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Upregulation of Lipopolysaccharide-Induced Interleukin-10 by Prostaglandin A_1 in Mouse Peritoneal Macrophages

Kim, Hyo Young[1], Jae Ryong Kim[2], and Hee Sun Kim[1]*

Department of [1]Microbiology, [2]Biochemistry and Molecular Biology, and Aging-associated Vascular Disease Research Center, College of Medicine, Yeungnam University, Daegu 705-717, Korea

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The cyclopentenone prostaglandins (cyPGs) prostaglandin A_1 (PGA_1) and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) have been reported to exhibit antiinflammatory activity in activated monocytes/macrophages. However, the effects of these two cyPGs on the expression of cytokine genes may differ. In this study, we investigated the mechanism of action of PGA_1 in lipopolysaccharide (LPS)-induced expression of interleukin (IL)-10 mRNA in mouse peritoneal macrophages. 15d-PGJ_2 inhibited expression of LPS-induced IL-10, whereas PGA_1 increased LPS-induced IL-10 expression. This synergistic effect of PGA_1 on LPS-induced IL-10 expression reached a maximum as early as 2 h after simultaneous PGA_1 and LPS treatment (PGA_1/LPS), and did not require new protein synthesis. The synergistic effect of PGA_1 was inhibited by GW9662, a specific peroxisome proliferator-activated receptor γ (PPARγ) antagonist, and Bay-11-7082, a NF-κB inhibitor. The extracellular signal-regulated kinases (ERK) inhibitor PD98059 increased the expression of PGA_1/LPS-induced IL-10 mRNA, rather than inhibiting the IL-10 expression. Moreover, PGA_1 inhibited LPS-induced ERK phosphorylation. The synergistic effect of PGA_1 on LPS-induced IL-10 mRNA and protein production was inhibited by p38 inhibitor PD169316, and PGA_1 increased LPS-induced p38 phosphorylation. In the case of stress-activated protein kinase/c-Jun NH_2-terminal kinase (SAPK/JNK), the SAPK/JNK inhibitor SP600125 did not inhibit IL-10 mRNA synthesis but inhibited the production of IL-10 protein remarkably. These results suggest that the synergistic effect of PGA_1 on LPS-induced IL-10 expression is NF-κB-dependent and mediated by mitogen-activated protein (MAP) kinases, p38, and SAPK/JNK signaling pathways, and also associated with the PPARγ pathway. Our data may provide more insight into the diverse mechanisms of PGA_1 effects on the expression of cytokine genes.

Keywords: IL-10, macrophage, PGA_1

*Corresponding author
Phone: 82-53-620-4363; Fax: 82-53-653-6628;
E-mail: heesun@med.yu.ac.kr

Excess production of cytokines from immune cells can have detrimental effects that can result in a number of inflammatory diseases. Thus, the control of cytokine production is important in regulating inflammatory reactions.

Antiinflammatory effects of cyPGs have been demonstrated *in vivo* and *in vitro* with diverse cell types [7, 11, 19, 22, 27]. The cyPGs PGA_1 and 15d-PGJ_2 are natural derivatives of PGE1 and PGD2 as arachidonic acid metabolites derived from cyclooxygenase [29]. These two cyPGs are known as representative natural ligands of PPARγ, a ligand-activated transcription factor that regulates target gene expression by heterodimerizing with the retinoid X receptor (RXR) [4, 5]. 15d-PGJ_2 attenuates the expression of proinflammatory mediators (nitric oxide, COX-2, TNF-α, IL-1, IL-6, CXCL10) in activated monocytes/macrophages and mesangial cells [7, 11, 19, 22, 27]. These observations have suggested that 15d-PGJ_2 may have therapeutic uses as an antiinflammatory agent. However, there is also evidence that 15d-PGJ_2 can promote inflammation [9, 10, 14, 15, 21]. For example, it enhances cyclooxygenase 2 in epithelial cells, inhibits LPS-induced IL-10 production in macrophages, and upregulates IL-8/CXCL8 and MIP-2/CXCL2 gene expression in human T cells and mouse monocytes/macrophages. Thus, the role of cyPGs in inflammation is complex and remains controversial. In our previous study [15], PGA_1 had similar effects to 15d-PGJ_2 on the expression of several chemokine genes. However, in contrast to that of 15d-PGJ_2, the role of PGA_1 in cytokine expression has not been well studied. Therefore, we investigated the mechanism of action of PGA_1 in LPS-induced IL-10 expression in mouse peritoneal macrophages.

Materials and Methods

Reagents

Trizol reagent for total RNA isolation was purchased from Invitrogen (Carlsbad, CA, U.S.A.). Dulbecco's phosphate-buffered saline (PBS), RPMI 1640, penicillin-streptomycin, and fetal bovine serum (FBS) were purchased from Gibco/BRL (Life Technologies, Gaithersburg, MD, U.S.A.). 15d-PGJ_2, prostaglandin A_1 (PGA_1), and

GW9662 were purchased from Biomol (Plymouth Meeting, PA, U.S.A.). MAPK inhibitors, 2'-amino-3'methoxyflavone (PD98059), anthral[1,9-cd]pyrazol-6(2H)-one (SP600125), 4-(4-fluorophenyl)-2-(40nitrophenyl)-5-(4-pyridyl)-1H-imidazole (PD169316), and NF-κB inhibitor, (E)3-[(4-methylphenyl)sulfonyl]-2-propenenitrile (Bay 11-7082) were purchased from Calbiochem (San Diego, CA, U.S.A.). Nitrocellulose transfer membrane was obtained from Schleicher & Schuell Bioscience (Dassel, Germany). [α-^{32}P]dCTP and [α-^{32}P]UTP were purchased from Dupont-New England Nuclear (Boston, MA, U.S.A.). The RiboQuant Multi-probe RNase Protection Assay System and the mCK-2b template set were purchased from Pharmingen (San Diego, CA, U.S.A.). Hank's balanced salt solution (HBSS), trihydroxymethyl aminomethane (Tris), sodium dodecyl sulfate (SDS), *Escherichia coli* lipopolysaccharide (LPS, O111:B4), dimethyl sulfoxide (DMSO), dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), pepstatin, leupeptin, autipain, and aprotinin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Oligonucleotide PCR primers for IL-10 and β_2-microglobulin were synthesized by Bionics (Seoul, Korea). The Lightcycler FastStart DNA SYBR Green I Mix was obtained from Roche (Mannheim, Germany), and pERK, pp38, pSAPK/JNK, phospho-IκBα, and γ-tubulin antibodies were obtained from Cell Signaling Technology (Danvers, MA, U.S.A.). All other reagents were pure-grade commercial preparations.

Mice

Specific pathogen-free, inbred C57BL/6 mice, 8 to 10 weeks of age, were purchased from Hyo-Chang (Daegu, Korea). The utmost precautions were taken to ensure that the mice remained infection-free, thereby ensuring that the degree of spontaneous activation of tissue macrophages would be minimal. The mice were cared for in accordance with the *Guide to the Care and Use of Experimental Animals* of the Yeungnam Medical Center.

Preparation of Mouse Peritoneal Macrophages

Thioglycollate-elicited peritoneal macrophages were obtained as previously described [18]. Briefly, macrophages in complete medium (RPMI 1640 supplemented with penicillin, streptomycin, and 10% FBS) were plated in 100-mm tissue culture dishes, incubated for 2 h at 37°C in a 5% CO_2 atmosphere, and then washed three times with HBSS to remove any non-adhering cells. Typically, this resulted in 95% macrophages as determined by morphological criteria. The macrophages were cultured overnight in complete medium at 37°C in 5% CO_2. The medium was then replaced with serum-free RPMI 1640 and the cells were cultured in the presence or absence of stimuli for the indicated times.

