To examine our hypothesis, we used palindromic CpG DNA in an unmodified form as a natural ligand for TLR9; such DNA sequences were first reported to exist in bacillus Calmette-Guérin DNA and to induce type I IFN in mice and humans (23, 24). The gene expression of chemokines CXCL10 (IFN-γ-inducible protein-10) and CCL3 (MIP-1α) were examined in relation to IRF-7, because they are all expressed in human pDC following stimulation with CpG DNA (7, 8) and in other cells stimulated with IFN-α (25, 26), and they also have a NF-κB binding site in their gene promoters (21, 22, 27, 28). In addition, the clarification of the mechanism of IRF-7 and CXCL10 expression may help to discriminate the TLR9 signaling pathway from the type I IFN signaling pathway, because their gene promoters possess ISRE, besides the NF-κB site (11–13, 28). We first examined the kinetics of the induction of these chemokines in comparison with those of IFN-α, then the involvement of NF-κB in relation to p38 MAPK, which is important for CpG DNA-triggered gene expression (14, 29, 30), and finally the pathway for IRF-7 activation to cause IFN-α expression. We demonstrate in this study that 1) human pDC express constitutively activated NF-κB p65 and p50, which are possibly involved in the constitutive expression of IRF-7 and CCL3, 2) the activation of NF-κB by CpG DNA seems to play a crucial role in the type I IFN-independent induction of IRF-7, CXCL10, and CCL3, in collaboration with p38 MAPK, and 3) the signal(s) generated upstream of the TLR9 activation of NF-κB/p38 MAPK would be required for the expression of IFN-α, most likely through the activation of IRF-7 in the chloroquine-sensitive regulatory machinery.

Materials and Methods

Oligonucleotides (ODN)

The ODN used in this study were purchased from Hokkaido System Science and added to the culture at 5 μM. Phosphodiester GGGGGGGGGG GACGATCGTCGGGGGGGGGG (denoted as palGACGA1010 hereafter), identical with g10gacga described previously (14), was used as the IFN-α-inducing-type of CpG DNA in an unmodified form to avoid nonspecific activity, which may be caused by the phosphorothioate modification, because the CG-conversion to GC in the phosphorothioate form continued to exhibit activity to some extent in signaling assays in our preliminary experiments. Phosphodiester GGGGGGGGGGGGAGCAT GCTCGGGGGGGGGG was used as the control of palGACGA1010 (denoted as "control ODN" in this study). Phosphodiester 30-mer G (G30) and T (T30) were used for poly(G) and poly(T), respectively.

Reagents

The reagents added to the culture were as follows: actinomycin D (Act D), cycloheximide (CHX), dextran sulfate, fucoidan, wortmannin, chloroquine, pyrrolidinedithiocarbamate (PDTC), caffeic acid phenethyl ester (CAPE), dexamethasone (DEX), polymixin B (PMX), and LPS from Sigma-Aldrich; anti-CD118 Ab and recombinant human IFN-αs and IFN-β from Pestka Biochemical Laboratories; SB203580, SB202474, PD98059, isohelenin, and chondroitin sulfate from Calbiochem.

Isolation and culture of pDC

PBMC were isolated from the peripheral blood of healthy volunteers (negative for HIV, hepatitis B virus, and hepatitis C virus; normal blood cell counts and sera enzymes/proteins; and without fever, medication, or symptomatic allergies) with informed consent, and a low-density fraction was separated on 47.5% Percoll (Amersham Pharmacia Biotech). pDC were then enriched as blood DC Ag (BDCA)4-positive cells by positive sorting with anti-BDCA4-Ab (Miltenyi Biotec) and Dynabeads M-450 goat anti-mouse IgG (Dynal Biotech), or as lineage marker⁻/CD11c⁻/CD4⁺ cells by depleting the cells which reacted with Dynabeads CD14 (Dynal Biotech), and then with the anti-CD3/CD19/CD16/CD56/CD11c mAb (BD Biosciences Pharmingen) followed by Dynabeads M-450 goat anti-mouse IgG (Dynal Biotech). The positively sorted fraction contained >98% BDCA4 cells when assessed microscopically and was used for most of the experiments. The lineage marker⁻/CD11c⁻/CD4⁺ fraction contained >85% BDCA4 cells when analyzed by flow cytometry and was used for the analysis of phosphorylated p38 MAPK/STAT1. pDC were cultured at 1–2 × 10⁶/ml in RPMI 1640 (Sigma-Aldrich) containing 10% heat-inactivated FCS (Equitech-Bio; endotoxins <0.03 ng/ml). In some experiments, PMX was added to prevent a response that would have otherwise been caused by a contamination of LPS in the reagents (31). Different donors' pDC were cultured individually, and if necessary, pooled together to obtain an adequate cell number for analysis.

Real-time RT-PCR

Total RNA was extracted with ISOGEN (Nippon Gene) and converted to cDNA using a SuperScript first-strand synthesis system for RT-PCR (Invitrogen Life Technologies) with oligo(dT) primer. For real-time RT-PCR, cDNA was analyzed for the expression of IRF-7, IκBα, and B2M genes using TaqMan Universal PCR Master Mix (Applied Biosystems) with TaqMan Gene Expression Assay Kits (Applied Biosystems) using an ABI Prism 7900 Sequence Detection System (Applied Biosystems). The expression of other genes was analyzed using SYBR Green PCR Master Mix (Applied Biosystems). The sequences for PCR primers were as follows: GAPDH (5'-CCG CGG GGC TCT CCA GAA CAT-3' and 5'-AAT GCC AGC CCC AGC GTC AAA-3'); IFN-α1/13 (5'-CTT CAA CCT CTT TAC CAC AAA AGA TTC-3' and 5'-TGC TGG TAG AGT TCG GTG CA-3'); IFN-α2 (5'-CCT GAT GAA GGA GGA CTC CAT T-3' and 5'-AAA AAG GTG AGC TGG CAT ACG-3'); IFN-α4 (5'-GAA GAG ACT CCC CTG ATG AAT GT-3' and 5'-GCA CAG GTA TAC ACC AAG CTT CTT C-3'); IFN-α5 (5'-TCC TCT GAT GAA TGT GGA CTC T-3' and 5'-GTA CTA GTC AAT GAG AAT CAT TTC G-3'); IFN-α6 (5'-CTG TCC TCC ATG AGG TAG TT-3' and 5'-GGT CTT ATT CCT TCC TCC TTA AC-3'); IFN-α7 (5'-CAG ACA TGA ATT CAG ATT CCC A-3' and 5'-TTT CCT CAC AGC CAG GAT GA-3'); IFN-α8 (5'-GTG ATA GAG TCT CCC CTG ATG TAC-3' and 5'-CTT CAA TCT TTT TTG CAA GTT GA-3'); IFN-α10 (5'-TGG CCC TGT CCT TTT CTT TAC TT-3' and 5'-TCA AAC TCC TCC TGG GGG AT-3'); IFN-α14 (5'-TGA ATT TCC CCA GGA GGA A-3' and 5'-TCC CAA GCA GCA GAT GAG TT-3'); IFN-α16 (5'-CAA AGA ATC ACT CTT TAT CTG ATG G-3' and 5'-CAA TGA GGA TCA TTT CCA TGT TGA AT-3'); IFN-α17 (5'-TGT GAT ACA GGA GGT TGG GA-3' and 5'-GTT TTC AAT CCT TCC TCC TTA ATA-3'); IFN-α21 (5'-ATC TCA AGT AGC CTA GCA ATA TTG-3' and 5'-AGG TCA TTC AGC TGC TGG TT-3'); IFN-β (5'-GTC TCC TCC AAA TTG CTC TC-3' and 5'-ACA GGA GCT TCT GAC ACT GA-3'); IFN-κ (5'-GCC CCA AGA GTT TCT GCA ATA C-3' and 5'-GGC CTG TAG GGA CAT TTG ATA GA-3'); IFN-ω (5'-GAG GTA CTT CCA GGG AAT CCG-3' and 5'-CAT TTC AAG ATG AGC CCA GGT C-3'); CXCL10 (5'-GAG CCT CAG CAG AGG AAC C-3' and 5'-GAG TCA GAA AGA TAA GGC AGC-3'); and CCL3 (5'-GCT GCT CAG AGA CAG GAA GTC TT-3' and 5'-ACA GGA ACT GCG GAG AGG AGT-3').

