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The Novel Cytokine p43 Induces IL-12 Production in Macrophages via NF-κB Activation, Leading to Enhanced IFN-γ Production in CD4$^+$ T Cells[1]

Eugene Kim,* Seung Hyun Kim,* Sunghoon Kim,† and Tae Sung Kim[2]*

Recently, we determined that p43, an auxiliary factor of mammalian multiaminoacyl-tRNA synthetases, is secreted, and functions as a novel pleiotropic cytokine. In this study, we have attempted to characterize the effects of p43 on the generation of IL-12 in mouse macrophages. p43 was determined to induce significant IL-12 production from mouse macrophages in a dose-dependent manner. The stimulatory effect of p43 on the activation of IL-12p40 promoter was mapped to a region harboring an NF-κB binding site. The nuclear extracts from the p43-stimulated macrophages exhibited profound NF-κB DNA-binding activity, as determined by the EMSA. In addition, the p43-stimulated IL-12 induction and NF-κB DNA-binding activity were significantly suppressed by caffeic acid phenethyl ester and BAY11-7082, both inhibitors of NF-κB activation, indicating that p43 induced the production of IL-12 in macrophages mainly via the activation of NF-κB. Importantly, p43 increased the level of IFN-γ production in the Ag-primed lymph node cells, but had no effect on IL-4 levels. The addition of a neutralizing anti-IL-12p40 mAb to the cell cultures resulted in a decrease of the production of p43-enhanced IFN-γ by the keyhole limpet hemocyanin-primed lymph node cells. Furthermore, coincubation with p43-pretreated macrophages enhanced the production of IFN-γ by the keyhole limpet hemocyanin-primed CD4$^+$ T cells, thereby indicating that p43 may enhance IFN-γ expression in CD4$^+$ T cells via the induction of IL-12 production in macrophages. These results indicate that p43 may play an essential role in the development of the Th1 immune responses associated with cancer immunotherapy and protective immunity against intracellular pathogens. ***The Journal of Immunology,*** **2006, 176: 256–264.**

Mammalian aminoacyl tRNA synthetases have been shown to form a macromolecular protein complex with three nonenzymatic cofactors: p43, p38, and p18 (1–3). Among these factors, p43 is secreted to act as a cytokine on both endothelial and immune cells (4, 5). p43 stimulates monocytes/macrophages to secrete a variety of proinflammatory cytokines, including IL-8, TNF-α, and MIP-1 (6, 7). p43 also controls a set of biological activities, including angiogenesis, inflammation, and wound repair (8, 9), thereby indicating that it functions as a novel pleiotropic cytokine. However, the effects of p43 on IL-12 production and Th cell-mediated responses remain unknown.

IL-12 is a heterodimeric cytokine composed of two disulfide-linked subunits of 35 (p35) and 40 (p40) kDa, encoded by two separate genes (10, 11). It is generated by phagocytic cells and other APCs in response to stimulation by a variety of microorganisms, as well as their products (12, 13). The expression of IL-12p40 has been demonstrated to be primarily regulated at the transcriptional level in monocytes exposed to LPS and IFN-γ, which involved at least two transcription factors that belong to the NF-κB and Ets families (14–16). Expression of IL-12p35 is also known to be subject to similar transcriptional regulation (17, 18). IL-12 appears to be critical for the development of Th1 cells and the initiation of cell-mediated immune responses. Th1 cells, characterized by secretion of IFN-γ and TNF-α, are primarily responsible for activating and regulating the development and persistence of CTL (19, 20). Recent studies have indicated that a polarized Th1 immune response may be effective in defense against intracellular pathogens, as well as in cancer immunotherapy (21). The nature of Th1-polarizing signals is not yet fully understood. However, at the onset of an immune response, IL-12 appears to induce polarized Th cell differentiation, resulting in Th1 responses (22).

In this study, we have determined that p43 significantly enhances the expression of IL-12 mRNA and protein secretion in mouse macrophages. Our experimental results indicate that the p43-mediated augmentation of IL-12 production from macrophages may, at least in part, result from the induction of interactions between NF-κB and DNA. Importantly, the elevated IL-12 production occurring in the p43-treated macrophages might result in the enhanced synthesis of IFN-γ, a Th1 cytokine, in CD4$^+$ T cells.

Materials and Methods

Mice, cell lines, and culture medium

RAW264.7 cells were cultured at 37°C in a 5% CO_2 humidified atmosphere in DMEM supplemented with 10% FBS (Invitrogen Life Technologies). The 6- to 8-wk-old female DBA/2 mice and BALB/c mice used in this study were obtained from Japan SLC and Daehan Animal, respectively. Cultures of the lymph node cells from the BALB/c mice and the spleen cells from the DBA/2 mice were maintained in RPMI 1640 supplemented with 10% FBS. The mice were maintained under specific viral pathogen-free conditions and were treated according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

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mAbs, cytokines, and reagents

Anti-IL-4 (BVD4 and BVD6), anti-IFN-γ (HB170 and XMG1.2), and anti-IL-12p40 (C17.8 and C15.6) mAbs were purified from ascitic fluid via ammonium sulfate precipitation, and then subjected to DEAE-Sephagel chromatography (Sigma-Aldrich). Anti-IL-12p35 mAb Red-T/G297-289, murine rIFN-γ, and rIL-4 were purchased from BD Pharmingen, and the murine rIL-12 was generously provided by S. Wolf (Genetics Institute, Cambridge, MA). The anti-p38 MAPK mAb and anti-phosphotyrosine mAb used in this study were purchased from Santa Cruz Biotechnology. The anti-p43 Ab was prepared as described previously (23). The LPS (from *Escherichia coli* 0111:B4) and phospholipase C (PLC)[3] inhibitor U73122 were purchased from Sigma-Aldrich. The keyhole limpet hemocyanin (KLH) was obtained from Calbiochem. The NF-κB inhibitors, caffeic acid phenethyl ester (CAPE) and BAY11-7082 ((E)-3-(4-methylphenylsulfonyl)-2-propenenitrile) were acquired from BIOMOL. The protein kinase C (PKC) inhibitor, chelerythrine, and the MEK inhibitor, PD98059 (2-(2-amino-3-methoxyphenyl)-oxanaphthalen-4-one), were purchased from Tocris Cookson. The p38 MAPK inhibitors, SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-imidazole) and SB202190 (4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-imidazole), as well as their inactive analog, SB202474 (4-ethyl-2-(4-acetylphenyl)-5-(4-pyridyl)-imidazole), were purchased from Calbiochem-Novabiochem.

