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Synergistic Effect of Interleukin-18 on the Expression of Lipopolysaccharide-Induced IP-10 (CXCL-10) mRNA in Mouse Peritoneal Macrophages

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Abstract Interleukin (IL)-18, a member of the family of IL-1 cytokine, is one of the principal inducers of interferon-γ (IFN-γ) in T lymphocytes and natural killer cells. The objective of the present study was to evaluate the effect of IL-18 on the expression of chemokine IP-10 (CXCL-10) mRNA in mouse peritoneal macrophages. IL-18 had very weak direct effect or synergistic effect with IL-12 on the expression of IP-10 mRNA in C57BL/6 mouse peritoneal macrophages. However, IL-18 pretreatment was found to play a cooperative role in the expression of lipopolysaccharide (LPS)-induced IP-10 mRNA. For the expression of LPS-induced IP-10 mRNA, the synergistic effect was detected after 16 h of IL-18 pretreatment prior to LPS stimulation. The expression level of CD14 in cells stimulated with LPS was not changed by IL-18 pretreatment, and the level of IFN-γ production during IL-18 pretreatment plus LPS stimulation was barely discernible (0.36±0.31 pg/ml). Namely, the synergistic effect of IL-18 pretreatment was not related to a change of LPS receptor, CD14 expression, and the production of IFN-γ by the interaction between IL-18 and LPS. The synergistic effect of IL-18 pretreatment on the expression of LPS-induced IP-10 was related to not NF-*k*B but AP-1 activation, and associated with the extracellular signal-regulated kinase (ERK) pathway, one of the mitogen-activated protein kinase signaling pathways. These results provide useful information that may elucidate the mechanisms underlying the effect of IL-18 on the expression of IP-10 mRNA.

Key words: Interleukin-18, IP-10, lipopolysaccharide

The inflammatory response to lipopolysaccharide (LPS) stimulation is mediated, at least in part, by the secretion of chemokines at incipient inflammation sites. Virtually all sorts of immune cells carry the potential to generate abundant amounts of different chemokines. Many studies have provided data that indicate that individual chemokine genes can be differentially regulated in response to LPS stimulation [12–14].

Interleukin (IL)-18 has been classified as a member of the IL-1 family, primarily by its structural similarity to IL-1β [4]. It represents one of the principal inducers of interferon-γ (IFN-γ) in natural killer cells and T lymphocytes. Until the discovery of IL-18, the dominant IFN-γ-inducing factor in macrophages was thought to be IL-12, a heterodimeric cytokine [21]. IL-18 acts synergistically with IL-12, inducing the generation of IFN-γ in a variety of immune cells [6, 16–18]. IFN-γ-inducible protein 10 kilodaltons (IP-10, CXCL-10) is a representative CXC chemokine that is induced by IFN-γ [5]. This chemokine has a relevant function in the inflammatory reactions, acting as a chemoattractant for lymphocytes.

Although it has been demonstrated that IL-18 induces IFN-γ production in a variety of ways, the effect of IL-18 on the expression of IFN-γ-inducible chemokine genes has not been clarified. We hypothesized that IL-18 would act on the expression of IP-10 in macrophages. Thus, the objective of the present study was to evaluate the effect of IL-18 on the expression of IP-10 in mouse peritoneal macrophages.

Materials and Methods

Reagents

Brewer's thioglycollate broth was obtained from Difco Laboratories (Detroit, MI, U.S.A.). RPMI 1640 medium, Hank's balanced salt solution (HBSS), Dulbecco's phosphate-buffered saline (PBS), and fetal bovine serum (FBS) were