Preparation of whole-cell, cytoplasmic, and nuclear extracts

Whole-cell, cytoplasmic, and nuclear extracts were prepared using TransAM Nuclear Extract Kits (Active Motif) according to the manufacturer's instructions.

NF-κB activity

pDC were harvested, and whole-cell lysates or nuclear extracts were prepared. NF-κB activities were measured using TransAM NF-κB Kits (Active Motif) according to the manufacturer's instructions.

Flow cytometry

Cells were fixed in PBS with 2% paraformaldehyde, and then permeabilized in 90% methanol. Cells were then stained with Alexa Fluor-conjugated anti-phospho-p38 MAPK Ab (BD Pharmingen), Alexa Fluor-conjugated anti-phospho-STAT1 Ab (BD Pharmingen), or isotype control, and analyzed using the EPICS XL ADC System (Beckman Coulter).

ELISA

Cytokine/chemokine concentrations of cell-free culture supernatants were measured using ELISA kits (BioSource International), according to the manufacturer's instructions.

Western blotting

The normalized amounts of cell extracts were electrophoresed on 10% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Millipore). The transblotted membranes were blocked with skim-milk and stained with a polyclonal Ab against IRF-7 (Santa Cruz Biotechnology), Histone 1 (Santa Cruz Biotechnology), or GAPDH (Chemicon International) followed by staining with HRP-conjugated anti-rabbit Ab

(Amersham Biosciences) or with HRP-conjugated anti-mouse Ab (Bio-Rad Laboratories). Signals were detected using ECL reagents (Amersham Biosciences) and exposed to films.

Statistical analysis

Statistical significance was evaluated using Student's or paired *t* test at *p* < 0.05.

Results

CpG DNA induces human pDCs to express CXCL10 and CCL3 in addition to IFN-α

The sequence of palGACGA1010 used in this study is composed of palindromic 5'-GACGATCGTC-3' as the core sequence, with 10-mer G at each 5' and 3' site as the flanking sequence. Because the GACGATCGTC sequence exists in the bacterial genomes, with various numbers of G on each side, we used the pal-GACGA1010 in unmodified phosphodiester form as a counterpart of bacterial DNA, which has previously been shown to have immunostimulatory activity in mice and humans (14, 32).

In pDC freshly isolated and mock-cultured, mRNAs for IFN-αs, β, and ω, and CXCL10 were barely detectable, whereas mRNAs for CCL3 and IFN-κ were observed at a substantial level. Consistent with the data reported by Coccia et al. (17), in which the prototype palindromic CpG DNA, AAC-30 (23), was used, pal-GACGA1010 up-regulated the expression of all of the genes with the exception of IFN-κ (Fig. 1A). Control ODN did not induce any of these genes. In the following experiments, the expression of IFN-α was represented by IFN-α1/13, and palGACGA1010 by CpG DNA, unless otherwise stated.

The time needed for up-regulation by CpG DNA of the gene expression varied somewhat among individuals, but the patterns were always similar between IFN-α, CXCL10, and CCL3 in a given pDC preparation. The data from three donors are shown in Fig. 1B. In general, the gene expression for IFN-α became obvious around 4–5 h later, with transient expression as early as 2–3 h, after the stimulation with CpG DNA. The time course of CXCL10 expression was nearly similar to that of IFN-α. The expression of CCL3 spontaneously increased, and this increase was observed regardless of the isolation method of pDC (i.e., positive or negative isolation) and regardless of the presence of a LPS inhibitor, PMX. The up-regulation of CCL3 mRNA by CpG DNA was weaker when compared with that of IFN-α and CXCL10. However, the 2.5- to 4.5-fold increase in the 8-h culture (*n* = 4; data not shown) showed that gene induction of CCL3 by CpG DNA is substantial. The extent of the up-regulation of CCL3 mRNA may have been masked due to the spontaneous expression, which rapidly increased by 2–3 h and decreased along with the culture period.

In accordance with the gene induction, the production of IFN-α, CXCL10, and CCL3 were also increased (Fig. 1C). Their up-regulation was not inhibited by the addition of PMX. Treatment of pDC with Act D abrogated the production of these proteins, indicating the de novo synthesis of CXCL10 and CCL3, in addition to IFN-α.

The expression of IFN-α, CXCL10, and CCL3 can be induced directly by the CpG DNA stimulation

Because IFN-α is capable of inducing CXCL10 and CCL3 (25, 26), type 1 IFNs induced by the CpG DNA stimulation may cause the expression of these chemokines through the activation of the IFN-αβ receptor. We stimulated pDC with representative type 1 IFN isotypes and confirmed that CXCL10 and CCL3 were expressed, as was IRF-7 that was used as a representative type 1 IFN-inducible gene (Fig. 2A). However, CpG DNA elicited the expression of these chemokines even when the IFN-αβ receptor was blocked with anti-CD118 Ab; no statistical difference in their
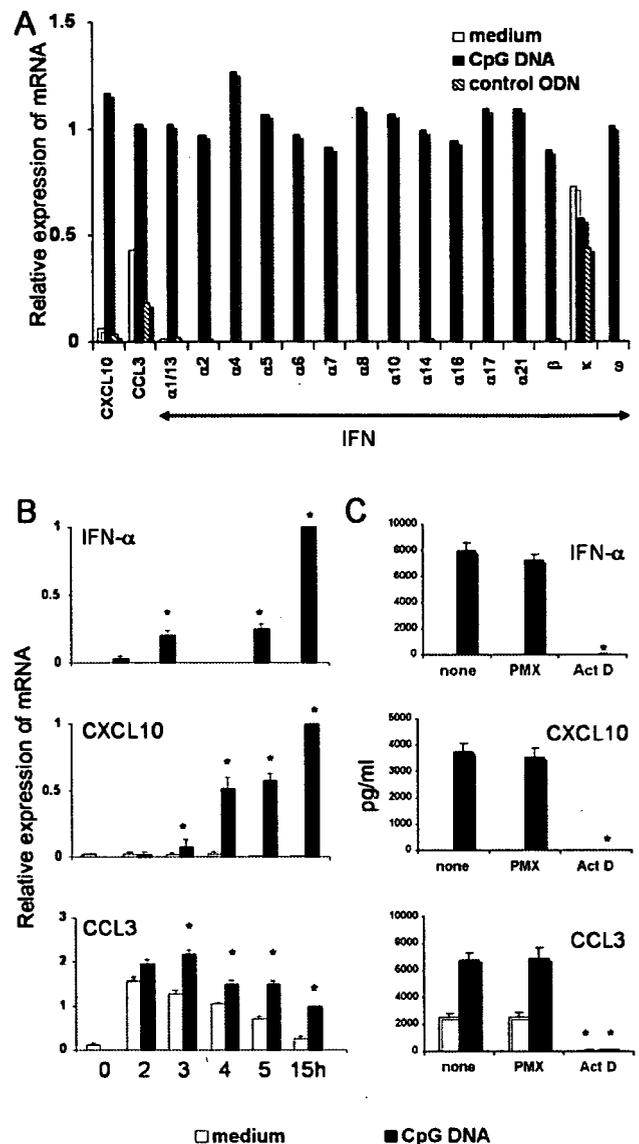