Expression and purification of p43

The p43 (312 aa) was expressed as a His tag fusion protein in *E. coli* BL21 (DE3), then purified via nickel affinity chromatography and Mono Q or S ion-exchange chromatography. The LPS was removed from the protein solution by dialysis in pyrogen-free buffer (10 mM potassium phosphate buffer (pH 6.0) and 100 mM NaCl). After dialysis, the p43 solution was loaded onto a polymyxin resin (Bio-Rad) that had been pre-equilibrated with the same buffer, incubated for 20 min, then eluted. To further remove any residual LPS, the protein solution was dialyzed against PBS containing 20% glycerol, and filtered using a Posidyne membrane. The concentration of the LPS in p43 was <20 pg/ml, as determined by a *Limulus* amebocyte lysate assay (QCL-1000 kit; BioWhittaker).

Preparation of primary macrophages

Spleen and lymph node cells were cultured at a concentration of 5×10^6 cells/ml for ~3 h at 37°C. Nonadherent cells were removed by washing with warm RPMI 1640 containing 10% FBS and antibiotics (Invitrogen Life Technologies). The adhering cells were removed from the plates via 15 min of incubation with ice-cold PBS solution containing 5 mM EDTA. The isolated adherent cell population was then treated with p43, or left untreated.

Cytokine assays

The quantities of IFN-γ, IL-4, and IL-12 in culture supernatants were determined via sandwich ELISA, using mAbs specific to each cytokine, as previously described (24). The mAbs used to coat the plates and the biotinylated second mAb were as follows: for IFN-γ, HB170 and XMG1.2; for IL-4, BVD4-11D11 and BVD6; for IL-12p70, Red-T/G297-289 and C17.8. Standard curves were generated with recombinant cytokines (BD Pharmingen). The lower limits of detection were as follows: 125 pg/ml for IFN-γ, 3 pg/ml for IL-4, and 50 pg/ml for IL-12p70.

RT-PCR

Total RNA was prepared from the cells, then reverse transcribed into cDNA, followed by PCR amplification of the cDNA. The sequences of PCR primers used in the experiments were as follows: mouse IL-12p40 (sense, 5′-CAGAAGCTAACCATCTCCTGGTTTG-3′; antisense, 5′-TCCGGAGTAATTTGGTGCTTCACAC-3′), IL-12p35 (sense, 5′-TCAGCGTTCCAACAGCCTC-3′; antisense, 5′-CGCAGAGTCTCGCCATTATG-3′), β-actin (sense, 5′-TGGAATCCTGTGGCATCCATGAAAC-3′; antisense, 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′). The PCR was run for 35 cycles of 94°C (30 s), 58°C (45 s), and 72°C (30 s) on an MJ Research thermal cycler. After the amplification, 6 μl of the RT-PCR products was separated on 1.5% (w/v) agarose gel, followed by ethidium bromide staining.

[3] Abbreviations used in this paper: PLC, phospholipase C; CAPE, caffeic acid phenethyl ester; ChIP, chromatin immunoprecipitation; KLH, keyhole limpet hemocyanin; PKC, protein kinase C.

IL-12 p40 promoter constructs and transient transfection

The −689/+98 fragment of the murine IL-12p40 promoter from pXP2 (15) was subcloned into the *Kpn*I/*Xho*I sites of the pGL3 basic luciferase vector (Promega). All of the deletion mutants were generated via PCR, using an upstream primer that harbored the *Bam*HI site. A linker-scanning mutant was generated via a two-step PCR procedure, with overlapping internal primers that contained mutated sequences for the NF-κB site. For the transfections, the cells were incubated for 24 h in 24-well plates with medium supplemented with 10% FBS, and transfected with the indicated plasmids, in the presence of Lipofectamine, according to the manufacturer's protocol (Invitrogen Life Technologies). After 20 h, the cells were washed, and DMEM containing 10% FBS was added. The cells were harvested 20 h later, and assayed with regard to luciferase activity.

EMSA

Nuclear extracts were prepared from the cells, as previously described (25). Oligonucleotides containing an NF-κB binding site within the IL-12p40 (5′-CTTCTTAAATTCCCCCAGA-3′) and the Ig γ-chain (5′-CCGGTTAACAGAGGGGGCTCCGAG-3′) were used as probes. Specific binding was verified by competition experiments with a 50-fold excess of unlabeled, identical oligonucleotides or with cAMP response element-containing oligonucleotides.

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was conducted as previously described (26). On the day before the experiment, 2×10^6 cells were plated in 0.5% BSA containing growth medium. The cells were stimulated for the indicated times, and sequentially cross-linked with disuccinimidyl glutarate and 1% formaldehyde in serum-free medium for 15 min at 37°C. The cells were washed, transferred to Eppendorf tubes, and finally solubilized in 400 μl of SDS lysis buffer (1% SDS, 10 mM Tris (pH 8.0), and 1 mM EDTA) with a protease inhibitor mixture (Sigma-Aldrich). The samples were then sonicated three times for 15 s on setting 2 until the DNA fragments measured 300–400 bp or less. Equal amounts of DNA were then immunoprecipitated overnight at 4°C in ChIP dilution buffer (50 mM NaCl, 1 mM HEPES (pH 7.4), 1% IGEPAL-630, 10% glycerol, and 1 mM DTT) with 20 μg of anti-p50, anti-p65, or anti-Rel B (Santa Cruz Biotechnology). The immunoprecipitates were collected using protein A magnetic beads (Dynal Biotech) and washed sequentially with ChIP dilution buffer, high salt buffer, LiCl buffer, and TE buffer (10 mM Tris (pH 8.0) and 1 mM EDTA). The DNA was eluted in 1 ml of elution buffer (1% SDS in 0.1 M $NaHCO_3$). The samples were decross-linked in 200 mM NaCl at 65°C for 1 h. The DNA was phenol extracted, ethanol precipitated, and used for PCR. The primer pairs used in this step were as follows: IL-12p40 (sense, 5′-AGTATCTCTGCCTCCTTCCTT-3′; antisense, 5′-GCAACACTGAAAACTAGTGTC-3′) and α-actin (sense, 5′-AGGGACTCTAGTGCCCAACACC-3′; antisense, CCCACCTCCACCCTACCTGC-3′). The PCR products were fractionated via agarose gel chromatography, followed by ethidium bromide staining.