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obtained from Gibco BRL (Life Technologies, Gaithersburg, MD, U.S.A.). The magna nylon transfer membrane was obtained from Micron Separation, Inc. (Westboro, KS, U.S.A.), and the nitrocellulose transfer membrane was obtained from Scheicher & Schueil Bioscience (Dassel, Germany). The high prime kit was acquired from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Dupont-New England Nuclear (Boston, MA, U.S.A.) was the source for the [α-^{32}P]dCTP. The recombinant mouse IFN-γ, IL-18, and IL-12 were all acquired from Bio-source (Camarillo, CA, U.S.A.). *Escherichia coli* LPS (O111:B4), trihydroxymethyl aminomethane (Tris), pyrrolidone dithiocarbamate (PDTC), dimethyl sulfoxide (DMSO), dithiothreitol (DTT), phenylmethyl sulfonylfluoride (PMSF), pepstatin, leupeptin, autipain, aprotinin, and sodium dodecyl sulfate (SDS) were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Curcumin was obtained from Acro (New Jersey, U.S.A.). MAPK inhibitor 2'-amino-3'methoxyflavone (PD98059) was obtained from Calbiochem (San Diego, CA, U.S.A.). RNA-bee for total RNA isolation was obtained from TEL-TEST (Friendswood, TX, U.S.A.). The plasmids encoding the IP-10 and GAPDH genes were kindly provided by Dr. Hamilton at the Department of Immunology, Lehner Research Institute, Cleveland Clinic Foundation, IL, U.S.A. Oligonucleotide primers of IP-10, CD14, and β_2-microglobulin for PCR were synthesized by Bionics (Seoul, Korea). The nuclear factor-*k*B (NF-*k*B), and activator protein (AP-1) oligonucleotides were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The Lightcycler FastStart DNA SYBR Green I Mix was obtained form Roche (Mannheim, Germany), and pERK, ERK, and GAPDH antibodies were obtained from Cell-signaling Technology (Danvers, MA, U.S.A.). All other reagents were commercial preparations of pure grade.

Mice

Specific pathogen-free (SPF) female inbred C57BL/6 mice, at 8 to 10 weeks of age, were purchased from Hyochang Science (Daegu, Korea) and maintained in microisolator cages at the animal center of Yeungnam Medical College. All experimental animals received autoclaved food and bedding in order to minimize the exposure to viral or microbial pathogens and the degree to which tissue macrophages were spontaneously activated.

Preparation of Mouse Peritoneal Macrophages and Cell Culture

Thioglycollate (TG)-elicited macrophages were obtained by the previously described method [13]. Peritoneal lavage from the C57BL/6 mice was performed using 10 ml of ice-cold HBSS containing 5 U/ml of heparin. Macrophages were plated in 100-mm dishes, incubated for 2 h at 37°C in an atmosphere containing 5% CO_2, and then washed three times with HBSS in order to remove any nonadherent cells. Macrophages were cultured overnight in RPMI 1640 containing 10% FBS at 37°C in an atmosphere containing 5% CO_2. After being allowed to stand overnight, the culture medium was replaced with serum-free RPMI 1640 medium. Cells were then cultured in the absence or presence of stimuli at the indicated dose for the indicated time.

Preparation of Total RNA and Northern Hybridization Analysis

Total cellular RNA was extracted with RNA-bee solution in accordance with the manufacturer's instructions.

For Northern blot analysis, equal amounts of RNA (7 μg/sample) were used in each lane of the gel. The RNA was denatured, separated by electrophoresis in 1% agarose/2.2 mol/l formaldehyde gel, and then transferred to a nylon membrane. The blots were prehybridized for 8 h at 42°C in prehybridization solution (50% formamide, 1% SDS, 1× Denhardt's, 0.25 mg/ml denatured salmon sperm DNA, and 50 mmol/l sodium phosphate). Hybridization was conducted for 16 to 18 h at 42°C with 1×10^7 counts per minute (cpm) of denatured plasmid DNA, which contained IP-10 inserts. The blots were rinsed for 30 min at 42°C and for 15 min at 65°C with a 0.5×SSC-0.1% SDS solution. The filters were dried and exposed to X-ray films (Agfa-Gevaert, Belgium) at −70°C.

Enzyme-Linked Immunosorbent Assay (ELISA) for IFN-γ and IP-10

The levels of IFN-γ protein in the cell supernatants were measured with an ELISA kit obtained from Bio-source (Camarillo, CA, U.S.A.), and the levels of IP-10 were also measured with an ELISA kit obtained from R&D Systems (Minneapolis, MN, U.S.A.). All procedures were performed in accordance with the manufacturer's instructions.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared by using the method described in Kim *et al.* [9]. Cells were washed three times with cold PBS. Thereafter, cells were scraped and harvested by centrifugation. Cell pellets were resuspended and incubated on ice for 15 min in 400 μl of hypotonic buffer A (10 mmol/l HEPES, 10 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 0.5 mmol/l DTT, 0.1 mmol/l 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 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 mmol/l HEPES, 25% glycerol, 0.4 mol/l NaCl, 1 mmol/l EDTA, 1 mmol/l EGTA, 0.5 mmol/l DTT, 0.1 mmol/l PMSF, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 10 μg/ml autipain, and 10 μg/ml aprotinin). Samples were sonicated at the 3 to 4 level