FIGURE 1. CpG DNA induces human pDC to express CXCL10 and CCL3, in addition to type 1 IFNs. *A*, pDC were cultured for 16 h with CpG DNA, control ODN, or medium alone, and RT-PCR was performed for the genes indicated. Data are from one of four separate experiments and shown as the mean relative expression levels in a triplicate assay. Three donors' pDC were used for one set of assay. The CpG DNA gene induction for chemokines and type 1 IFNs, except that for IFN-κ, was statistically significant when compared with the respective controls. *B*, pDC were cultured for the time indicated, with medium alone or CpG DNA, and mRNA for each gene was assayed by RT-PCR. The expressions at 0, 2, 3, 4, and 5-h cultures were shown as the levels relative to that of 15-h culture with CpG DNA. Data shown are the mean ± SE of three independent experiments using different donors' pDC. *, Statistically significant when compared with the respective controls with medium alone. *C*, pDC were cultured for 12 h with or without CpG DNA in the presence or absence of PMX (50 U/ml) or Act D (5 μg/ml). The concentrations of IFN-α, CXCL10, and CCL3 in the culture supernatants were measured by ELISA. Data shown are representative of three independent experiments using pDC from different donors and shown as the mean ± SD in a triplicate assay. *, Statistically significant when compared with the respective controls.

expression between the culture with and without Ab was observed (Fig. 2B). Up-regulation of CXCL10 and CCL3 by CpG DNA thus appears to be initiated in a manner independent of the type 1 IFN
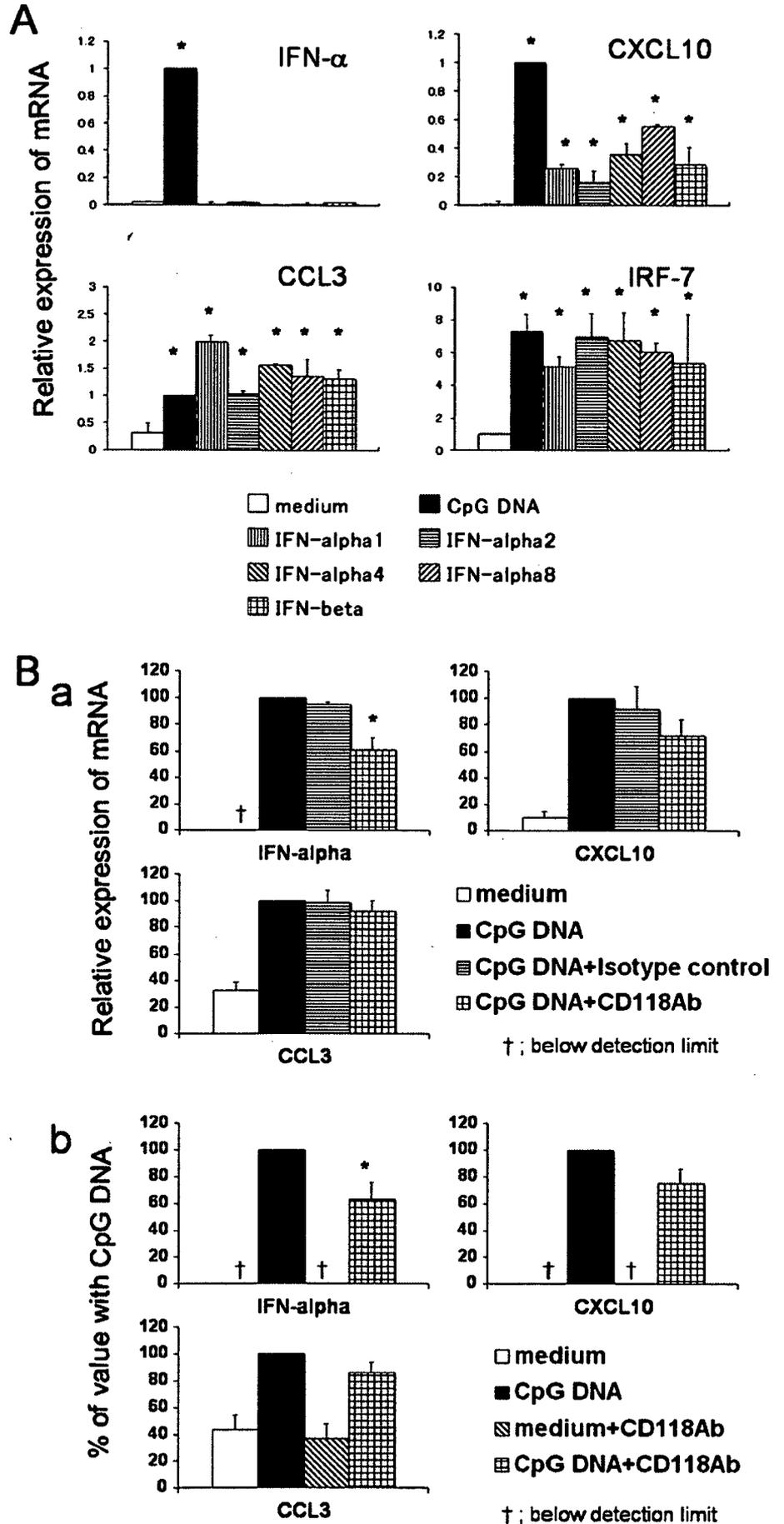
FIGURE 2. Type I IFNs induce pDC to express CXCL10 and CCL3, but not IFN-α, whereas CpG DNA induces them to express IFN-α, CXCL10, and CCL3 in the presence of CD118 Ab. A, pDC were cultured overnight with medium alone, CpG DNA, control ODN, or 1000 IU/ml IFN-α1, α2, α4, α8, or β, and the genes for IFN-α, CXCL10, and CCL3 were measured, along with that for IRF-7 as a positive control. Data shown are the mean ± SE of four independent experiments. *, Statistically significant vs medium alone. B, pDC were pretreated for medium, 1 μg/ml mouse IgG2a (isotype control), or 1 μg/ml anti-CD118 Ab, which we confirmed blocked IFN-α-induced STAT1 phosphorylation in preliminary experiments, and cultured for 12 h (a) or 18 h (b) with or without CpG DNA. PCR (a) and ELISA (b) were performed to assess IFN-α, CXCL10, and CCL3 induction. Data are shown as the mean ± SE of the percentage of the values with CpG DNA in three to six experiments using different donors' pDC. The level of IFN-α, CXCL10, and CCL3 mRNA or the amounts of each protein in the culture with CpG DNA+CD118 Ab were statistically higher when compared with the respective controls with medium alone. *, Statistically lower when compared with the respective values in the culture with CpG DNA alone. No effect of the isotype control on the CpG DNA-induced IFN-α/CXCL10/CCL3 production was observed in the separate set of experiments (data not shown).

response in pDC. This is consistent with the IFN-α-independent induction of IRF-7 and IFN-α, which we (14) and Kerkmann et al. (15) have previously reported, and we again show the data regarding the IFN-α expression in Fig. 2B. For the prolonged expression of IFN-α, however, an autocrine response to type I IFN may be required, because the treatment of pDC with CD118 Ab partially suppressed the induction of IFN-α by CpG DNA.