Preparation of RNA and Ribonuclease Protection Assay (RPA)

Total RNA was extracted using Trizol reagent according to the manufacturer's instructions. The quantity of RNA obtained was determined by measuring its optical density (OD) at 260 and 280 nm.

RPA for nine cytokines, IL-12p35, IL-12p40, IL-10, IL-1α, IL-1β, IL-1Rα, IL-18/IGIF, IL-6, IFN-γ, MIF, L32, and GAPDH, were performed according to the instructions of the Multi-probe RNase Protection Assay System using RiboQuant. Briefly, mCK-2b was used to obtain radiolabeled antisense RNA probes. *In vitro* transcription was carried out by incubation in a transcription buffer containing 10 mM ATP, 10 mM CTP, 10 mM GTP, 250 μCi [α-^{32}P]UTP, and T7 RNA polymerase. The mixture was incubated at 37°C for 60 min and then treated with DNase I at 37°C for 30 min. The mixture was then extracted with phenol and chloroform. Extracted RNA was precipitated with ethanol, collected by centrifugation at 4°C, and then resuspended in 50 μl of hybridization buffer and diluted to 3×10^5 cpm/μl. Two μl of resuspended probe was used for the reaction. The RNA samples (10 μg RNA/sample) were dried in a vacuum evaporator and resuspended in 8 μl of hybridization buffer. The RNA was annealed to the probe by successive incubation at 95°C for 3 min and at 56°C overnight in a total volume of 10 μl. RNase was added to each sample and incubated at 30°C for 45 min to remove single-stranded RNA; the protected RNA duplexes were then purified by phenol/chloroform extraction and ethanol precipitation. After the pelleted RNA was resuspended in 5–6 μl of gel loading buffer and incubated at 95°C for 3 min, the RNA was quickly quenched on ice and analyzed by electrophoresis on 5% polyacrylamide/8 M urea gels. The gel was adsorbed to filter paper, dried under vacuum, and exposed on X-ray film (Agfa-Gevaert N.V., Belgium) with intensifying screens at −70°C.

Real-Time Polymerase Chain Reaction (Real-Time PCR)

Real-time PCR for IL-10 in mouse peritoneal macrophages was performed using the LightCycler (Roche, Mannheim, Germany). RNA was reverse transcribed to cDNA from 1 μg of total RNA. Real-time PCR was performed in triplicate in a total volume of 20 μl of LightCycler FastStart DNA SYBR Green I mix (Roche) containing primer and 2 μl of cDNA. Prior to PCR amplification, the mixture was incubated at 95°C for 10 min, and the amplification step consisted of 45 cycles of denaturation (10 s at 95°C), annealing (5 s at 60°C), and extension (72°C for 10 s) with fluorescence detection at 72°C after each cycle. After the final cycle, melting point analyses were performed on all samples over the range from 65 to 95°C with continuous fluorescence detection. Expression levels of β_2-microglobulin were used for sample normalization. Results for each gene are expressed as the relative expression level compared with β_2-microglobulin. The following primers were used: IL-10 (256 bp): sense, 5'-tacctggtagaagtgatgcc-3'; antisense, 5'-catcatgtatgcttctatgc-3'; β_2-microglobulin (300 bp): sense, 5'-ggctcgctcggtgaccctagtcttt-3'; antisense, 5'-tctgcaggcgtatgtatcagtctca-3'. IL-10 mRNA levels were determined by comparing experimental levels to the standard curves and are expressed as fold of relative expression.

Enzyme-Linked Immunosorbent Assay (ELISA) for IL-10 Production

The IL-10 protein levels in cell media were measured with an ELISA kit that was obtained from eBioscience (San Diego, CA, U.S.A.). All procedures were performed in accordance with the manufacturer's instructions.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared as previously described [16]. Cells were washed three times with cold PBS, and then scraped and harvested by centrifugation. Cell pellets were resuspended and incubated on ice for 15 min in 400 μl of hypotonic buffer A (10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM PMSF, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 10 μg/ml autipain, and 10 μg/ml aprotinin). Nonidet P-40 was then added to a final concentration of 2.5% and the cells were vortexed for 10 s. Nuclei were separated from the cytosol by centrifugation at 12,000 ×*g* for 15 s. Pellets were resuspended in 40 μl of hypotonic buffer C (20 mM HEPES, 25% glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 0.5 mM

DTT, 0.1 mM PMSF, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 10 μg/ml autipain, and 10 μg/mL aprotinin). Samples were sonicated at level 3–4 for 2–3 s, and then centrifuged for 10 min at 4°C. Nuclear protein concentration was measured using the Bradford assay (Bio-Rad, Richmond, CA, U.S.A.). A consensus sequence for the NF-κB DNA binding site (5'-AGTTGAGGGGACTTTAGGC-3') (*sc*-2505, Santa Cruz Biotechnology) was labeled with [α-^{32}P]dCTP using a random-primed DNA labeling kit (Roche, Germany). A mutant binding sequence for NF-κB was identical to *sc*-2505 except for a "G"→"C" substitution in the NF-κB DNA binding motif (*sc*-2511, Santa Cruz Biotechnology). Labeled DNA was purified over a S-200 HR column (Pharmacia, Piscataway, NJ, U.S.A.) to remove unbound nucleotides. Nuclear extracts were incubated at room temperature for 20 min with approximately 50,000 cpm of labeled oligonucleotide suspended in binding buffer [200 mM HEPES, 500 mM KCl, 10 mM EDTA, 50% glycerol, 10 mM DTT, 1 mg/ml BSA, 1 μg/μl poly(dI-dC)]. Following this incubation, samples were resolved on 4% polyacrylamide gels at 140 V and exposed to film.

Protein Extraction and Western Blot Analysis

Total lysates were prepared as described by Kim *et al.* [17]. The protein concentrations were determined by the Bradford assay (Bio-Rad, Richmond, CA, U.S.A.) using bovine serum albumin as a standard. Nuclei were separated from the cytosol by centrifugation at 12,000 ×*g* for 5 min. Pellets were resuspended in 50 μl of buffer C (20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 10% NP-40). Samples were centrifuged at 12,000 ×*g* for 10 min. Thirty-μg samples of protein were separated on 10% SDS-polyacrylamide gels, and then transferred to nitrocellulose membranes. The membranes were soaked in 5% nonfat dried milk in TTBS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween-20) for 1 h and then incubated for 16–18 h with primary antibodies against pERK, pp38, pSAPK/JNK, phospho-IκBα, and γ-tubulin at 4°C. Membranes were washed three times with TTBS for 10 min and then incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h at 4°C. The membranes were rinsed three times with TTBS for 10 min and antigen-antibody complex was detected using the enhanced chemiluminescence detection system (LAS-3000, Fujifilm, Japan).

Statistical Analysis

Data are expressed as mean±SEM. Statistical differences were analyzed by Wilcoxon signed-rank test. A level of $P<0.05$ was considered statistically significant.