Immunoprecipitation and Western blot analysis

The cell lysates were incubated overnight with protein A-Sepharose beads (Pharmacia) coupled with anti-phosphotyrosine mAb, after which the beads were washed and the samples were eluted under nonreducing conditions, as previously described (27). The immunoprecipitates were analyzed via electrophoresis on 12% SDS-polyacrylamide gel before being transferred to poly(vinylidene fluoride) membranes, using a SemiPhor (Hoefer Scientific Instrument). The membranes were then incubated with washing buffer (PBS containing 0.1% Tween 20) containing 2% BSA for at least 1 h, to block any nonspecific protein binding. Primary mAb was diluted up to 1/1000 in washing buffer, and applied for 1 h to the membrane at room temperature. After washing, the blots were incubated with the appropriate HRP-conjugated secondary mAb (diluted up to 1/3000 in washing buffer) for 1 h at room temperature. The immunoreactive bands were visualized using an ECL system (Amersham).

Preparation of lymph node cells and isolation of $CD4^+$ T cells

Draining axillary, popliteal, and inguinal lymph nodes were removed from mice 7 days after priming with 100 μg of KLH absorbed to aluminum hydroxide (alum) adjuvant, which had been injected into the footpads, as previously described (28). To obtain $CD4^+$ T cells, single cell suspensions of lymph nodes were labeled with microbeads conjugated to anti-CD4 mAb (Miltenyi Biotec). The labeled cells were then separated from the unlabeled cells via magnetic cell sorting, using the VarioMACS (Miltenyi Biotec). The purified $CD4^+$ T cells were then prepared and cultured in vitro with KLH (100 μg/ml) in the absence or presence of p43. At the specified

times, the cell supernatants were harvested for IL-4 and IFN-γ protein determinations.

Statistical analyses

Student's *t* test and one-way ANOVA were used to determine the statistical differences between the various experimental and control groups. A p value of <0.01 was considered to be significant.

Results

p43 induces IL-12 production in mouse macrophages

The effects of p43 on IL-12 production were evaluated via the treatment of splenic macrophages with p43 (10–1000 nM) for 48 h. Afterward, the IL-12 levels in the culture supernatants were determined by a sandwich ELISA. As indicated (Fig. 1*A*), p43 strongly induced IL-12 production in a dose-dependent manner. The treatment of macrophages with 500 nM p43 induced levels of IL-12 comparable to those in macrophages that had been treated with 0.5 μg/ml LPS, a well-known inducer of IL-12. Macrophage viability remained constant throughout the incubation period in the presence of the p43 concentrations used in the experiments, as determined by MTT assay.

To determine whether the induction of IL-12 secretion by p43 at the protein level was associated with the expression of IL-12 mRNA, we conducted RT-PCR for the IL-12p40 and IL-12p35 subunit genes in the p43-treated mouse macrophages. The levels of RT-PCR product for both IL-12p40 and IL-12p35 genes were shown to have increased significantly in a dose-dependent manner, when the macrophages were exposed for 6 h to p43 (100–1000 nM), thereby indicating that the IL-12 production induced by p43 might have occurred at the transcriptional level (Fig. 1*B*). p43 treatment had no effects on the expression of β-actin mRNA in the macrophages, suggesting that the inductive effect of p43 on IL-12 production was not the result of a general cellular activation event. p43 treatment was also shown to induce IL-12-production in the RAW164.7 monocytic cell line, in a dose-dependent manner (data now shown). Furthermore, the addition of neutralizing anti-p43 Abs to p43-treated macrophage cultures resulted in a significant inhibition of IL-12 production, to levels commensurate with those observed in the untreated control cultures (Fig. 1*C*). These results indicate that p43 was a major factor in the induction of IL-12 production by macrophages.

We used several different approaches to exclude the possibility of endotoxin contamination in recombinant p43-mediated production of IL-12. First, LPS and p43 were incubated for 30 min at 100°C before the stimulation of RAW264.7 cells. As expected, incubation had no effect on the LPS-mediated production of IL-12. In contrast, incubation at 100°C resulted in the abolition of p43-induced IL-12 production in RAW264.7 cells (Fig. 2). Second, treatment of p43 with proteinase K before the stimulation of RAW264.7 cells resulted in the complete abrogation of p43-mediated IL-12 production. LPS-mediated IL-12 production, however, remained unchanged under these conditions (Fig. 2). The recombinant p43 concentrations were directly measured. Less than 0.05 endotoxin U/10 μg of protein (1 endotoxin unit = 0.1 ng/ml *E. coli* LPS) was detected. These endotoxin concentrations proved insufficient for the activation of either the primary macrophage cells or RAW264.7 cells.

p43 induces the NF-κB-mediated activation of the IL-12p40 promoter

The IL-12p40 subunit is known as the highly inducible and tightly regulated component of IL-12. The NF-κB transcription factor is essential for the production of IL-12p40. The inducible promoter activity of the p40 gene was first localized to a novel sequence for binding of the NF-κB/Rel family. Subsequently, C/EBP and Ets elements were determined to exhibit functional synergy with the NF-κB site. To identify the region involved in these activities of p43, a series of luciferase reporter constructs were generated, harboring the p40 promoter sequences from positions −689, −231,