Scavenger receptor (SR)-A ligand-sensitive pathway and endosomal maturation is required for the induction of CXCL10 and CCL3 as it is for IFN-α

To confirm that the CXCL10 and CCL3 are induced via endosomal maturation, a pathway which is essential for the CpG DNA induction of IFN-α expression (14), pDC were preincubated for 1 h with chloroquine, an inhibitor of endosomal maturation (29), and cultured with CpG DNA for 12–18 h. As shown in Fig. 3, the production of these chemokines was significantly inhibited, as was that of IFN-α, indicating that CXCL10 and CCL3 are induced through the endosomal maturation of CpG DNA. We then tested wortmannin, an inhibitor of the class I/III PI3 kinases that facilitate phagocytosis, endocytosis, and endosomal maturation (33), and fucoidan, dextran sulfate, or poly-G, a ligand for SR-A, which is a likely candidate involved in the endocytosis of CpG DNA (34, 35), following a similar protocol to that described above. CpG DNA inductions of IFN-α, CXCL10, and CCL3 were all abrogated, without decreasing cell viability, by the pretreatment of pDC with wortmannin or SR-A ligands. The equivalent amount of the solvent DMSO, or chondroitin sulfate or poly-T used as a control of dextran sulfate or poly-G, respectively, did not alter the levels of

FIGURE 3. SR-A ligand-sensitive pathway and endosomal maturation are required for the CpG DNA-induction of CXCL10 and CCL3 as it is for IFN-α induction. pDC were cultured for 12–18 h with medium or CpG DNA in the presence or absence of chloroquine (0.2 μg/ml), wortmannin (1 and 0.1 μM), fucoidan (5 and 0.5 μg/ml), dextran sulfate (10 μg/ml), chondroitin sulfate (10 μg/ml), poly-G (5 μM), or poly-T (5 μM). The reagents' concentrations used in this experiment did not cause cell death. The amounts of IFN-α, CXCL10, and CCL3 in culture supernatants were measured by ELISA and expressed as the percentage of the values with CpG DNA alone. Data from three independent experiments were shown in the mean ± SE. The values with chloroquine, wortmannin, fucoidan, dextran sulfate, and poly-G were statistically small when compared with the respective controls with CpG DNA alone.

the cytokines produced. These results suggest that the SR-A ligand-sensitive pathway is involved in the CpG DNA-induced IFN-α/chemokine production in pDC.

The p38 MAPK pathway involved in the induction of IFN-α is also required for that of CXCL10 and CCL3

We previously demonstrated that p38 MAPK is involved in the induction of IFN-α expression by CpG DNA (14). To determine the signaling pathway through which the chemokine production is induced, the effect of p38 MAPK inhibitor SB203580 was examined. As shown in Fig. 4A, the production of both CXCL10 and CCL3 by CpG DNA was decreased by the treatment of pDC with this agent, as was the IFN-α production, with the degree of inhibition being weaker in CXCL10. The IC_{50} for IFN-α, CXCL10, and CCL3 production were ~2, 3, and 6 μM, respectively. The control analog SB202474 did not alter these chemokine productions at the concentrations equivalent to the effective concentrations of SB203580. No decrease in the viability of pDC treated with the agent or the equivalent amount of its solvent DMSO (<0.1%) was observed during the time of incubation. In a murine macrophage-like cell line, ERK pathway is indispensable for the CpG DNA signaling (36). However, in human pDC, treatment with PD98059 did not produce substantial inhibition for these cytokine productions (Fig. 4B). p38 MAPK thus seems to be required not only for the CpG DNA induction of IFN-α, but also for that of CXCL10 and CCL3, in human pDC. In the experiments described hereafter, 10–20 μM of SB203580 was used because this agent sufficiently inhibited (>70% inhibition) IFN-α/CXCL10/CCL3 production with these concentrations.

NF-κB p65 and p50 are constitutively activated in pDC, and their activities are up-regulated by CpG DNA

There would be a common pathway, in CpG DNA-stimulated pDC, between the gene induction for IFN-α and those for the chemokines CXCL10 and CCL3, because their expressions were all independent of type I IFN receptor, but mediated by endosomal processing and p38 MAPK pathways (Figs. 2–4). We therefore examined the involvement of NF-κB, which is a terminus of TLR9 signaling pathway, in the CpG DNA-induced CXCL10, CCL3, and IFN-α.

Because little information regarding the characteristics of NF-κB in human pDC has been reported, we first examined the activation status of various NF-κB family members, p65, p50, p52, cRel, and RelB, in fresh pDC, using an ELISA kit that allowed us to measure their binding to the consensus ODN. Although the binding activities varied greatly among various preparations of pDC, those of p65 and p50 were considered to be significant, because their activities disappeared by the addition of wild type, but not the mutated probe (Fig. 5A). Those of p52, cRel, and RelB were hardly detectable. When pDC were treated for 3 h with CpG DNA, only the binding activities of p65 and p50, but not those of p52, cRel, or RelB, were enhanced (Fig. 5B). Control ODN did not increase the activities for any members of the NF-κB family. Among the individual pDC tested, the increments of the p65 and p50 activities were moderate with 1.25–1.51 ($n = 5$) and 1.25–2.84 ($n = 7$) fold, respectively, but were statistically significant when analyzed across the experiments. Because the IκB gene is immediately accessible to NF-κB and is transcribed immediately after NF-κB recruitment (37), the expression of IκBα mRNA was analyzed as an alternative way to evaluate functional activity of NF-κB; the increase by CpG DNA was shown as 3-fold on average (Fig. 5C). The modest increment of the binding activities by CpG DNA stimulation may be accounted for by their constitutive activation.
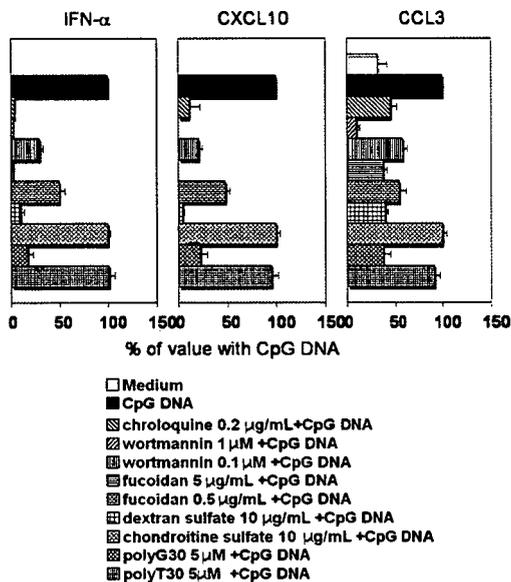
A