RESULTS

Effect of PGA_1 on LPS-Induced Cytokine Gene Expression in Mouse Peritoneal Macrophages

We first compared the effect of PGA_1 on LPS-induced cytokine gene expression to that of 15d-PGJ_2. After thioglycollate-elicited peritoneal macrophages (TG-PeMϕ)

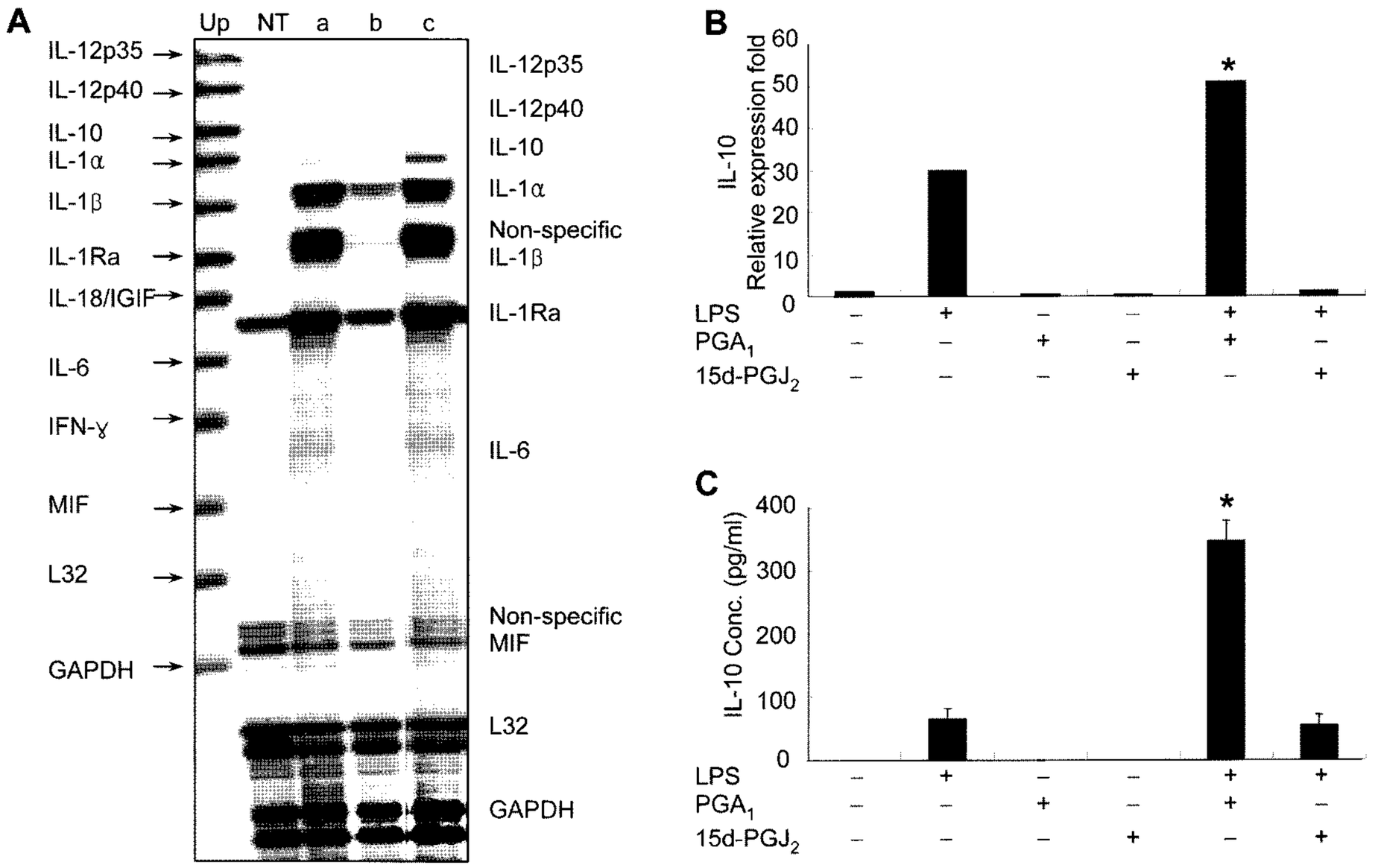

Fig. 1. Effect of PGA_1 on the expression of LPS-induced IL-10 in mouse peritoneal macrophages.

A. TG-PeMϕ were untreated (NT) or treated with LPS (a, 100 ng/ml), LPS plus 15d-PGJ_2 (b, 10 μM), or LPS plus PGA_1 (c, 25 μM) for 2 h. Total RNA was extracted, and 10 μg of isolated RNA per sample was subjected to analysis by RPA. GAPDH and L32 were used as internal standards. uP: unprotected probe. These data are representative of three independent experiments. **B**, **C**. TG-PeMϕ were untreated (NT) or treated with LPS and/or PGA_1 or 15d-PGJ_2 for 2 h. Total RNAs and cell supernatants were isolated and real-time PCR and ELISA were performed. Bars represent mean±SEM from three separate experiments. *$P<0.05$ *vs.* cells treated with LPS alone.

were stimulated with LPS (100 ng/ml), LPS plus PGA_1 (25 μM), or LPS plus 15d-PGJ_2 (10 μM) simultaneously for 2 h, RPA was performed. As shown in Fig. 1A, 15d-PGJ_2 had an inhibitory effect on LPS-induced expression of IL-12p35, IL-12p40, IL-10, IL-1α, IL-1β, and IL-6 mRNA. However, the effects of PGA_1 differed from those of 15d-PGJ_2 on the expression of these cytokine genes, with the exception of IL-12p40. PGA_1 especially increased the expression of LPS-induced IL-10 mRNA. This synergistic effect of PGA_1 on IL-10 expression was confirmed by real-time PCR and ELISA for IL-10 production (Figs. 1B and 1C).

We subsequently examined the dose effect and time course of the synergistic effect of PGA_1 on LPS-induced IL-10 expression. TG-PeMϕ were treated with LPS (100 ng/ml) or LPS plus PGA_1 (5, 12, 25, or 50 μM) simultaneously for 2 h. For the time course of PGA_1 synergistic effect, TG-PeMϕ were treated with LPS or LPS plus PGA_1 (12 μM) simultaneously (PGA_1/LPS) for 0.5, 2, or 4 h. PGA_1 at 5 or 12 μM had the most synergistic effect on LPS-induced IL-10 expression (Fig. 2A). Maximum synergistic expression of PGA_1/LPS-induced IL-10 mRNA occurred at 2 h after treatment, but disappeared at 4 h after treatment (Fig. 2B).

If the synergy of PGA_1/LPS on IL-10 mRNA expression is due to newly synthesized protein, the synergistic action would be blocked in macrophages cotreated with a protein synthesis inhibitor such as cycloheximide (CHX). To test this possibility, TG-PeMϕ were treated with LPS alone or in combination with PGA_1 in the presence or absence of CHX (10 μg/ml) for 2 h. The PGA1/LPS-induced synergistic effect was not attenuated by CHX; rather,