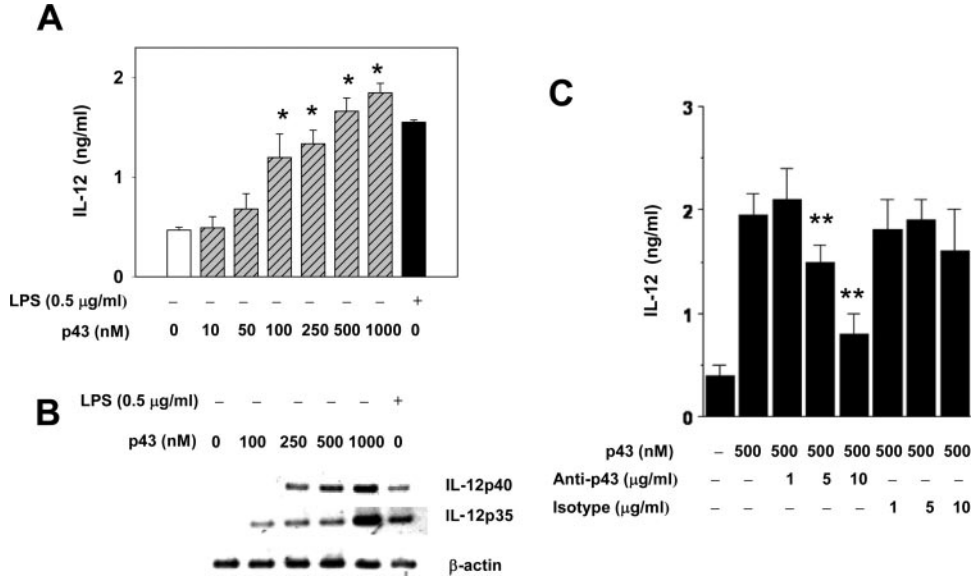

FIGURE 1. p43 induces IL-12 production in primary mouse macrophages. *A*, Primary macrophages were incubated for 48 h with various concentrations of p43 (10, 50, 100, 250, 500, and 1000 nM) or LPS. Culture supernatants were harvested, and IL-12 levels were evaluated by ELISA. The results are expressed as means ± SEM ($n = 3$). *, $p < 0.01$, relative to an untreated group. *B*, Effect of p43 on the expression of IL-12p40 and IL-12p35 genes at the mRNA level. The macrophages were incubated for 6 h with p43 (500 nM) or LPS (0.5 μg/ml), and total RNA was prepared from the cells. RT-PCR products for IL-12p40, IL-12p35, and β-actin were analyzed on 1.5% agarose gels. *C*, Effect of a neutralizing anti-p43 Ab on IL-12 production in p43-treated macrophages. Primary mouse macrophages were cultured with p43 (500 nM) in the presence of anti-p43 Ab. Culture supernatants were harvested 48 h later and assayed for IL-12 levels by ELISA. The results are presented as means ± SEM ($n = 3$). **, $p < 0.01$, relative to p43-treated group in the absence of anti-p43.

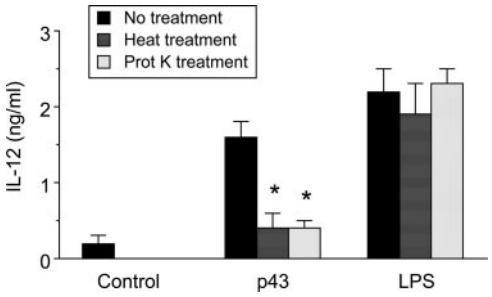

FIGURE 2. p43-induced IL-12 production is not due to endotoxin contamination. RAW264.7 cells were stimulated for 48 h with heat-treated or proteinase K-digested p43 (500 nM) or LPS (0.5 μg/ml), and IL-12 levels were determined by ELISA. The results are presented as means ± SEM ($n = 3$). *, $p < 0.001$, relative to the untreated group.

and −185 to + 98, relative to the transcription initiation site (Fig. 3*A*). Mouse RAW264.7 monocytic cells were then transfected with each of these constructs and treated with p43, and assessed with regard to luciferase activity. Each of these constructs showed strong stimulation in response to p43 exposure (Fig. 3*C*). The deletion of sequences to −185 (p40/185) did not diminish these p43-dependent promoter activities, thereby suggesting that the target site for p43 is located within this region. To directly determine the role of the NF-κB site located between −121 and −131 of the p40 promoter with regard to the activity of p43, a linker-scanning mutation was introduced into the NF-κB site within the context of the −689/+98 construct (p40/LS). The p43-induced promoter activation was, in this case, reduced substantially (Fig. 3*C*), suggesting that p43-induced IL-12 production was mediated via the NF-κB site.

Involvement of NF-κB DNA-binding activity in p43-stimulated IL-12 production

To obtain further insight into the mechanisms underlying the p43-mediated induction of p40-κB function, we analyzed the NF-κB DNA-binding activity manifesting in the nuclear extracts of either unstimulated or p43-stimulated RAW264.7 cells. As expected, the nuclear extracts from the p43-stimulated cells exhibited profound κB-binding activity, as determined by the EMSA using labeled oligonucleotides containing a consensus NF-κB site and an IL-12p40 NF-κB site, respectively (Fig. 4). The binding was specific because it could be inhibited by an unlabeled, identical oligonucleotide, but not by an unrelated, nonspecific oligonucleotide. It was absent in the nuclear extracts from the nonstimulated cells. The nuclear extracts from RAW264.7 cells treated with p43 showed increased NF-κB-binding activity in a dose- and time-dependent manner (Fig. 4, *A* and *B*), thereby suggesting that p43 may modulate the interactions occurring between NF-κB and DNA. Furthermore, to characterize the in vivo nuclear protein binding of NF-κB to the IL-12p40 promoter region, we conducted a ChIP assay with Abs against p50, p65, or Rel B. The data indicated that NF-κB clearly bound to the IL-12p40 promoter after p43 treatment, and the NF-κB complex might be a heterodimer of p65 and p50 (Fig. 4*C*). No binding to the α-actin promoter used as a negative control was observed, indicating that NF-κB bound specifically to the IL-12p40 promoter.

p43-induced κB-binding activity was inhibited by CAPE and BAY11-7082, inhibitors of NF-κB activation (Fig. 5*A*). To further investigate whether NF-κB activation is required for the increased IL-12 production induced by p43, we evaluated the effects of these NF-κB inhibitors on p43-induced IL-12 production in RAW264.7 cells. Both BAY11-7082 and CAPE were observed to significantly inhibit p43-induced IL-12 production, in a dose-dependent manner (Fig. 5*B*). These results (Fig. 5) indicated that the induction of IL-12 by p43 might be mediated via the NF-κB signaling pathway.

Involvement of p38 MAPK and PKC in p43-stimulated IL-12 production

To determine whether or not p38 MAPK activation was required for the induction of IL-12 by p43, we evaluated the effects of p38 MAPK inhibitors on p43-induced IL-12 production in mouse macrophages. SB203580 and SB202190, two inhibitors specific for p38 MAPK, partially inhibited p43-induced IL-12 production, whereas SB202474, a chemical used as negative control, did not (Fig. 6*A*). SB202474 is structurally related to two well-known pyridinyl inhibitors of p38 MAPK inhibitors, SB203580 and SB202190, but does not inhibit p38 MAPK (29).

To further characterize the involvement of other signaling components in the induction of IL-12 production by p43, the macrophages were first treated with inhibitors of various signaling components, including PKC, PLC, and MEK, followed by incubation in a medium containing p43. The IL-12 levels in the cell culture supernatants were subsequently determined. As shown (Fig. 6*A*), the PKC inhibitor chelerythrin partially inhibited the p43-induced production of IL-12 in a concentration-dependent manner. In contrast, the PLC inhibitor (U73122) and the MEK inhibitor