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.). Consensus sequences for the nuclear factor (NF)-*k*B DNA binding site (5'-agttgaggggactttaggc-3') and activator protein (AP)-1 DNA binding site (5'-cgcttgatgactcagccggaa-3') (Santa Cruz Biotechnology) were labeled with [α-^{32}P]dCTP using the random primed DNA labeling kit (Roche, Germany). Labeled DNA was purified over a S-200 HR column (Pharmacia, Piscataway, NJ, U.S.A.) to remove unbound nucleotides. Nuclear protein extracts, at a concentration of 10 μg, were incubated at room temperature for 20 min with ~50,000 cpm of the labeled oligonucleotide suspended in binding buffer (200 mmol/l HEPES, 500 mmol/l KCl, 10 mmol/l EDTA, 50% glycerol, 10 mmol/l DTT, 1 mg/ml BSA, and 1 μg/μl poly[dI-dC]). Then, samples were resolved on a 4% polyacrylamide gel at 150 V and exposed to films.

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

Real-time PCR for IP-10 and CD14 in mouse peritoneal macrophages was performed using a LightCycler (Roche, Mannheim, Germany). RNA was reverse-transcribed to cDNA from 1 μg of total RNA, which was then subjected to real-time PCR performed essentially according to the manufacturer's instructions. 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. PCR amplification was preceded by incubation of the mixture for 10 min at 95°C, and the amplification step consisted of 45 cycles of denaturation, annealing, and extension. Denaturation was performed for 10 s at 95°C, annealing was performed for 5 s at 60°C, and extension was performed at 72°C for 10 s, with fluorescence detection at 72°C after each cycle. After the final cycle, melting point analyses of all samples were performed within the range of 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 primers were as follows: IP-10 (431 bp): sense primer, 5'-cctatcctgcccacgtgttgag-3'; antisense primer, 5'-cgcacctc-cacatagcttacag-3'; CD14 (500 bp): sense primer, 5'-acatctt-gaacctccgcaac-3'; antisense primer, 5'-agggttcctatccagcctgt-3'; β_2-microglobulin (300 bp): sense primer, 5'-ggctcgctcg-gtgaccctagtcttt-3'; antisense primer, 5'-tctgcaggcgtatgtatca-gtctca-3'. The levels of IP-10 and CD14 mRNA were determined by comparing experimental levels with the standard curves and expressed as arbitrary units.

Protein Extraction and Western Blot Analysis

Cells were lysed on ice using RIPA lysis buffer (25 mmol/l Tris [pH 7.4], 150 mmol/l KCl, 5 mmol/l EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/l Na_3VO_4, 5 mmol/l NaF, and 1 mmol/l PMSF). Cell disruption was completed by sonicating for 15 s on ice. Following centrifugation at 13,600 ×*g* for 15 min at 4°C, protein concentrations in the supernatants were quantified by the Bradford assay (Bio-Rad, Richmond, CA, U.S.A.) using bovine serum albumin as a standard. Thirty-μg samples of protein were separated on 10% SDS-polyacrylamide gels, and then transferred to nitrocellulose membranes. Membranes were soaked in 5% nonfat dried milk in TTBS (10 mmol/l Tris-HCl [pH 7.5], 150 mmol/l NaCl, and 0.05% Tween-20) for 30 min and then incubated for 1 h with primary antibodies against ERK, pERK, and GAPDH at 4°C. After washing three times with TTBS for 10 min, membranes were incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h at 4°C. Membranes were rinsed three times with TTBS for 10 min and the antigen-antibody complex was detected using the enhanced chemiluminescence detection system (LAS-3000, Fujifilm, Japan).

Statistical Analysis

Data are expressed as mean±SEM. Statistical analysis of differences was compared by the Mann-Whitney test. A level of $P<0.05$ was considered statistically significant.

RESULTS

Effects of IL-18 on the Expression of IP-10 (CXCL-10) mRNA in Mouse Peritoneal Macrophages

IFN-γ and LPS were used for positive induction of the IP-10 mRNA expression. After the TG-elicited C57BL/6 peritoneal macrophages (PeMϕ) were stimulated with LPS (100 ng/ml), IFN-γ (100 U/ml), IL-18 (50 ng/ml), or IL-12 (25 ng/ml) for 4 h, Northern analysis was conducted on all samples. As shown in Fig. 1A, IL-18 or IL-12 alone exerted no effect on the IP-10 mRNA expression. Next, we attempted to evaluate the synergistic effect of IL-18 plus IL-12 at several concentrations on the expression of IP-10 mRNA. After the TG-elicited PeMϕ was stimulated with LPS (100 ng/ml) alone, IL-18 (50 ng/ml) plus various concentrations of IL-12 (5, 25, and 125 ng/ml), or IL-12 (25 ng/ml) plus various concentrations of IL-18 (5, 50, and 250 ng/ml) simultaneously for 4 h, total RNAs were analyzed by Northern blot analysis. Although the IP-10 mRNA expression was detected in all cells to which IL-18 plus IL-12 stimulation was applied, no enhanced IP-10 mRNA expression was detected, as compared with the expression of IP-10 in cells stimulated with LPS (Fig. 1B). Next, the synergistic effect of IL-18 on the time course of LPS-induced IP-10 mRNA expression at a variety of time points was examined. The TG-elicited PeMϕ were stimulated with LPS or LPS plus IL-18 for 2, 4, 8, and 16 h. The overall time-course pattern and levels of the IP-10 mRNA expression in cells stimulated with LPS plus IL-18 were almost identical with those of the IP-10 mRNA expression