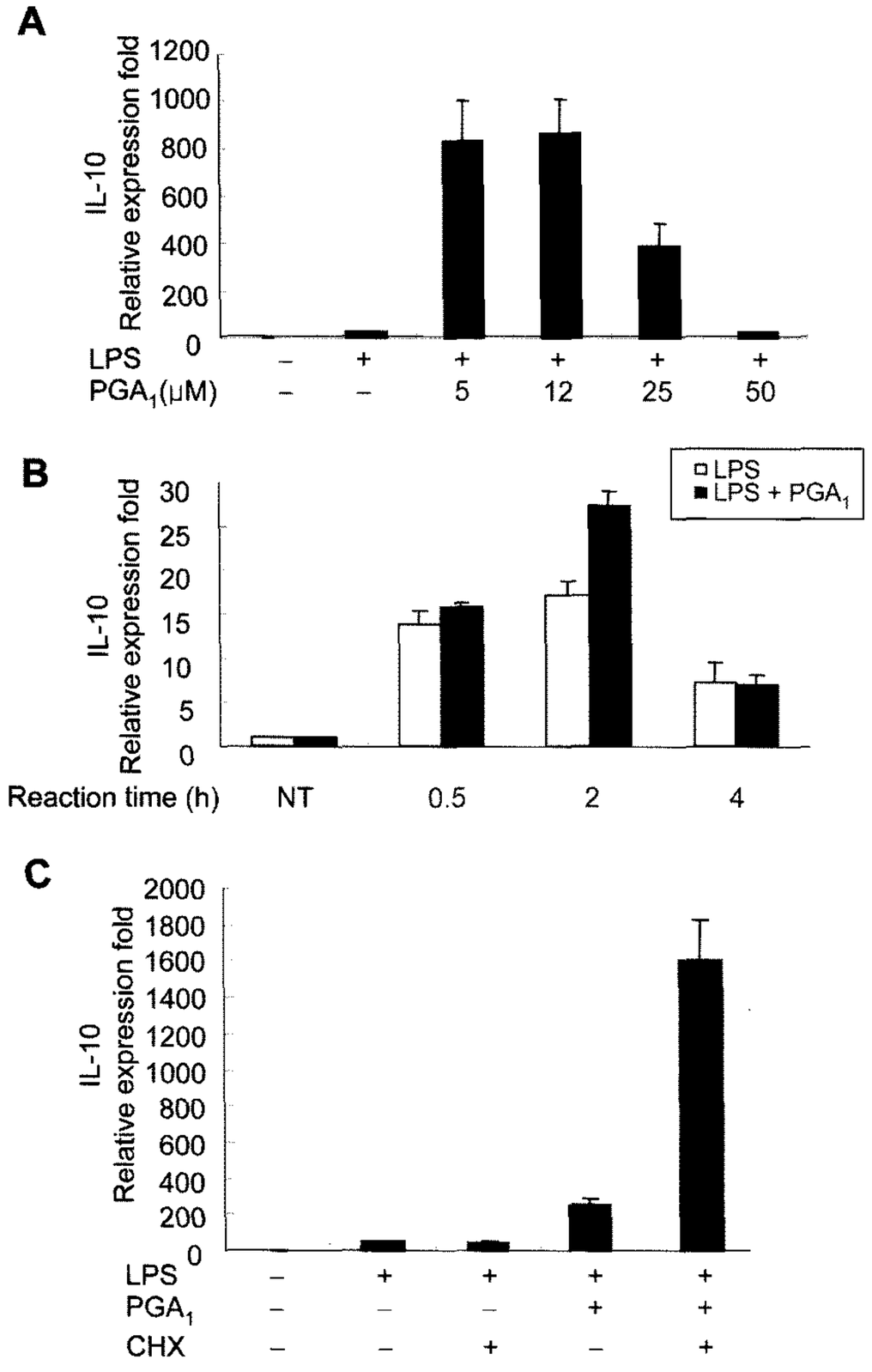

Fig. 2. Dose effect of PGA_1 on LPS-induced IL-10 expression and time course of PGA_1/LPS-induced IL-10 mRNA expression. **A**. TG-PeMϕ were treated with LPS (100 ng/ml) or simultaneously with LPS plus PGA_1 (5, 12, 25, or 50 μM PGA_1) for 2 h. Total RNAs were isolated and real-time PCR was performed. Bars represent mean±SEM from three separate experiments. *P<0.05 *vs.* cells treated with LPS alone. **B**. TG-PeMϕ were treated with LPS or LPS plus PGA_1 (12 μM) simultaneously for 0.5, 2, or 4 h. Total RNA was isolated and real-time PCR was performed. **C**. Synergistic effect of PGA_1 on LPS-induced IL-10 expression is not prevented by a protein synthesis inhibitor. TG-PeMϕ were untreated (NT) or treated with LPS and/or PGA_1 (12 μM) in the absence or presence of cycloheximide (CHX, 10 μg/ml) for 2 h. Total RNA was isolated and real-time PCR was performed. Bars represent mean±SEM from three separate experiments. *P<0.05 *vs.* cells treated with PGA_1/LPS.

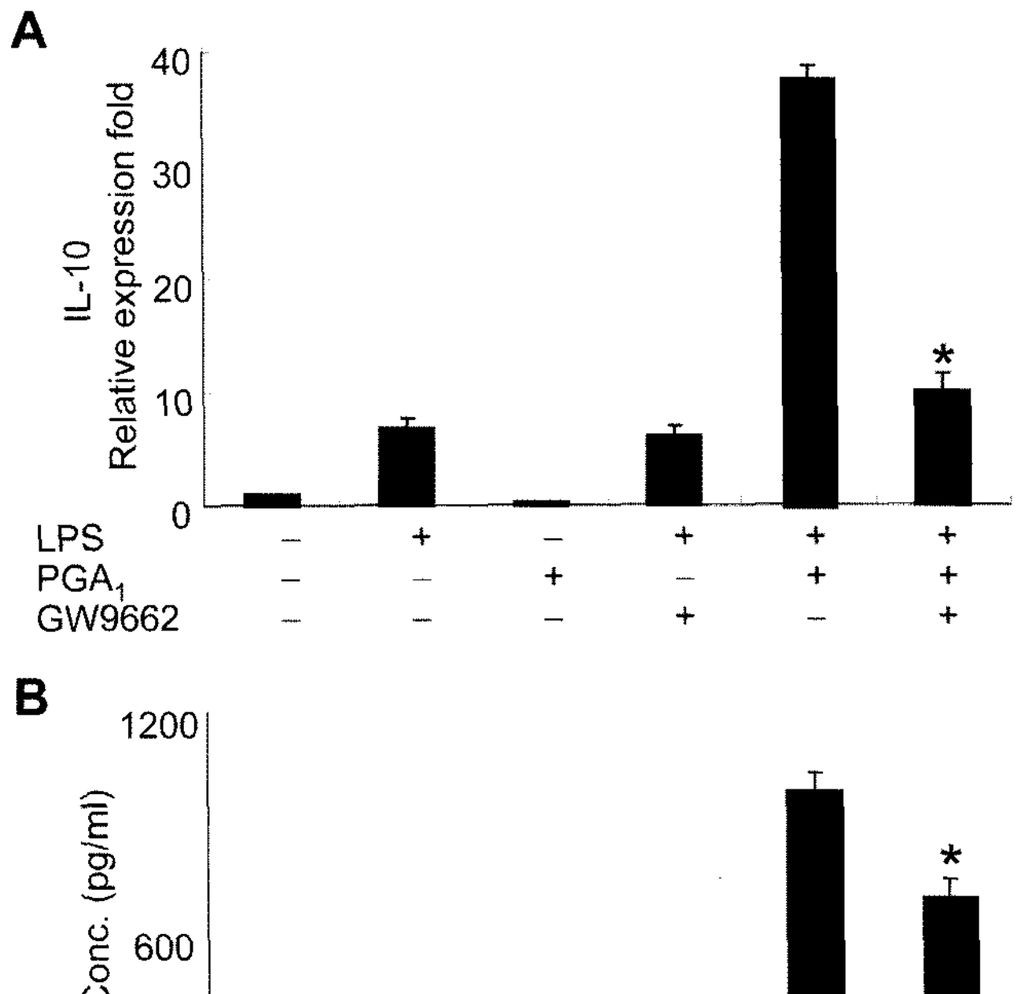

Fig. 3. PGA_1-mediated synergy of LPS-induced IL-10 mRNA expression is dependent on PPARγ.
TG-PeMϕ were untreated or treated with LPS (100 ng/ml) and/or PGA_1 (12 μM) in the absence or presence of GW9662 (10 μM) for 2 h. Total RNAs and cell supernatants were prepared, and real-time PCR (**A**) and ELISA (**B**) were performed. Bars represent mean±SEM from three separate experiments. *P<0.05 *vs.* cells treated with PGA_1/LPS.

the PGA_1/LPS-induced IL-10 expression was increased remarkably by CHX (Fig. 2C).