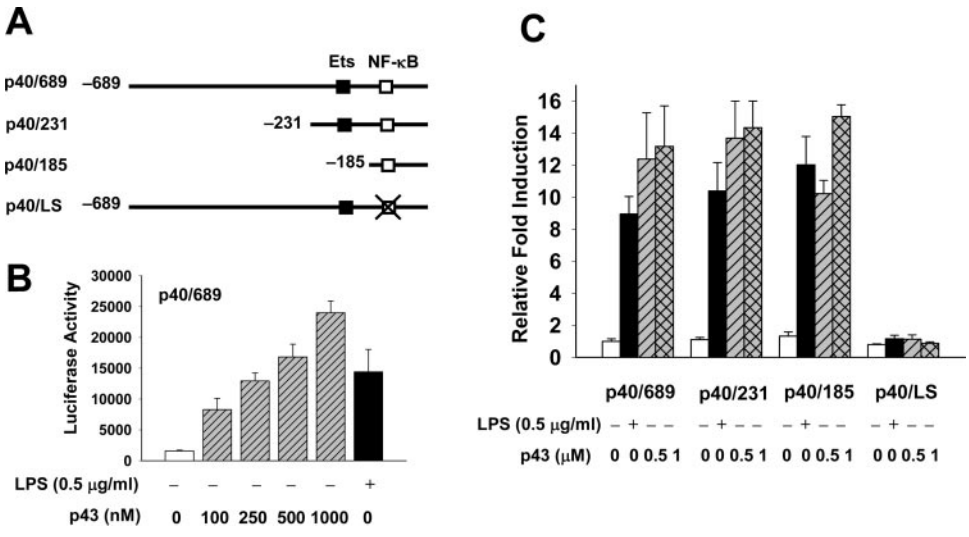

FIGURE 3. Analysis of p43-mediated transcriptional induction of IL-12 p40 promoter constructs. *A*, Mouse IL-12 p40 promoter constructs and a linker-scanning mutant of the NF-κB site are depicted schematically, along with the Ets and NF-κB binding sites. The nucleotide sequence numbers are represented for each of the constructs. *B* and *C*, Transient transfection of RAW264.7 cells with each of the p40 promoter constructs, followed by treatment with p43 (0.5 and 1.0 μM) or LPS (0.5 μg/ml). The results are expressed as induction over the values obtained with the unstimulated RAW264.7 cells transfected with each of the promoter constructs, which was assigned an arbitrary value of 1. The data are representative of three similar experiments.

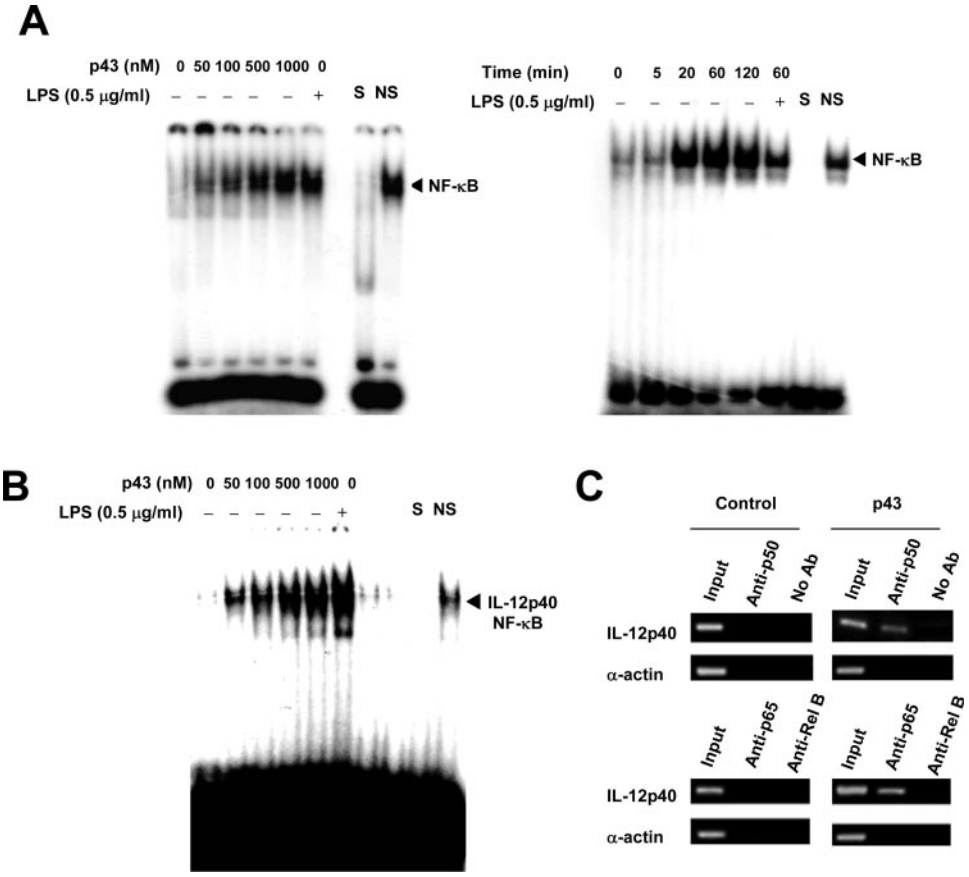

FIGURE 4. p43 increase NF-κB DNA-binding activity. RAW264.7 cells were incubated for 2 h with various concentrations (50–1000 nM) of p43, or with 500 nM p43 at various time periods (5, 20, 60, 120 min), and the nuclear extracts from the treated cells were evaluated with regard to NF-κB-binding activity in EMSA using labeled oligonucleotides containing a consensus NF-κB site (*A*) or an IL-12p40 NF-κB site (*B*), as indicated. S and NS designate the presence of an unlabeled, identical oligonucleotide and nonspecific oligonucleotide, respectively. The specific NF-κB complexes are as indicated. *C*, In vivo NF-κB binding to the IL-12p40 promoter. RAW264.7 cells were incubated for 2 h with 500 nM p43, and cross-linked chromatin was subjected to immunoprecipitation with anti-p65, anti-p50, anti-Rel B, or without Ab (No Ab). Immunoprecipitated DNA was amplified by PCR using primers specific for IL-12p40 or α-actin promoter as a negative control. The data are representative of two independent experiments.

(PD98059) exerted only minimal effects on p43-induced IL-12 production. Furthermore, the PKC inhibitor clearly suppressed the p43-induced activation of p38 MAPK (Fig. 6*B*), indicating that PKC might be an upstream component of p38 MAPK activation in p43-induced IL-12 production. The inhibition of IL-12 production by inhibitors for each of the kinases did not result from any general cytotoxic effects, as the cells' viability in all cultures remained constant throughout the incubation period in the presence of inhibitors used in the experiment, as demonstrated by the results of trypan blue exclusion tests.

p43-treated macrophages enhanced IFN-γ production in KLH-primed CD4$^+$ T cells via IL-12 induction

To determine whether or not p43 affected the production of other cytokines by lymph node cells primed with KLH, the footpads of BALB/c mice were initially injected with KLH (100 μg) in alum. Seven days later, lymph node cells from these immunized mice were stimulated in vitro for 4 days with KLH in the presence of p43, after which the production of IFN-γ and IL-4 was determined. As indicated (Fig. 7*A*), p43 increased the production of IFN-γ in a dose-dependent manner, with the highest induction of IFN-γ production occurring at 500 nM. In contrast, p43 had no effect on the production of IL-4, a Th2 cytokine, in the KLH-stimulated lymph node cells.