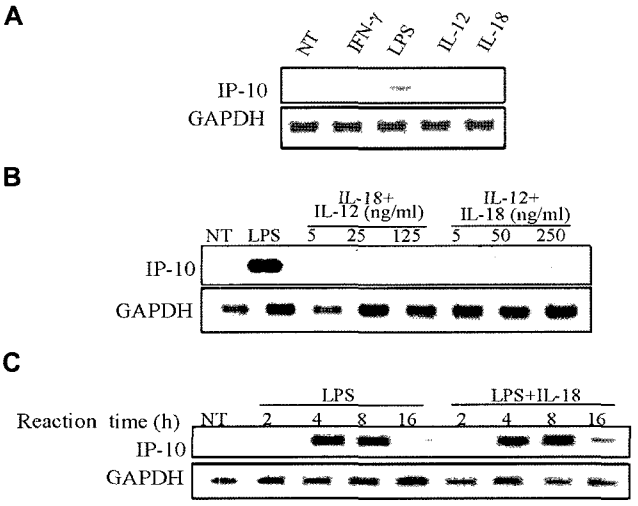

Fig. 1. Expression of chemokine IP-10 mRNA in mouse peritoneal macrophages.

A. Thioglycollate (TG)-elicited C57BL/6 mouse peritoneal macrophages (PeMϕ) were untreated (NT) or treated with IFN-γ (100 U/ml), LPS (100 ng/ml), IL-12 (25 ng/ml), or IL-18 (50 ng/ml) for 4 h. Total RNA was isolated and the level of the IP-10 mRNA was analyzed by Northern hybridization. **B.** TG-elicited PeMϕ were untreated (NT) or treated with LPS (100 ng/ml) alone, IL-18 (50 ng/ml) with various concentrations of IL-12 (5, 25, 125 ng/ml), or IL-12 (25 ng/ml) with various concentrations of IL-18 (5, 50, 250 ng/ml) simultaneously for 4 h. Total RNA was prepared, and Northern blot analysis was performed. **C.** Time course of LPS/IL-18-induced IP-10 mRNA expression in mouse peritoneal macrophages. TG-elicited peritoneal macrophages were untreated (NT) or treated with LPS (100 ng/ml) alone or LPS plus IL-18 (50 ng/ml) simultaneously for the indicated times. Total RNA was prepared and the level of IP-10 mRNA was analyzed by Northern hybridization. These data are a representative of three similar experiments.

induced by LPS alone. The expression of IP-10 mRNA induced by LPS alone or by the combination of LPS and IL-18 reached a peak 4 h after treatment, and this level persisted for 8 h (Fig. 1C).

Effect of IL-18 Pretreatment on the IP-10 (CXCL-10) mRNA Expression

We attempted to evaluate the effect of IL-18 pretreatment on the LPS-induced IP-10 mRNA expression. The TG-elicited PeMϕ were treated with IL-18 (or LPS) at 16, 8, 4, and 0 h (simultaneously) prior to administration of LPS (or IL-18). After 2 h, Northern blot analysis was performed. The most effective synergy of IL-18 pretreatment with LPS stimulation on the expression of IP-10 mRNA was found when IL-18 pretreatment was applied 16 h prior to LPS stimulation, and the most effective synergy between LPS pretreatment and IL-18 stimulation was detected when LPS was applied 4 h prior to addition of IL-18 (Fig. 2A). In cells in which LPS was treated prior to IL-18 stimulation, the pattern of the IP-10 mRNA expression at each of the pretreatment time points was similar to the time-course result of LPS in Fig. 1C. This can be attributed to the fact that the expression of IP-10 mRNA in cells with LPS pretreatment is not due to the synergistic reaction between LPS and IL-18, but due to the effect of LPS stimulation itself.