Synergistic Effect of PGA_1 on LPS-Induced IL-10 Expression is Mediated by PPARγ and NF-κB Activation

To evaluate whether the mechanism of the synergistic effect of PGA_1 was mediated by PPARγ, GW9662, a PPARγ antagonist, was tested in mouse peritoneal macrophages. TG-PeMϕ were stimulated with PGA_1/LPS in the presence or absence of GW9662 (10 μM) for 2 h. GW9662 remarkably inhibited the synergistic effect of PGA_1 on LPS-induced IL-10 expression (Fig. 3A) and inhibited IL-10 protein production (Fig. 3B). Therefore, the synergistic increase in IL-10 mRNA expression following PGA_1/LPS treatment is related to PPARγ activation.

Next, the role of NF-κB activation on PGA_1/LPS-induced IL-10 expression was investigated. Bay-11-7082 selectively blocks the phosphorylation of IκBα, thus preventing the activation and nuclear translocation of NF-κB. After TG-PeMϕ were treated with PGA_1 and/or LPS in the presence or absence of Bay-11-7082 (10 μM) for 2 h, real-time PCR and ELISA for IL-10 gene expression and protein production were performed. Bay-11-7082 blocked the synergistic effect of PGA_1 on LPS-induced IL-10 mRNA expression remarkably (Fig. 4A) and also inhibited the production of PGA_1/LPS-induced IL-10 protein (Fig. 4B). To further confirm the association between NF-κB activity and IL-10 expression, EMSA for binding activity of NF-κB and Western blotting for IκBα phosphorylation were performed. In cells treated with PGA_1/LPS, we could not detect strong increase of NF-κB activity; however, IκBα

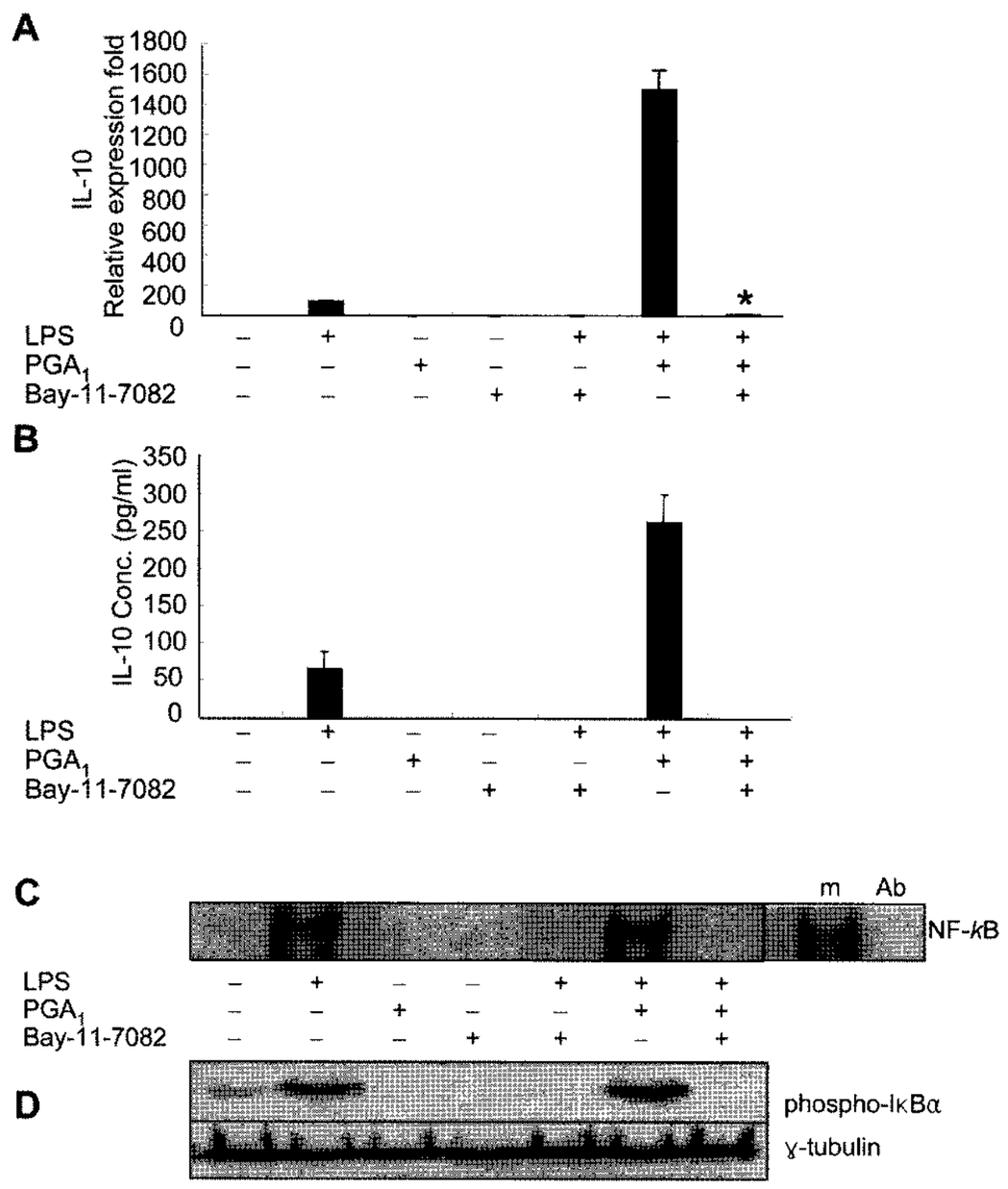

Fig. 4. Synergistic action of PGA_1 on LPS-induced IL-10 mRNA expression is dependent on NF-κB activation.
TG-PeMϕ were untreated or treated with LPS (100 ng/ml) or LPS plus PGA_1 (12 μM) in the absence or presence of Bay-11-7082 (10 μM) for 2 h. Total RNAs and cell supernatants were prepared, and real-time PCR (**A**) and ELISA (**B**) were performed. **C**. Specific binding activity of NF-κB from nuclear extracts was assessed by electrophoretic mobility shift assay (EMSA). Aliquots of nuclear extract were incubated with a 100-fold excess of mutant probe (m) or with 2 μg of anti NF-κB Ig (Ab) before EMSA. **D**. Cell lysates were separated on 10% SDS-polyacrylamide gels and then immunoblotted with phospho-IκBα antibodies. Data shown are representative of three independent experiments. Bars represent mean± SEM from three independent experiments. *$P<0.05$ *vs.* cells treated with PGA_1/LPS.