Legend:
- CpG-IFN, SB203580
- CpG-CCL3, SB203580
- CpG-CXCL10, SB203580
- CpG-IFN, SB202474
- CpG-CCL3, SB202474
- CpG-CXCL10, SB202474
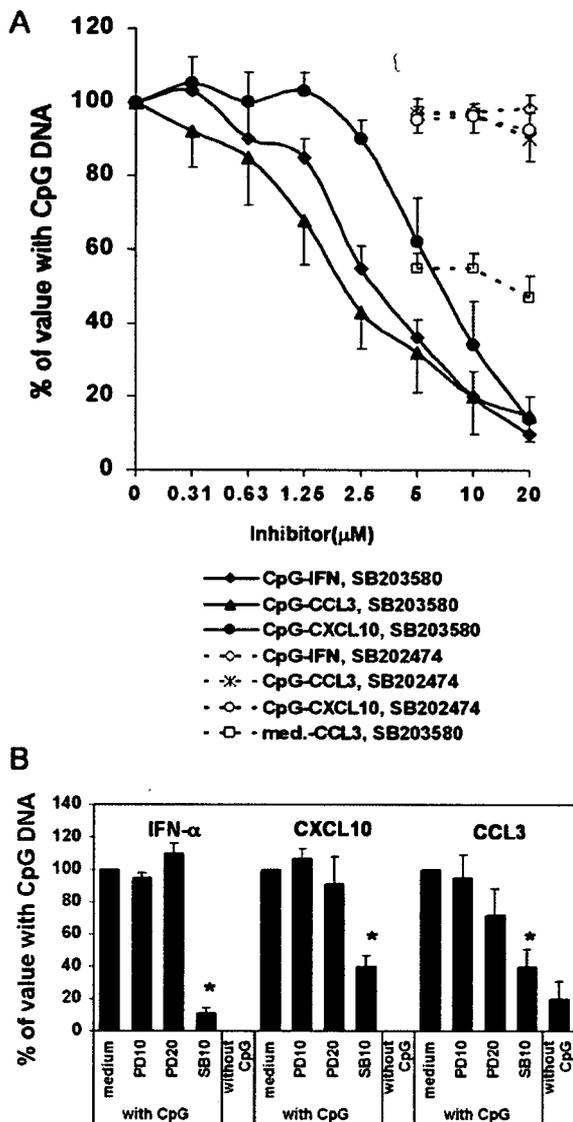- med.-CCL3, SB203580

B

FIGURE 4. The p38 MAPK pathway involved in the induction of IFN-α is also required for that of CXCL10 and CCL3. *A*, pDC were pre-cultured for 1 h with or without various concentrations of SB208530 or SB202474, and cultured for 14 h with medium (med.) or CpG DNA (CpG). The amounts of IFN-α, CXCL10, and CCL3 in the culture supernatants were measured by ELISA, and the effects of the reagents were expressed by the percentages of the values in the culture with CpG DNA alone (in the case of CpG DNA-induced CCL3, the spontaneously produced amounts were subtracted). The equivalent amount of their solvent DMSO did not alter the levels of the cytokine productions (data not shown). Data shown are the mean ± SE of three independent experiments using different donor's pDC. *B*, pDC were treated for 2 h with or without 10 μM of SB203580 (SB), or 10 or 20 μM of PD98059 (PD), and cultured for 14 h with or without CpG DNA (CpG). The amounts of cytokines in the culture supernatants were measured by ELISA and expressed by the percentages of the values in the culture with CpG DNA alone. Data are shown as the mean ± SE of three independent experiments using different donor's pDC. *, Significantly decreased compared with the respective controls with CpG DNA alone.

CpG DNA activation of NF-κB mediates the up-regulation of CXCL10 and CCL3 as well as that of IFN-α via the up-regulation of IRF-7

To examine the involvement of NF-κB activation in the induction of IFN-α/CXCL10/CCL3, we performed a pharmacological inhib-

itor-inhibition test, because the biological natures of human pDC, such as a tiny population in blood, a loss of TLR9 during the culture, and an expression of IFN-α in response to short-interfering RNA (38), restrict the experimental designs.

pDC were pretreated with PDTC (an antioxidant that inhibits IκB phosphorylation (39)) or CAPE (which prevents the translocation of NF-κB and its binding to DNA, but not IκB degradation (40)), and cultured for 14–16 h with CpG DNA. The CpG DNA induction of IFN-α/CXCL10/CCL3 was inhibited by each inhibitor with almost the same concentrations of both a half maximum and a maximum inhibition: these values obtained from three donor's pDC were around 0.1 and 1.0 μM for PDTC, and 1.0 and 3.0 μg/ml for CAPE. The inhibitions were also observed with the non-antioxidant inhibitor of NF-κB, isohelenin, which has been reported to act as a highly specific inhibitor of NF-κB activation by preventing IκBα degradation (41), at 1 μM of half maximum and 5 μM of maximum inhibitory concentrations. These inhibitors did not cause cell death even at the maximum inhibition concentrations during the time of incubation in this study. Their effectiveness was confirmed in our preliminary experiments using other cells and/or pDC. DMSO, at the concentrations carried in the culture as a vehicle for CAPE/isohelenin, did not alter the level of these cytokine productions.

Because IFN-α gene does not have NF-κB binding site, the involvement of NF-κB in the IFN-α induction may possibly be through the NF-κB-mediated expression of IRF-7 (21, 22, 31). Indeed, in pDC pretreated with PDTC, a marked reduction in the CpG DNA-induced expression of IRF-7 was observed with concurrent inhibition in that of IFN-α/CXCL10/CCL3 (Fig. 5C). Type I IFN induced IRF-7 expression in pDC (Fig. 2A), and this pathway was in part involved in the augmentation of IFN-α production by CpG DNA (Fig. 2B). Noteworthy, however, is the fact that no contribution or if any, only partial, of NF-κB to the type I IFN-induced IRF-7 expression was observed (Fig. 5D, *left*). This was also the case for CXCL10 (Fig. 5D, *right*), which is known as the type I IFN-inducible gene. The expression of type I IFN-inducible genes seem to be triggered via the NF-κB pathway when pDC are activated by CpG DNA.

IRF-7 and CCL3 are constitutively expressed in pDC (Figs. 1–3 and 5). These expressions may be associated with the constitutively activated NF-κB (Fig. 5A). We cultured pDC with NF-κB inhibitors and found that the basal levels of IRF-7 and CCL3 mRNA decreased during the culture (Figs. 5, *C* and *D*, and 8B). Our results indicate that NF-κB is involved in both the constitutive and CpG DNA-induced gene expressions.