As IL-12 has been determined to profoundly enhance IFN-γ in CD4$^+$ T cells, it was expected that the levels of IFN-γ production would be altered by the reduced IL-12 levels in the lymph node cell cultures. The addition of anti-IL-12 p40 mAb (0.01–5 μg/ml) to the cultures of p43-treated lymph node cells resulted in a significant inhibition of IFN-γ production, whereas the isotype control Ab of IgG2a did not affect IFN-γ production (Fig. 7*B*).

To directly investigate whether or not p43-treated macrophages enhanced the production of IFN-γ in CD4$^+$ T cells, macrophages (2×10^5 cells/well) were pretreated with 500 nM p43. After 6 h, the cells were washed and incubated with KLH-primed CD4$^+$ T cells (8×10^5 cells/well). As shown (Fig. 7*C*), the p43-pretreated macrophages enhanced the production of IFN-γ from the purified CD4$^+$ T cells. These results suggested that the induction of IL-12 synthesis by the p43-treated macrophages constituted a major effect, modulating the ability of macrophages to regulate IFN-γ synthesis in CD4$^+$ T cells.

Discussion

The p43 protein, a cofactor of tRNA synthetase secreted from endothelial and immune cells, is a newly discovered cytokine, which up-regulates a variety of inflammation-related gene products, and also controls various biological activities, including angiogenesis, inflammation, and wound repair (3, 30). In this study, we determined that p43 significantly induced the production of IL-12 by mouse macrophages in a dose-dependent manner. It also potently increased the levels of IL-12 production when the cells were simultaneously treated with LPS (data now shown). The levels of IL-12 mRNA expression were significantly increased in the presence of p43, suggesting that the induction of IL-12 by p43 occurred at the transcriptional level (Fig. 1). The enhanced production of IL-12 in the p43-treated macrophages resulted in an increase of IFN-γ production by the Ag-primed CD4$^+$ T cells. These findings suggested that the p43-mediated induction of IL-12 formation led to the enhancement of Th1 cytokine synthesis by CD4$^+$ T cells.

IL-12 performs an essential function in the optimal generation of IFN-γ-secreting Th1 cells. Through this activation, and through

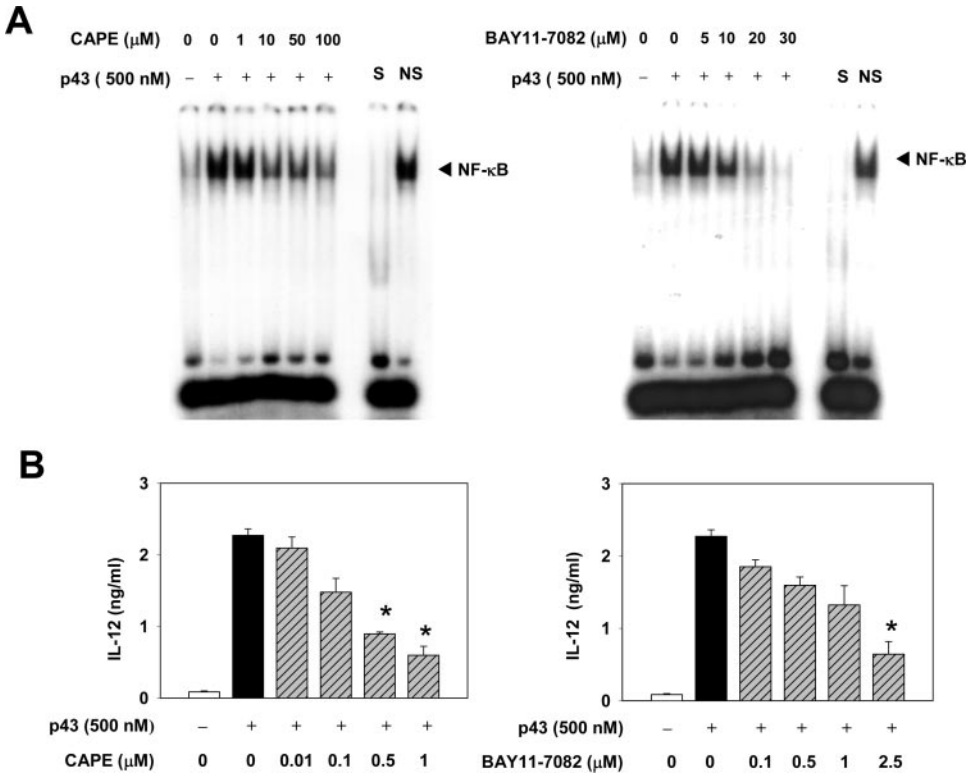

FIGURE 5. NF-κB inhibitors suppress p43-induced NF-κB-binding activity and IL-12 production in macrophages. *A*, RAW264.7 cells were cultured for 2 h in the absence or presence of various concentrations of CAPE (1–100 μM) or BAY11-7082 (5–30 μM), and then treated with 500 nM p43. After 1 h of culture, nuclear extracts were prepared from the RAW264.7 cells, and examined for NF-κB-binding activity in EMSAs, using a labeled oligonucleotide containing a consensus NF-κB site, as indicated. S and NS indicate the presence of an unlabeled, identical oligonucleotide and nonspecific oligonucleotide, respectively. The specific NF-κB complexes are as indicated. *B*, RAW264.7 cells were cultured for 2 h in the absence or presence of various concentrations of CAPE (0.01–1 μM) or BAY11-7082 (0.1–2.5 μM), and then treated with 500 nM p43. After 48 h of culture, IL-12 levels in the cell supernatants were evaluated via sandwich ELISA. The results are presented as means ± SEM ($n = 3$). *, $p < 0.0005$, relative to the p43-treated group in the absence of NF-κB inhibitors.

its ability to directly induce IFN-γ secretion from both T and NK cells, IL-12 plays a central role in both innate and adaptive immunity, and is clearly relevant to host defenses against intracellular pathogens. rIL-12 has striking therapeutic effects in mouse models of tumor (31), infectious diseases (32), and airway inflammation (33). On the basis of these results, clinical trials investigating the potential therapeutic effects of IL-12 have been initiated in human cancer patients, HIV-infected patients, and patients suffering from chronic viral hepatitis. IL-12 may also prove useful as a vaccine adjuvant (34). Clinical trials using direct administration and gene therapy approaches have yielded some promising results. These findings have raised a great deal of interest in the identification of enhancers of IL-12 production, for the treatment of diseases associated with pathologic Th2 responses, most notably allergic disorders and asthma. Conversely, p43 may play a role in Th1-dominated diseases, such as autoimmune diseases. p43 has been determined to be expressed abundantly in the foam cells of atherosclerosis lesions, and in the microglial cells found in lesions associated with experimental autoimmune encephalomyelitis, neuritis, and uveitis (35). In addition, we reported in a recent study

