different concentrations of LiCl for 3 h, followed by RNA preparation. The expression of CCL3 and CCL4 was measured by real time PCR. C. BM-DCs were treated with LiCl for 1 h, and nuclear extracts were prepared. EMSA was carried out with labeled CDP probe. One representative experiment out of three (for A and B) and one out of two (for C) are shown.
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FIG. 11. Model for the PGE<sub>2</sub> signaling in DC leading to the inhibition of CCL3 and CCL4 expression. PGE<sub>2</sub> binds to EP-2 receptors and activates adenylate cyclase (AC). Intracellular cAMP activates the EPAC → PKB → P3K pathway resulting in phosphorylation of PKB and subsequent phosphorylation of p38. Nonphosphorylated p38 binds to DNA and promotes CDP binding. Phosphorylated p38 does not bind to DNA, and loses repressor activity. The final consequence of PGE<sub>2</sub> binding to the EP-2 receptors is the inhibition of GSK-3, allowing CDP to act as a repressor for CCL3/4 gene transcription.

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