CpG DNA causes collaborative activation in p38 MAPK and NF-κB

Because the CXCL10, CCL3, and IFN-α inductions were all suppressed by either p38 MAPK or NF-κB inhibitor, the relationship between these pathways was examined using their respective inhibitors. As shown in Fig. 6A, the CpG DNA enhancement of p65 and p50 activities was abrogated following the treatment with SB203580. The CpG DNA induction of phosphorylation of p38 MAPK was also prevented by NF-κB inhibitors (Fig. 6B, *left*). We previously reported that the induction of STAT1 phosphorylation by CpG DNA, but not by type I IFN, is mediated by p38 MAPK (14). Therefore, given that p38 MAPK activation is mediated by NF-κB, the activation of NF-κB should increase the STAT1 phosphorylation as well. Indeed, PDTC treatment of pDC decreased STAT1 phosphorylation, which was up-regulated by the short-term culture with CpG DNA (Fig. 6B, *right*). These results suggest

A

OD 450

0.5 — 0.4 — 0.3 — 0.2 — 0.1 — 0

□ p50
■ p65
▦ p52
▤ cRel
▥ RelB

* * ND ND ND   ND ND ND

Lysate alone   Lysate with wild-type probe   Lysate with mutated probe

B

Fold increase

2 — 1.8 — 1.6 — 1.4 — 1.2 — 1

* † ; below detection limit

† † †   † † †   † † †

p50   p65   p52   cRel   RelB

Relative expression of IκBα

5 — 4 — 3 — 2 — 1 — 0

*

□ medium   ■ CpG DNA   ▦ control ODN

C

Relative expression

IFN-α   CXCL10   CCL3   IRF-7

□ medium   ■ CpG DNA   ▦ PDTC+CpG DNA

D

Relative expression

IRF-7   CXCL10

Medium   IFN-α   CpG DNA   Medium   IFN-α   CpG DNA

□ medium alone   ■ with CAPE

FIGURE 5. NF-κB p65 and p50 are constitutively activated in pDC, and their activities are up-regulated by CpG DNA. *A*, Different donors' pDC were pooled, and their nuclear proteins were analyzed in triplicate for the binding activity of NF-κB family members using TransAM NFκB Kits. Values (mean ± SD) are representative data from one of five independent sets of experiments. *, Statistically significant when compared with the respective controls. ND, not done. *B, Left*: pDC were stimulated with CpG DNA for 3 h, and the whole-cell lysates were analyzed for the binding activity of NF-κB. Data are shown as the fold increase (mean ± SE) of seven (1.25- to 2.84-fold) and five (1.25- to 1.51-fold) separate experiments, for p50 and p65 activities, respectively. *, Statistically significant when compared with the respective controls. *Right*, pDC from two different donors were individually cultured for 3 h with medium, CpG DNA, or control ODN, the cells cultured under the same conditions were pooled, and expression of IκBα was analyzed by real-time PCR in triplicate (mean ± SD). Another set of experiments using different donors' pDC resulted in the similar result. *, Statistically significant when compared with the control with medium alone. *C*, pDC were preincubated with 1 μM of PDTC for 1 h and subsequently cultured for 5 h with CpG DNA or medium. The expression of mRNA for IRF-7/IFN-α/CXCL10/CCL3 was measured by RT-PCR. Data are the mean ± SE from three independent experiments. *, Significantly decreased compared with the respective controls with CpG DNA alone. *D*, pDC were precultured for 1 h with or without 5 μg/ml CAPE and subsequently cultured for 6 h with medium, IFN-α2a (200 IU/ml), or CpG DNA. The expression of IRF-7 and CXCL10 was analyzed by RT-PCR for three different donors' pDC (mean ± SE). *, Statistically significant when compared with the respective controls without CAPE. Inhibition of IFN-α-induced IRF-7 and CXCL10 by CAPE was, if any, partial and did not reach a significant level. In three other individual experiments using different NF-κB inhibitors and different concentrations of IFN-α2a (100–2000 IU/ml), the inhibition was 5–53% in IRF-7 and 8–43% in CXCL10.
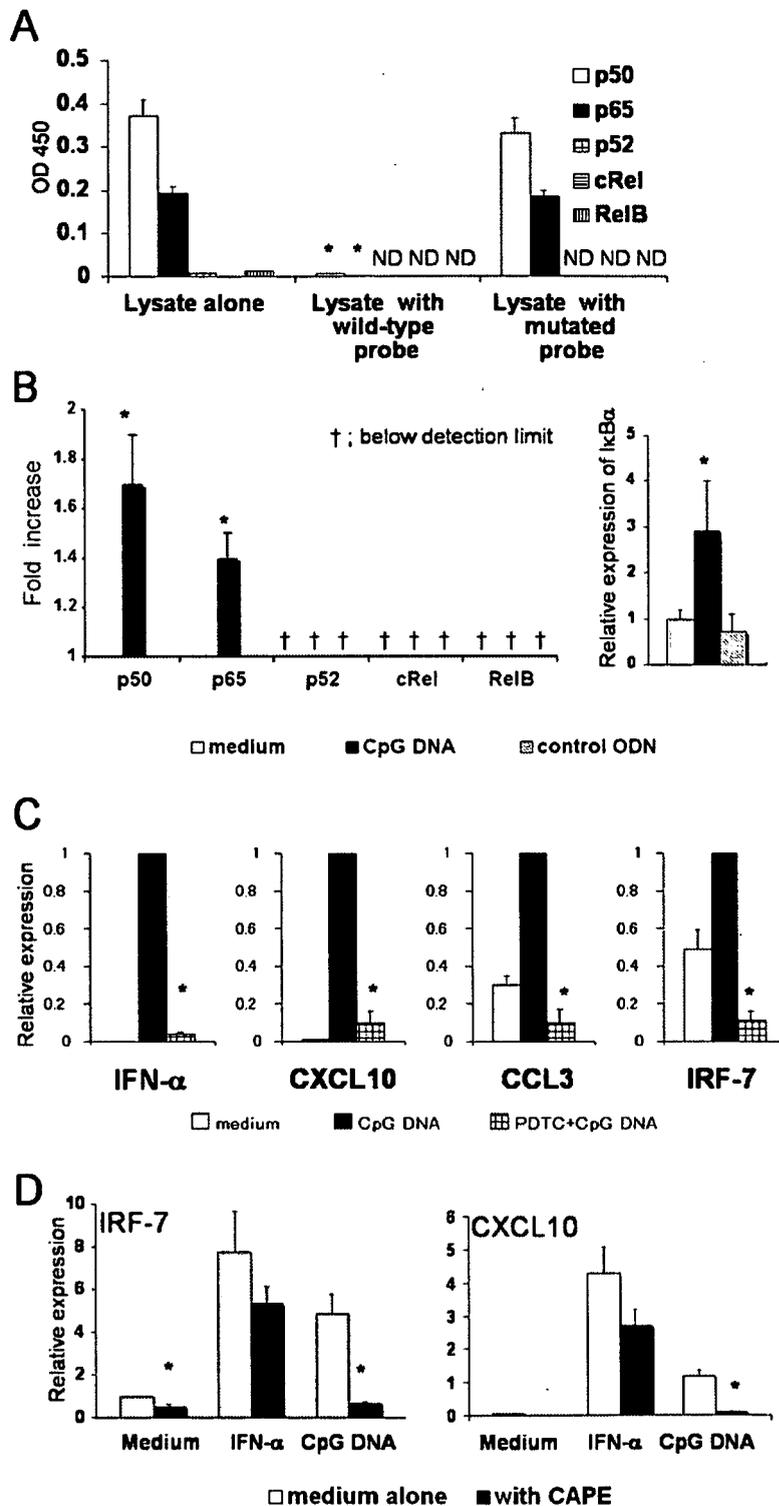
that CpG DNA activates both the NF-κB and p38 MAPK pathways, through which NF-κB-dependent and/or p38 MAPK-dependent gene expression is induced.