To confirm the synergistic effect of IL-18 pretreatment on LPS-induced IP-10 mRNA expression, we performed real-time PCR for IP-10 mRNA expression and ELISA for the production of IP-10 protein. In the previous Northern

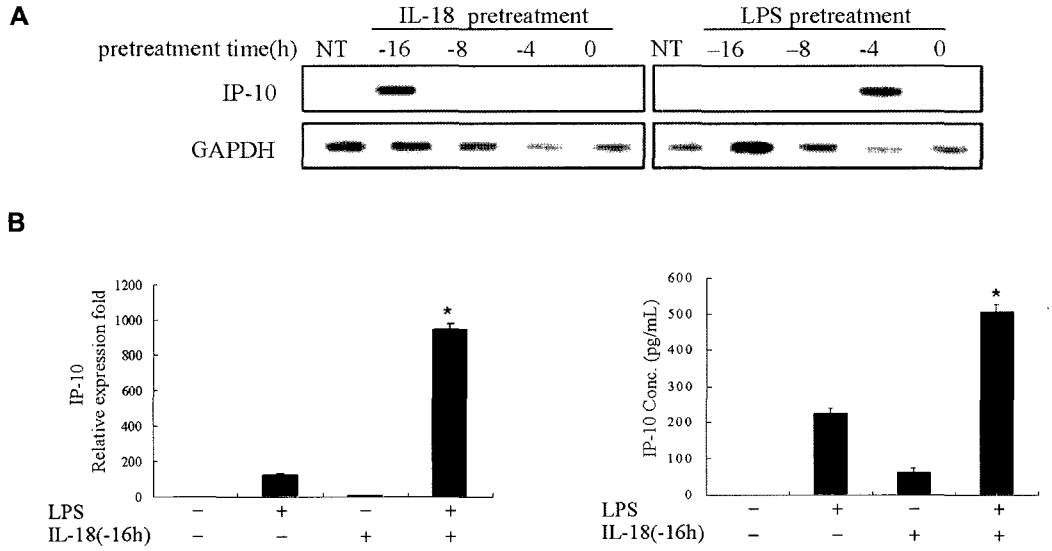

Fig. 2. Pretreatment effect of IL-18 on the expression of LPS-induced IP-10 mRNA in mouse peritoneal macrophages.

A. TG-elicited PeMϕ were treated with IL-18 at 16 (-16), 8 (-8), 4 (-4), or 0 h prior to the stimulation with LPS or LPS (100 ng/ml) at 16 (-16), 8 (-8), 4 (-4), or 0 h prior to the stimulation with IL-18 (50 ng/ml) for 2 h. These data are a representative of three similar experiments. **B.** TG-elicited PeMϕ were untreated (NT) or treated with LPS (100 ng/ml), IL-18 (50 ng/ml) at 16 (-16) h prior to the stimulation with LPS (100 ng/ml), or pretreated with IL-18 for 16 h only. After 2 h reaction time, total RNA and cell supernatants were isolated, and real-time PCR and ELISA for IP-10 production were performed. Bars represent mean±SEM from three separate experiments. $*P<0.05$ compared with cells treated with LPS alone.

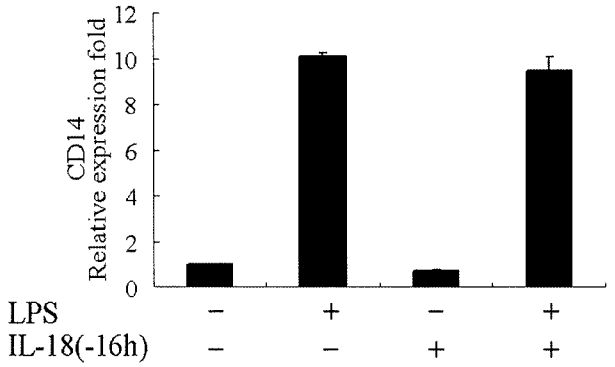

Fig. 3. The pretreatment of IL-18 does not effect the expression of LPS receptor, CD14. TG-elicited PeMϕ were untreated (NT) or treated with LPS (100 ng/ml), IL-18 (50 ng/ml) at 16 (-16) h prior to the stimulation with or without LPS.
After 2-h reaction time, total RNA was isolated and the level of CD14 mRNA was analyzed by real-time PCR. Bars represent mean±SEM from two separate experiments.

blot analysis (Fig. 1A), we could not detect the expression of IP-10 mRNA by IL-18. However, although the expression of IP-10 mRNA was very weak, IP-10 mRNA and protein induced by IL-18 were detected in the results of real-time PCR and ELISA (Fig. 2B). We observed significant increase of IP-10 mRNA expression and protein production in cells stimulated with IL-18 at 16 h prior to the stimulation with LPS (IL-18 pretreatment/LPS), compared with those in cells stimulated with LPS alone (Fig. 2B, $P<0.05$).