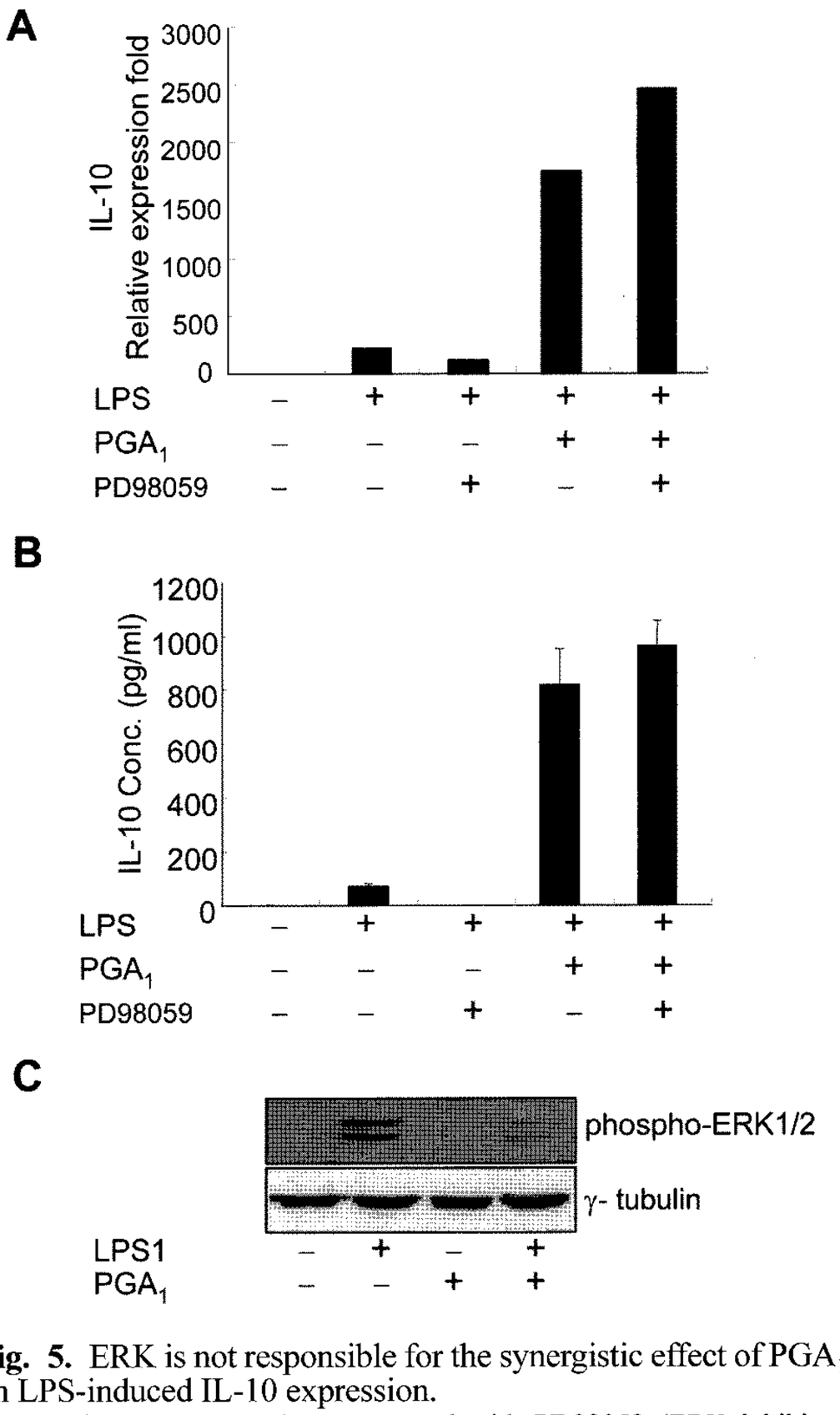

Fig. 5. ERK is not responsible for the synergistic effect of PGA_1 on LPS-induced IL-10 expression.
TG-PeMϕ were untreated or pretreated with PD98059 (ERK inhibitor, 10 μM) for 30 min. Cells were left untreated or treated with LPS (100 ng/ml) and/or PGA_1 (12 μM) for 2 h. After total mRNAs and cell supernatants were isolated, real-time PCR (**A**) and ELISA (**B**) were performed. **C**. TG-PeMϕ were untreated (NT) or treated with LPS (100 ng/ml) and/or PGA_1 (12 μM) for 10 min. Thereafter, cell lysates were separated on 10% SDS-polyacrylamide gels and then immunoblotted with ERK1/2 and phospho-ERK1/2 antibody.

phosphorylation was increased compared with those in cells treated with LPS alone (Figs. 4C and 4D). We therefore concluded that the synergistic effect of PGA_1 on LPS-induced IL-10 expression is mediated *via* NF-κB activation.

Synergistic Effect of PGA_1 on LPS-Induced IL-10 mRNA Expression is Related to MAPK Signaling Pathways

We investigated whether MAPK signaling pathways are involved in the synergistic effect of PGA_1 on LPS-induced IL-10 expression. After TG-PeMϕ were pretreated with the ERK1/2 inhibitor PD98059 (10 μM), the p38 MAP kinase inhibitor PD169316 (10 μM), or the SAPK/JNK inhibitor SP600125 (25 μM) for 0.5 h, cells were treated with PGA_1 and/or LPS for 2 h. Real-time PCR and ELISA were then performed. In addition, to further confirm these results, we investigated the phosphorylation of MAP kinases in cells treated with PGA_1/LPS. PD98059 increased the IL-10 mRNA expression in cells stimulated with PGA_1/LPS, rather than inhibiting IL-10 expression. The ELISA result showed the same pattern as the gene expression (Figs. 5A and 5B). Moreover, phosphorylation of ERK was inhibited in cells treated with PGA_1/LPS (Fig. 5C). The expression of PGA_1/LPS-induced IL-10 mRNA was decreased by the p38 MAP kinase inhibitor PD169316, and production of