CpG DNA can directly up-regulate the expression of IRF-7

We previously reported that constitutively expressed IRF-7 moves to the nuclei in response to CpG DNA (14). This observation suggested that IFN-α induction by CpG DNA is triggered by the activation of constitutively expressed IRF-7. Indeed, CHX-treated

pDC were able to express IFN-α in response to CpG DNA (Fig. 7A). However, the data shown in Figs. 2B and 5D suggest that type 1 IFN signal-independent IRF-7 contributes to the expression of IFN-α. We then tested whether IRF-7 is induced directly by CpG DNA or not (Fig. 7, *B* and *C*). Time kinetics for the IRF-7 induction by CpG DNA was first analyzed showing that the up-regulation started from 3 h, continuously increased for up to 16 h, and declined thereafter. The up-regulation of IRF-7 was observed even in the presence of CHX to an extent comparable to that in the
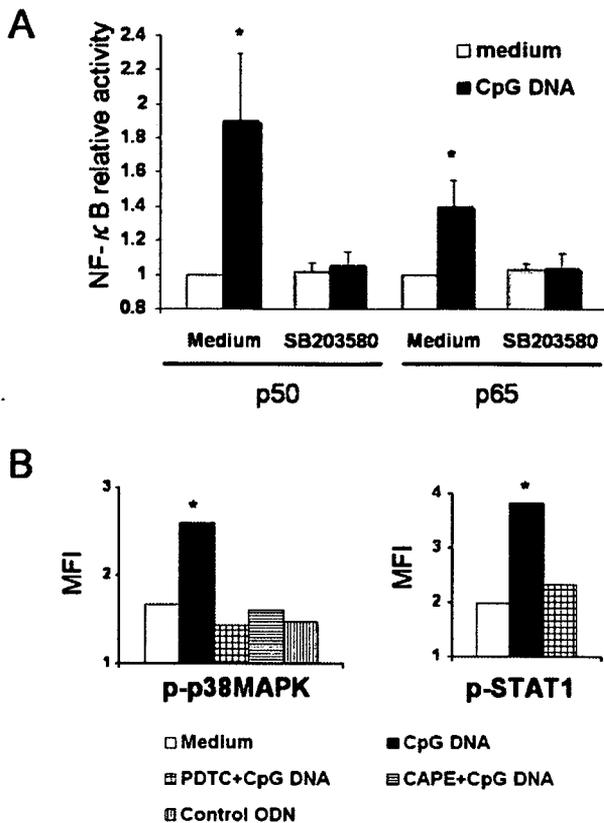
FIGURE 6. CpG DNA causes collaborative activation in p38 MAPK and NF-κB. *A*, pDC were treated for 1 h with or without 10 μM SB203580, and cultured for 3 h with or without CpG DNA. The activities of NF-κB p50 and p65 were analyzed as described in Fig. 5. Data are the mean ± SE of four separate experiments, where both or either of p50 and p65 was analyzed. *, Statistically significant when compared with the respective controls without CpG DNA. *B*, pDC were treated for 1 h with medium, PDTC (1 μM), or CAPE (5 μg/ml), and cultured for 3 h with CpG DNA, control ODN, or medium. Phosphorylation of p38 MAPK and STAT1 was analyzed by flow cytometry using Abs against their phosphorylated forms, and expressed as mean fluorescent intensity (MFI). Four independent experiments were repeated with similar results with different donors' pDC. *, Statistically significant when compared with the respective controls with medium alone.

absence of CHX, in a 5-h culture, by which time point the IFN-α gene is re-expressed (Fig. 1*B*). At 12 h with CHX, the IRF-7 was continually expressed, although the level was lower than that in the absence of CHX, where the IRF-7 expression approaches a plateau through the autocrine response to type I IFN (Fig. 2*B*). Addition of PMX to the culture did not prevent the induction of IRF-7 (data not shown). These results indicate that CpG DNA induces pDC to express IRF-7 in a manner independent of type I IFN signaling.

Signal(s) generated in chloroquine-sensitive machinery independently of NF-κB/p38 MAPK activation is required for the expression of IFN-α via the activation of IRF-7

It has recently been reported in mice that the TLR7/9-induction of IFN-α needs the phosphorylation of IRF-7, by IL-1 receptor-associated kinase-1, in a complex with MyD88 and TRAF6 (42). To prove the notion that, in CpG DNA-stimulated human pDC, IRF-7 is activated through signals upstream of NF-κB activation and, consequently, IFN-α is expressed, pDC were treated or not treated with IFN-β for 3 h, then incubated for 1 h with or without the inhibitors of p38 MAPK, NF-κB, or endosomal maturation, and
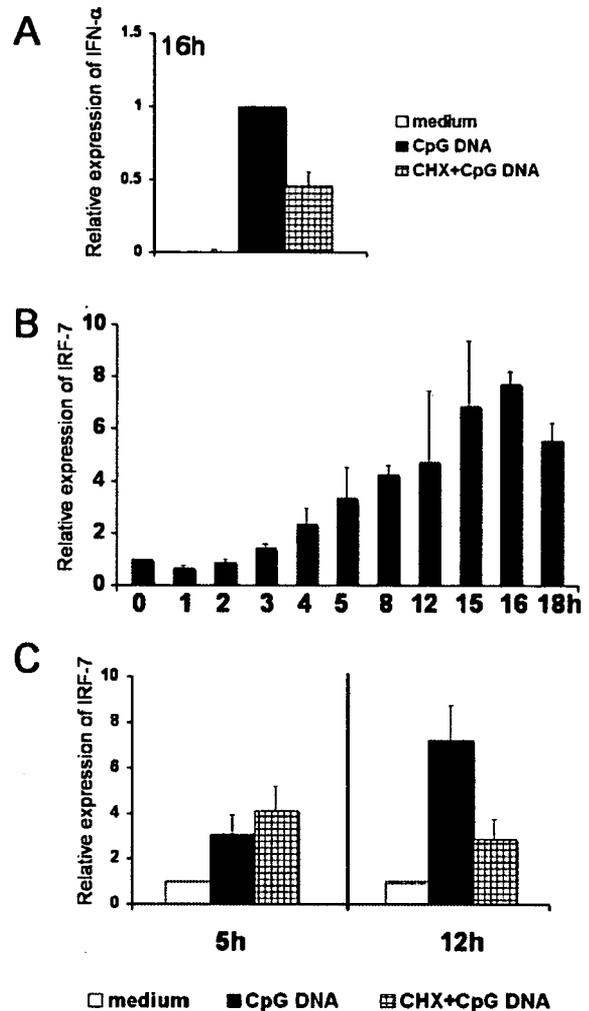