Synergistic Mechanisms of IL-18 Pretreatment on LPS-Induced IP-10 mRNA Expression

In order to elucidate the mechanisms underlying the synergistic effect of IL-18 pretreatment on the expression of LPS-induced IP-10 mRNA, we initially examine the effect of IL-18 pretreatment on the expression of the LPS receptor, CD14. The expression level of CD14 in cells stimulated with IL-18 pretreatment/LPS was almost the same as that in cells stimulated with LPS alone (Fig. 3). Next, we attempted to determine the levels of IFN-γ production

Table 1. The production of IFN-γ induced by IL-18 pretreatment in cells stimulated with LPS.

	IFN-γ concentration±SEM (pg/ml)*
Control	0.15±0.26
LPS	0.03±0.05
IL-18	0.00±0.00
IL-18 (0)+LPS	0.06±0.10
IL-18 (-16)+LPS	0.36±0.31

TG-elicited peritoneal macrophages were treated with LPS, IL-18, LPS plus IL-18 simultaneously, or IL-18 (50 ng/ml) at 16 (-16) h prior to the stimulation with LPS. After 2-h reaction time, the levels of IFN-γ in cell supernatants were measured using ELISA. *Values shown are the mean± SEM from triplicate wells.

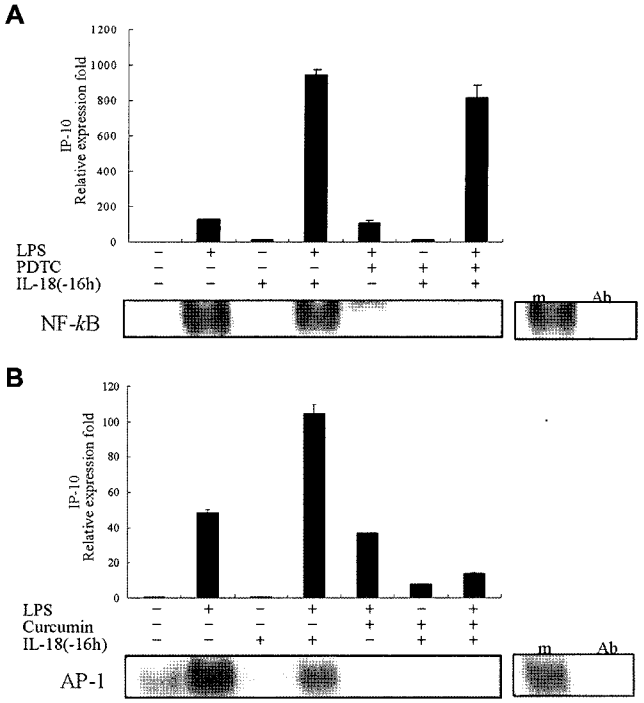

Fig. 4. Synergistic expression of IP-10 in cells stimulated with IL-18 pretreatment/LPS is not related to the activation of NF-κB and AP-1.
TG-elicited PeMϕ were untreated (NT) or treated with LPS (100 ng/ml) or IL-18 (50 ng/ml) at 16 (-16) h prior to the stimulation with or without LPS (100 ng/ml) in the absence or presence of PDTC (10 μmol/l), or curcumin (10 μmol/l). After 2 h, total mRNA and nuclear extracts were prepared, and real-time PCR and electrophoretic mobility shift assay (EMSA) for specific binding activity of NF-κB (**A**) or AP-1 (**B**) were performed. Part of the nuclear extract was incubated with a 100-fold excess of mutant probe (m), or with 2 μg of anti-(NF-κB or AP-1) Ig (Ab) before the EMSA. Bars represent the mean±SEM from three separate experiments. Similar results were obtained in three separate experiments.

in mouse peritoneal macrophages during the reaction time associated with IL-18 pretreatment. This was to determine whether the synergistic effect of IL-18 pretreatment on the LPS-induced IP-10 mRNA expression could be correlated with IFN-γ production during the reaction time. The TG-elicited PeMϕ were treated with LPS, IL-18, IL-18 plus LPS simultaneously, or IL-18 at 16 h prior to stimulation of LPS for 2 h, and ELISA was performed for the production of IFN-γ. Although the highest concentration of IFN-γ was detected at the 16-h time point of IL-18 pretreatment, the levels of IFN-γ production were quite weak (0.36±0.31 pg/ml) (Table 1).