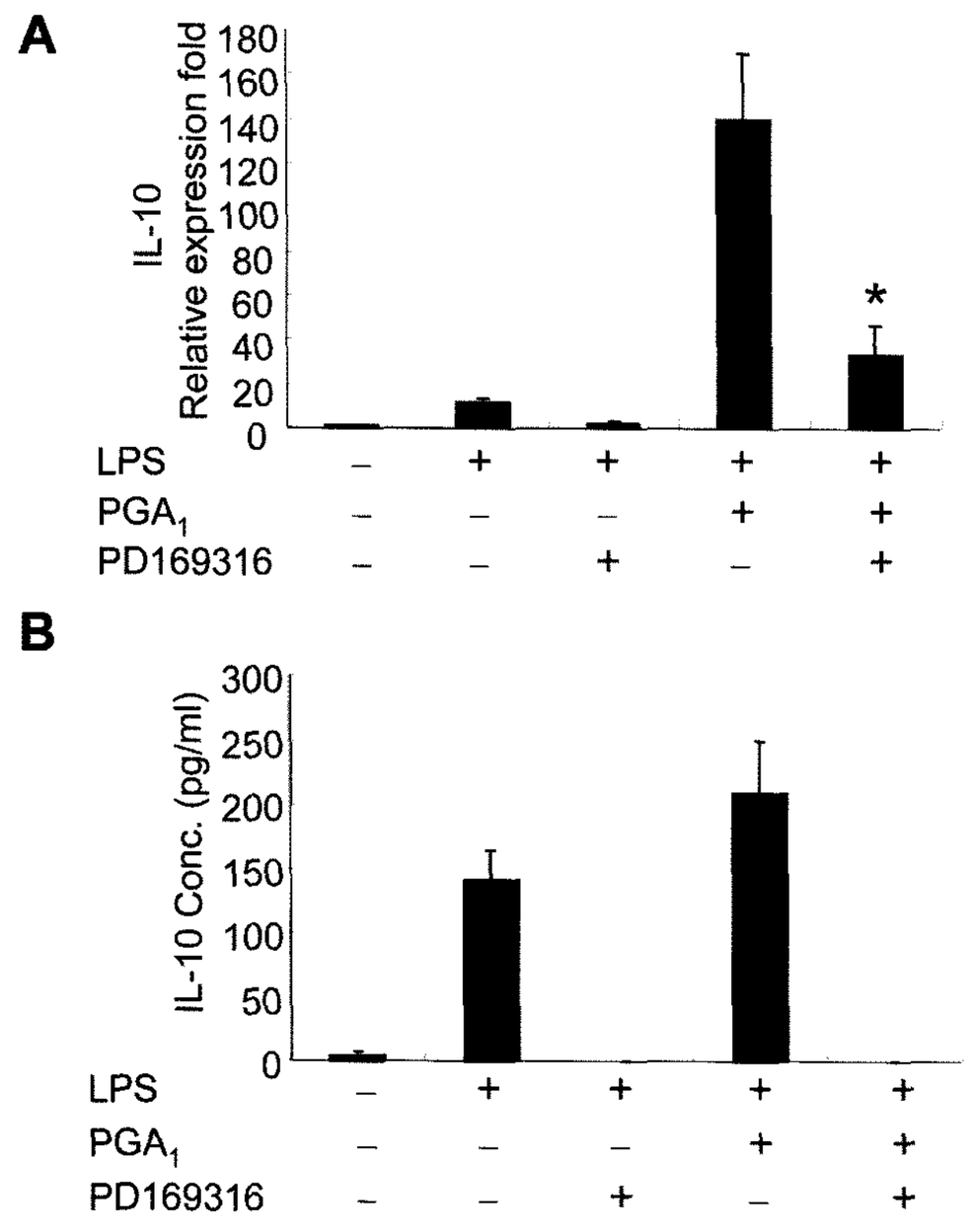

Fig. 6. Synergistic effect of PGA_1 on LPS-induced IL-10 expression is decreased by a p38 inhibitor.

TG-PeMϕ were untreated or pretreated with PD169316 (p38 inhibitor, 10 μM) for 30 min. Cells were left untreated or treated with LPS (100 ng/ml) and/or PGA_1 (12 μM) for 2 h. After total mRNAs and cell supernatants were isolated, real-time PCR (**A**) and ELISA (**B**) were performed. **C**. TG-PeMϕ were untreated or treated with LPS (100 ng/ml) and/or PGA_1 (12 μM) for 10 min. Thereafter, cell lysates were separated on 10% SDS-polyacrylamide gels and then immunoblotted with phospho-p38 antibody. Bars represent mean±SEM from three separate experiments. *P<0.05 *vs.* cells treated with PGA_1/LPS.

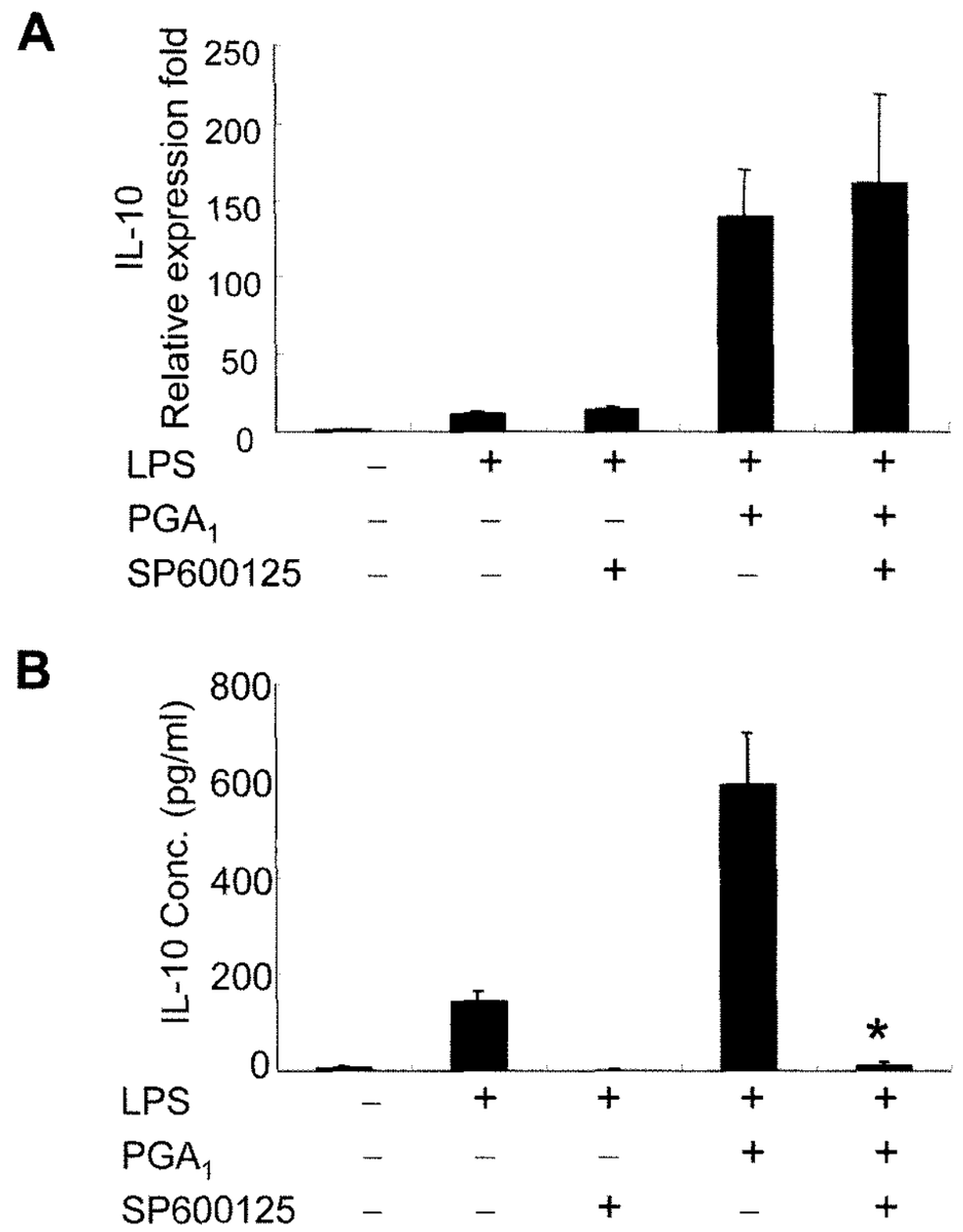

Fig. 7. SAPK/JNK is responsible for the synergistic effect of PGA_1 on LPS-induced IL-10 expression.

TG-PeMϕ were untreated or pretreated with SP600125 (SAPK/JNK inhibitor, 25 μM) for 30 min. Cells were left untreated or treated with LPS (100 ng/ml) and/or PGA_1 (12 μM) for 2 h. After total mRNAs and cell supernatants were isolated, real-time PCR (**A**) and ELISA (**B**) were performed. **C**. TG-PeMϕ were untreated or treated with LPS (100 ng/ml) and/or PGA_1 (12 μM) for 10 min. Thereafter, cell lysates were separated on 10% SDS-polyacrylamide gels and then immunoblotted with phospho-SAPK/JNK antibody. Bars represent mean±SEM from three separate experiments. *P<0.05 *vs.* cells treated with PGA_1/LPS.