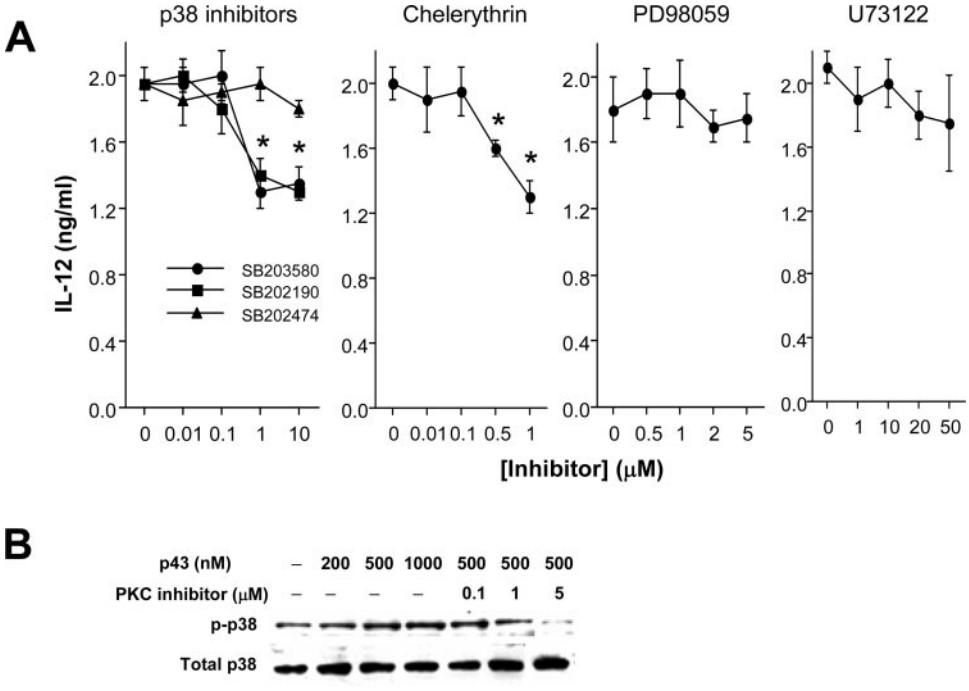

FIGURE 6. Effects of p38-, PKC-, PLC-, and MEK-specific inhibitors on p43-stimulated IL-12 production. *A*, RAW264.7 cells were cultured in the absence or presence of the p38 inhibitors (SB203580, SB202190) and their inactive analog (SB202474), the PKC inhibitor (chelerythrin), the PLC inhibitor (U73122), and the MEK inhibitor (PD98059) for 2 h, followed by treatment with 500 nM p43. After 48 h of culture, the IL-12 protein levels in the cell supernatants were determined via sandwich ELISA. The results are presented as means ± SEM ($n = 3$). *, $p < 0.05$, relative to a group incubated with p43 alone. *B*, The p38 MAPK levels were determined via Western blot analyses at 45 min after the treatment with p43. The cell lysates were then immunoprecipitated using anti-phosphorylated tyrosine mAb and blotted with anti-p38 MAPK mAb (p-p38), or directly probed with anti-p38 MAPK mAb (p38).