Next, we assessed the activities of transcription factors, NF-κB and AP-1. To evaluate the effect of NF-κB activity in synergistic action of IL-18 pretreatment/LPS on the expression of IP-10 mRNA, pyrrolidine dithiocarbamate (PDTC) was used as a specific inhibitor of NF-κB activation. After TG-elicited PeMϕ were treated with IL-18 at 16 h prior to administration of LPS with or without PDTC (10 μmol/l), real-time PCR and EMSA were conducted for

IP-10 mRNA and NF-*k*B activities. As shown in Fig. 4A, the expression of IP-10 mRNA in cells stimulated with LPS alone or IL-18 pretreatment/LPS was slightly decreased by PDTC. In EMSA, the activation of NF-*k*B in cells stimulated with IL-18 pretreatment/LPS was not enhanced, compared with that of NF-*k*B in cells stimulated with LPS alone. Furthermore, no activation of NF-*k*B was detected in cells stimulated with IL-18 pretreatment alone. To evaluate the effect of AP-1 activity in synergistic action of IL-18 pretreatment/LPS on the expression of IP-10 mRNA, curcumin was used as a specific inhibitor of AP-1 activation. As shown in Fig. 4B, the expression of IP-10 mRNA in cells stimulated with IL-18 pretreatment/LPS was decreased remarkably by curcumin; however, expression of IP-10 mRNA in cells with IL-18 pretreatment alone was increased by curcumin. In EMSA, the activation of AP-1 in cells stimulated with IL-18 pretreatment/LPS was diminished by curcumin.

We next determined whether the mitogen-activated protein kinase (MAPK) signaling pathway would be involved in the synergistic effect of IL-18 pretreatment on the LPS-induced IP-10 expression. Phosphorylation of extracellular signal-regulated kinase (ERK) was examined as an index of MAPK activation. The TG-elicited PeMϕ were untreated or pretreated with PD98059 (100 μmol/l), a highly selective inhibitor of ERK in the MAPK signaling pathway, for 1 h.

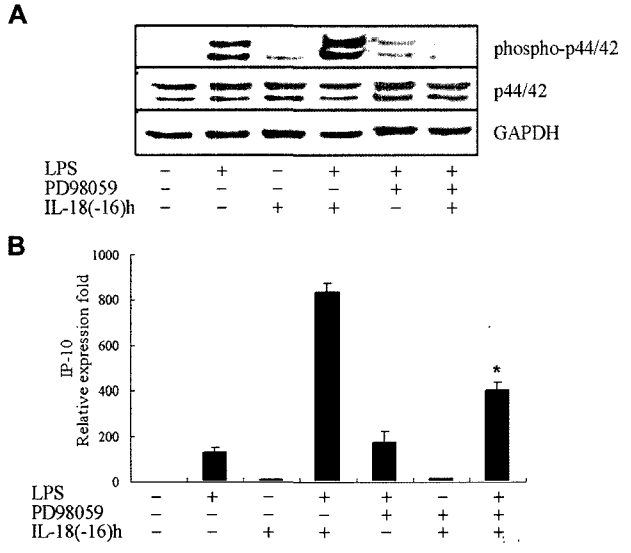

Fig. 5. Synergistic action of IL-18 pretreatment on the expression of LPS-induced IP-10 mRNA is related to the ERK pathway.
TG-elicited PeMϕ were either not pretreated, or pretreated with PD98059 (100 μmol/l) for 1 h, and then treated with LPS (100 ng/ml) or IL-18 (50 ng/ml) at 16 (-16) prior to stimulation with or without LPS (100 ng/ml). After 2 h, cell lysates were separated on a 10% SDS-polyacrylamide gel and then immunoblotted with pERK and ERK antibodies (**A**). Total RNAs for real-time PCR (**B**) were isolated. Bars represent mean±SEM from three separate experiments. Similar results were obtained in three separate experiments. *$P<0.05$ compared with cells treated with IL-18 pretreatment/LPS.

After that, cells were untreated or treated with LPS or IL-18 at 16 h prior to administration of LPS for 2 h. Phosphorylation of ERK in cells stimulated with LPS was increased by IL-18 pretreatment (Fig. 5A). The expression of LPS-induced IP-10 mRNA was not diminished by PD98059, but the expression of IP-10 mRNA in cells stimulated with IL-18 pretreatment/LPS was significantly diminished by PD98059 (Fig. 5B).