IL-10 protein in PGA_1/LPS-treated cell media was also blocked by PD169316 (Figs. 6A and 6B). An increase of p38 phosphorylation in cells treated with PGA_1/LPS was also detected (Fig. 6C). In the case of SAPK/JNK, SP600125 did not inhibit IL-10 mRNA expression in cells treated with PGA_1/LPS, and the phosphorylation of SAPK/JNK was not increased in cells stimulated with PGA_1/LPS compared with that in cells treated with LPS alone (Figs. 7A and 7C). However, SP600125 inhibited IL-10 protein production remarkably (Fig. 7B). Taken together, these results suggest the upregulation of IL-10 by PGA_1/LPS is mediated through p38 and SAPK/JNK signaling pathways.

Discussion

Prostaglandins (PGs) have diverse biologic actions depending on the target cell type and the PG type. Among PGs, cyclopentenone prostaglandins (cyPGs) can interact with specific cellular signaling molecules and transcription factors [6] and exhibit complex regulatory mechanisms on cytokine gene expression [1, 2, 11, 19].

In the present study, PGA_1 had a synergistic effect on LPS-induced IL-10 mRNA expression, in contrast to 15d-PGJ_2. Although 15d-PGJ_2 had suppressive effects on the LPS-induced expression of IL-12, IL-10, IL-1, and IL-8 mRNA, PGA_1 had no effect on LPS-induced IL-12p35, IL-1α, IL-1β, and IL-8 mRNA expression. In our previous chemokine study [15], the expression patterns of several LPS-induced chemokines in mouse peritoneal macrophages in response to PGA_1 and 15d-PGJ_2 were very similar; both of these cyPGs suppressed the LPS-induced expression of RANTES (CCL5), MIP-1α (CCL3), MIP-1β (CCL4), IP-10 (CXCL10), and MCP-1 (CCL2) mRNA. However, overall, 15d-PGJ_2 activities were more effective on cytokine expression than those of PGA_1 [15, 20, 31]. Thus, both of these cy-PGs have a different, complex effect on cytokine gene expression depending on the cell type or stimulant.

PPARγ is highly expressed in adipose tissues and thought to play an important role in adipocyte differentiation [25]. Peritoneal macrophages express low levels of PPARγ, but activated macrophages express high levels of PPARγ [23]. cyPGs are high-affinity natural ligands for PPARγ and are known to exert effects on cytokine genes through PPARγ-dependent and PPARγ-independent mechanisms [13, 24, 33]. Ricote *et al.* [23] demonstrated that 15d-PGJ_2 suppressed NF-κB activity *via* PPARγ-dependent and PPARγ-independent pathways in mouse peritoneal macrophages. In our previous studies [14, 15], the synergistic effects of 15d-PGJ_2 on LPS-induced MIP-2(CXCL2) and KC(CXCL1) gene expression were not mediated by the PPARγ pathway. The PPARγ antagonist GW9662 exhibited a remarkable inhibitory effect on the synergistic activity of PGA_1 on LPS-induced IL-10 mRNA expression and IL-10 protein production. We also attempted to confirm this PPARγ dependence by investigating PPARγ translocation from the cytosol to the nucleus in cells treated with PGA_1/LPS. However, the general mode of PPARγ activation, translocation from the cytosol to the nucleus, was not detected (data not shown). This is consistent with reports from Erl *et al.* [8] and Jostarndt *et al.* [12], who reported that PPARγ expression is located in the nucleus regardless of stimulation in monocytic cells and that classical translocation from the cytosol to the nucleus does not occur in cyPG-treated HUVEC. Although we did not observe the classical translocation of PPARγ, our result suggests that PPARγ is related to the effect of PGA_1 on LPS-induced IL-10 expression.

It is widely accepted that cyPGs exert their effects on inflammatory-mediated genes in cells by either inhibiting or activating NF-κB signaling [3, 15, 23, 30]. The antiinflammatory activity of cyPGs is mediated mainly through inhibition of NF-κB activation, but 15d-PGJ_2 has been also reported to upregulate IL-8 and MIP-2(CXCL2) expressions through NF-κB activation. Most of the previous studies with cyPGs have focused on 15d-PGJ_2, and few studies of the signaling pathways of PGA_1 or its effects on NF-κB activity have been reported. In our NF-κB result, NF-κB activity was not increased remarkably in cells treated with PGA_1/LPS. However, Bay-11-7082 blocked the synergistic PGA_1/LPS-induced IL-10 mRNA expression and protein production, and the increase of IκBα phosphorylation was detected in cells treated with PGA_1/LPS. Therefore, the synergistic effect of PGA_1 on LPS-induced IL-10 mRNA expression is mediated by NF-κB activation. Rossi *et al.* [26] demonstrated an inhibitory effect of PGA_1 on NF-κB activation in various human cell types. However, they used PGA_1 at high concentration (24 μM) and different cell types (Jurkat T cell, T lymphoid cell, and HeLa cell). Bureau *et al.* [3], who examined the pro-inflammatory role of cyPGs at low micromolar concentration in A549 epithelial cells, reported that PGA_1 at high concentration (≥24 μM) inhibits NF-κB activation, but low concentrations (≤12 μM) do not inhibit NF-κB activation in A549 epithelial cells.

Although many reports [6, 20, 28, 30, 34] have shown that cyPGs inhibit MAP kinase activation, this inhibition appears to be target gene-, cell type-, and stimulation condition-dependent. Wilmer *et al.* [32] reported that 15d-PGJ_2 dose-dependently increases ERK activity in human mesangial cells. Among MAPK signaling pathways, the ERK pathway is known to be associated with the synergistic activity of 15d-PGJ_2 on the expression of some cytokine genes [9, 10, 12]. In our previous MIP-2(CXCL2) study [15], p38 MAP kinase and SAPK/JNK pathways were associated with the synergistic effect of 15d-PGJ_2 on LPS-induced MIP-2 (CXCL2) expression. In the present study, p38 and SAPK/JNK signaling pathways were associated with the synergistic effect of PGA_1 on LPS-induced IL-10

expression. PGA_1 increased the phosphorylation of p38 in cells treated with LPS. SP600125, a SAPK/JNK inhibitor, inhibited the production of IL-10 protein but did not inhibit IL-10 mRNA synthesis in cells treated with PGA_1/LPS. SP600125 seems to have no effect on the transcription of IL-10 synthesis but plays an unknown inhibitory action on the translation process. Thus, IL-10 synthesis seems to be regulated at the transcriptional and translational levels in mouse peritoneal macrophages. Therefore, it is possible that upregulation of IL-10 by PGA_1/LPS is mediated through the SAPK/JNK pathway.

This is the first report demonstrating that PGA_1 has a synergistic effect on LPS-induced IL-10 expression in mouse peritoneal macrophages. This synergistic effect is mediated by MAP kinases, p38, and SAPK/JNK signaling pathways, and through a PPARγ pathway. IL-10 is a well-known antiinflammatory cytokine and has an important regulatory role in limiting the duration and extent of acute inflammatory response. Therefore, PGA_1 may play a regulatory role as a naturally occurring feedback inhibitor of inflammation.

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