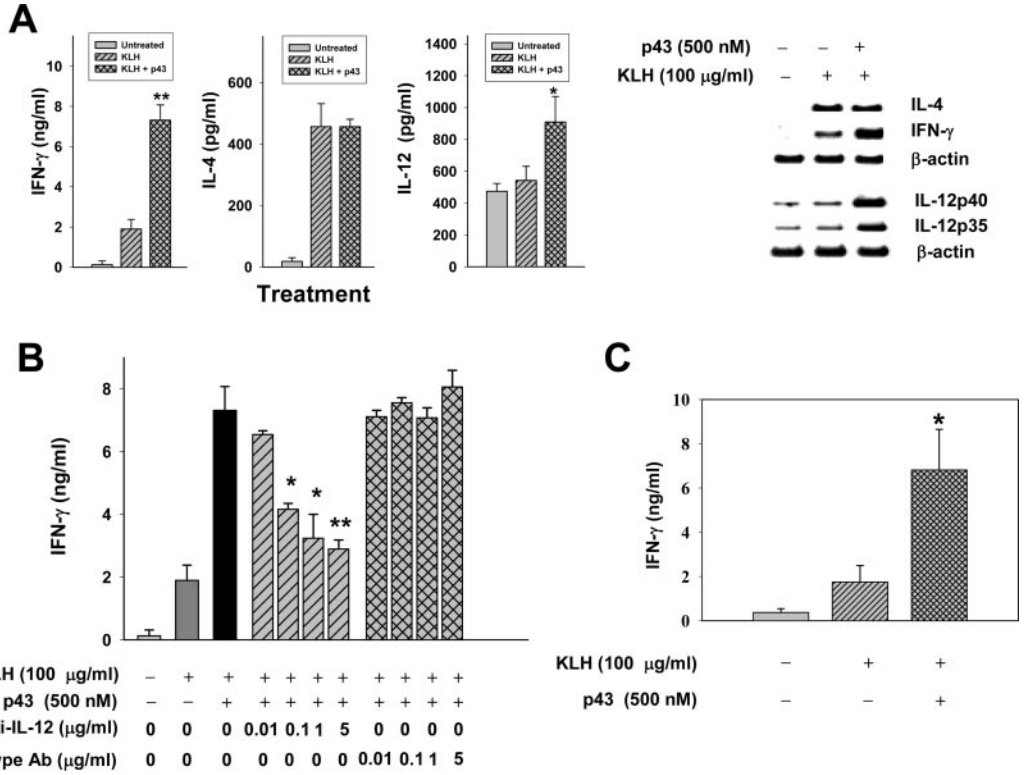

FIGURE 7. p43-mediated IFN-γ production in $CD4^+$ T cells is mediated by IL-12 in Ag-primed lymph node cells. *A*, Effects of p43 on the production of IFN-γ, IL-4, and IL-12 in KLH-primed lymph node cells. The footpads of mice were injected with KLH (100 μg) in alum. Seven days later, the lymph node cells were collected and stimulated in vitro for 4 days with KLH (100 μg/ml), in the presence of p43 (500 nM). The cell culture supernatants were then harvested and assayed for IFN-γ, IL-4, and IL-12 by ELISA. The KLH-primed lymph node cells were treated for 6 h with 500 nM p43, after which RT-PCR was conducted. The results are presented as means ± SEM ($n = 3$). ∗, $p < 0.01$; ∗∗, $p < 0.0001$, relative to an untreated group. *B*, The addition of neutralizing IL-12p40 mAb reduces p43-induced IFN-γ production in KLH-primed lymph cell cultures. The KLH-primed cells were cultured with 500 nM p43 in the presence of anti-IL-12p40 (C17.8; 0.01–5 μg/ml) or isotype control mAb (JES3-19F1; 0.01–5 μg/ml). The culture supernatants were harvested 4 days later, and assayed for IFN-γ by ELISA. The results are presented as means ± SEM ($n = 3$). ∗, $p < 0.005$; ∗∗, $p < 0.001$, relative to the p43-treated group in the absence of anti-IL-12 mAb. *C*, Macrophages pretreated with p43 enhance IFN-γ production by KLH-primed $CD4^+$ T cells. Macrophages (2×10^5 cells/well) were pretreated with 500 nM p43. After 6 h, the cells were washed and incubated for 4 days with KLH-primed $CD4^+$ T cells (1×10^6 cells/well). The culture supernatants were harvested, and the IFN-γ levels were determined by ELISA. The results are presented as the mean ± SEM ($n = 3$). ∗, $p < 0.005$, relative to an untreated group.

that, in wounded regions of skin, TNF-α induced p43 expression and secretion from macrophages that had been recruited to the site. As p43 itself can induce the expression of TNF-α in macrophages, p43 and TNF-α appear to form a positive feedback loop with one another, thereby amplifying inflammatory responses to tissue injury (9).

The mechanism(s) by which p43 induces IL-12 production in macrophages is not known. One possibility, however, is that p43 may enhance the production of IL-12 in mouse macrophages via the up-regulation of NF-κB activity in the IL-12 gene promoter. Our studies revealed that the inductive effects of p43 on a series of 5′ deletions of the IL-12p40 promoter were retained within −185 bp upstream of the transcription initiation site. This finding suggests that p43 may induce NF-κB-mediated activation at position −121/-131 bp (NF-κB site) in the IL-12p40 promoter. Linker scan mutation of the p40-κB site abolished the inductive effects of p43 on the p40 promoter, indicating that this site plays a central role in the transcriptional repression of the p40 gene (Fig. 3*C*). In addition, p43 significantly increased the binding of the NF-κB transcription factor to the κB site in macrophages, as demonstrated by the results of an electrophoretic mobility assay (Fig. 4). This result suggests that p43 may induce IL-12 production via an increase in the binding activity of NF-κB to κB sites. The NF-κB-mediated induction of IL-12 production is consistent with previous observations that LPS and CpG DNA induced the secretion of IL-12 in mouse macrophages via the up-regulation of NF-κB activity (36). In the previous study, we determined that p43 also activated other NF-κB-dependent cytokine genes, including TNF-α, IL-8, IL-1β, MIP-1, and MCP-1 (6).

Further detailed work will be required to elucidate the mechanism(s) by which p43 activates NF-κB in macrophages. One known mechanism underlying the regulation of NF-κB activation is its binding in the cytoplasm to members of a family of ankyrin-containing molecules, the IκBs. In the majority of cells, IκBα is the predominant inhibitory molecule, and the activation and translocation of NF-κB are therefore contingent upon its release from IκB. In vivo, IκB is rapidly degraded in response to a variety of stimuli, including phorbol ester, bacterial LPS, and TNF-α (37).

We have also demonstrated that the PKC/p38 MAPK pathway may be partially involved in the p43-induced production of IL-12 in macrophages. Treatment with the p38 MAPK inhibitors and the PKC inhibitor suppressed IL-12 production approximately by 30% (Fig. 6*A*). The PKC inhibitor was also shown to suppress the p38 MAPK activation induced by p43 (Fig. 6*B*), suggesting that PKC may be an upstream component of p38 activation in p43-treated macrophages. p38 MAPK has been reported to be involved in IL-12 production (21, 38). Recent reports have also revealed that

SB202190, a p38 MAPK inhibitor, inhibits CpG oligodeoxynucleotide-induced IL-12 production in macrophages (39). However, the p38 MAPK inhibitors did not affect the p43-induced NF-κB DNA-binding activity (data now shown), suggesting that p43 induced IL-12 production in macrophages via two independent pathways, the NF-κB and PKC/p38 MAPK pathways.

Although p43 may affect cytokine production in $CD4^+$ T cells in several ways and p43 can increase the expression of other cytokines (IL-23 and IL-27) of the IL-12 gene family (data not shown), the induction of IL-12 production in macrophages is a major mechanism by which p43 increases IFN-γ production in $CD4^+$ T cells, particularly because IL-12 exhibits a remarkable ability to enhance IFN-γ and inhibit IL-4 in $CD4^+$ T cells (40). In an experiment involving the inhibition of p43-treated macrophages with anti-IL-12 mAb, a neutralizing anti-IL-12 mAb was determined to effectively inhibit IFN-γ production by the activated $CD4^+$ T cells, if it was present at the initiation of the cell cultures (Fig. 7). The addition of anti-IL-12 mAb to mannose-binding lectin-treated macrophage cultures restored the induction of IL-4 and inhibited the induction of IFN-γ synthesis by T cells (41). In contrast, p43 had no effect on the production of IL-4 in lymph node cells (Fig. 7). Therefore, the induction of IL-12 production by p43 was concluded to be a primary immunoregulatory mechanism, which results in the enhancement of the Th1 cytokine profile in $CD4^+$ T cells.

In conclusion, p43 stimulates the induction of IL-12 production mainly via NF-κB. When combined with LPS, p43 additively stimulates IL-12 production. As IL-12 plays a pivotal role in the targeting of immunity toward cell-mediated Th1 responses, these findings suggest that p43 may also function as a potential immunomodulator, which generates cell-mediated immunity via induction of IL-12 production. Recent evidence appears to point toward a critical role for IL-12 in cancer immunotherapy, as well as in the promotion of immune responses against infectious diseases. Local delivery of IL-12 and the administration of IL-12-inducing agents such as CpG DNA are currently being investigated with regard to cancer immunotherapy (42, 43). Furthermore, IL-12 may be a key component of a novel strategy for the treatment of Th2-dominated diseases, most notably allergic diseases. Recent studies have described the role of CpG DNA motifs as endogenous IL-12 inducers, and have reported some encouraging results with regard to immunotherapeutic treatments for asthma (44).

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Disclosures

The authors have no financial conflict of interest.

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