Discussion

The aim of the present study was to determine whether IL-18 is able to directly induce chemokine IP-10 in mouse peritoneal macrophages. Although IL-18 itself had very weak effect on the IP-10 mRNA expression in mouse peritoneal macrophages, the synergistic induction of IP-10 mRNA was detected in cells that had been pretreated with IL-18 for 16 h prior to LPS stimulation.

IL-18 and IL-12 synergistically induce the production of IFN-γ [16–18]. Thus, we postulated that IFN-γ induced by combined stimulation with IL-18 and IL-12 could play a role in the autocrine feedback loop of macrophage activation on the IP-10 mRNA expression; however, the combined stimulation with IL-18 and IL-12 could not effectively induce the expression of IP-10 mRNA in mouse peritoneal macrophages. The combination of low concentrations of LPS plus IL-18 induces a 3- to 5-fold greater production of IFN-γ than did either stimulant alone in human whole blood cells [20], and although macrophages are not a main producer of IFN-γ, they are also able to produce IFN-γ according to the stimulators [19]. Therefore, we conducted an ELISA for IFN-γ production in order to determine whether the synergistic effect of IL-18 pretreatment on the LPS-induced IP-10 mRNA expression is related to the production of IFN-γ during the reaction time of IL-18 pretreatment/LPS. However, the overall levels of IFN-γ production were quite low (0.06–0.36 pg/ml). Consequently, the synergistic effect of IL-18 pretreatment on the LPS-induced IP-10 mRNA expression is mediated by mechanisms other than direct activation of IFN-γ.

The expression of chemokine genes appears to be initiated by activation of a variety of transcription factors. NF-*k*B and AP-1 are both transcription factors that are primarily involved in the transactivation of proinflammatory genes [3]. The actual significance of NF-*k*B activation in the regulation of IFN-γ expression by IL-18 in KG-1 cells [11] and murine T helper type I cells [22] has been reported. Matsumoto *et al.* [15] also detected DNA binding activity of NF-*k*B (p65/p65 or p65/p50) in the nucleus in murine THP-1 cells stimulated with IL-18. The functional importance of AP-1 with regard to IL-18-dependent IFN-γ promoter activity has already been well elucidated [2]. Therefore, the activation and/or cooperation of NF-*k*B or AP-1 may

help to explain the mechanism underlying the synergistic effect of IL-18 pretreatment on the LPS-induced IP-10 mRNA expression. We could not detect enhanced activation of NF-*k*B in cells stimulated with IL-18 pretreatment/LPS, compared with that of NF-*k*B in cells stimulated with LPS alone. In addition, the activation of NF-*k*B was not detected in cells stimulated with IL-18 pretreatment alone. Curcumin, a specific inhibitor of AP-1, was able to stimulate the expression of IL-18 pretreatment-induced IP-10 mRNA in an AP-1-independent manner. However, curcumin diminished remarkably the IP-10 expression in cells stimulated with IL-18 pretreatment/LPS, and AP-1 activation in cells stimulated with IL-18 pretreatment/LPS disappeared with curcumin treatment. In other words, the synergistic action of IL-18 pretreatment on LPS-induced IP-10 expression was dependent on not NF-*k*B but AP-1 activation.

The signaling pathway of IL-18 involves the recruitment of myeloid differentiation 88 (MyD88) and IL-2R-associated kinase (IRAK) to the IL-18 receptor complex, and the interaction between IRAK and the adaptor protein tumor necrosis factor receptor-associated factor-6 (TRAF-6) [1, 8, 10]. In addition to IRAK/TRAF-6 signaling, a recent study suggests a role of MARK in IL-18 signaling. Kalina *et al.* [7] reported that the production of IL-18-induced IFN-γ in the human NK cell line 92 is associated with activation of MAPK p38, ERK. In the present study, although phosphorylation of ERK was not related to the expression of LPS-induced IP-10 mRNA, the synergistic action of IL-18 pretreatment on LPS-induced IP-10 was diminished by PD98059. Furthermore, phosphorylation of ERK in cells stimulated with LPS was increased by IL-18 pretreatment, and phosphorylation of ERK in cells stimulated with IL-18 pretreatment alone was also detected. These results suggest that the ERK pathway is related to the synergistic action of IL-18 pretreatment on LPS-induced IP-10 expression.

The present study is, to the best of our knowledge, the first one to focus on the chemokine IP-10 expression induced by IL-18 in C57BL/6 mouse peritoneal macrophages. Although IL-18 alone has very weak direct effect on the expression of IP-10 mRNA, a definite period of IL-18 pretreatment synergistically enhances the LPS-induced IP-10 mRNA expression, and the ERK pathway probably plays a role in the synergistic effect of IL-18 pretreatment on the expression of IP-10 mRNA.

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