FIGURE 7. CpG DNA can up-regulate the expression of IRF-7 independently of protein synthesis. *A*, pDC were preincubated with or without 1 μg/ml CHX, which we confirmed blocked the CpG DNA-induced production of IFN-α, CXCL10, and CCL3, and cultured for 16 h with CpG DNA or medium alone. The expression of mRNA for IFN-α was analyzed by RT-PCR in three independent experiments. Data are shown in the mean ± SE; the level of mRNA for IFN-α in the culture with CpG DNA+CHX was statistically higher when compared with that with medium alone, but lower when compared with that with CpG DNA alone. *B*, pDC were preincubated with or without CpG DNA for the time indicated, and the mRNA for IRF-7 was analyzed by RT-PCR. The level of mRNA in the culture with CpG DNA was expressed as a value relative to the respective control in the culture with medium alone, and summarized in the mean ± SE at each time point, based on the data from five experiments. *C*, pDC were preincubated for 1 h with or without CHX, and cultured for 5 and 12 h with CpG DNA or medium. The expression of IRF-7 was analyzed by RT-PCR. Data are the mean ± SE of three independent experiments. No difference was observed between 5 and 12 h, in the levels of mRNA expressed in the culture with CHX+CpG DNA.

successively cultured for 8 h with or without CpG DNA (Fig. 8*Aa*). Treatment of pDC with 200 IU/ml IFN-β amplified IRF-7 expression 2- to 8-fold, which was enough to induce IFN-α to an extent detectable by ELISA. No alteration in the level of TLR9 mRNA was observed (data not shown). DEX was used to prevent the CpG DNA induction of IRF-7, because a synthetic glucocorticoid has been demonstrated to block the binding of NF-κB to the κB binding site as well as to interfere with STAT1 and p38 MAPK function (43–45). The inhibitory effect of DEX on the induction of
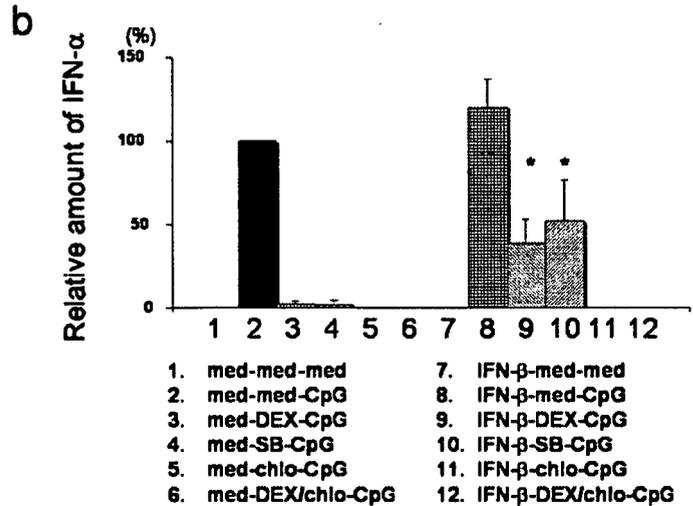
A a

time 0h ___ 3h 4h _____|12h

IFN-β   inhibitor CpG DNA

b

1. med-med-med
2. med-med-CpG
3. med-DEX-CpG
4. med-SB-CpG
5. med-chlo-CpG
6. med-DEX/chlo-CpG

7. IFN-β-med-med
8. IFN-β-med-CpG
9. IFN-β-DEX-CpG
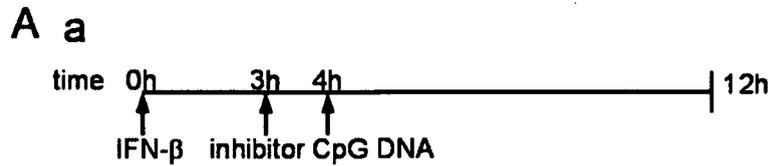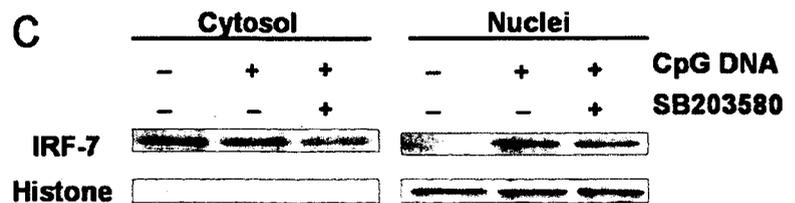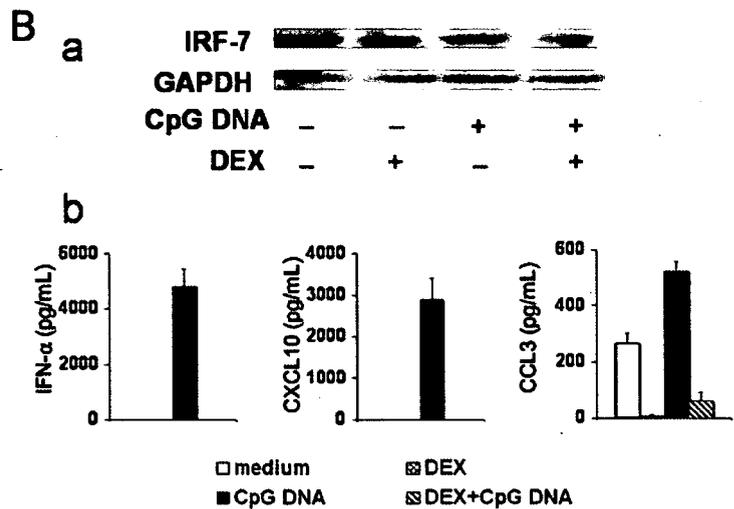10. IFN-β-SB-CpG
11. IFN-β-chlo-CpG
12. IFN-β-DEX/chlo-CpG

FIGURE 8. Machinery sensitive to chloroquine but not to NF-κB/p38 MAPK is involved in the expression of IFN-α via the activation of IRF-7. *A*, pDC were pre-cultured for 3 h with or without 200 IU/ml IFN-β, and then for 1 h with DEX (0.5 μM), SB203580 (SB, 20 μM), chloroquine (chlo, 0.2 μg/ml), DEX + chlo, or medium. The culture was further continued for 8 h with or without CpG DNA. The amount of IFN-α in the culture supernatant was measured by ELISA, and converted to a value relative to the amount in the culture with CpG DNA alone. The experimental design is shown in *a* and the data in *b* with the mean ± SE from five experiments. The amounts of IFN-α in no. 9 and no. 10 were statistically higher than those in no. 3 and no. 4, respectively. *B*, pDC were pretreated for 1 h with DEX or medium, and cultured with medium or CpG DNA for 5 h (*a*) and 12 h (*b*). Expression of IRF-7 was analyzed by Western blot (*a*) and that of IFN-α, CXCL10, and CCL3 by ELISA (*b*), showing that the concentration of DEX used in *A* is effective to inhibit the CpG DNA induction of these proteins. *C*, pDC were pretreated for 1 h with or without SB203580, and cultured for 3 h with or without CpG DNA. The cytoplasmic and nuclear IRF-7 was analyzed by Western blot. Densitometry analysis showed that the density of IRF-7 in the cytoplasmic and nuclear fraction was 36 and 9 in unstimulated pDC, 28 and 57 in CpG DNA-stimulated pDC, and 17 and 28 in SB203580-terated pDC, respectively.

B a

IRF-7
GAPDH
CpG DNA   _   _   +   +
DEX   _   +   _   +

b

☐ medium
■ CpG DNA
▨ DEX
▨ DEX+CpG DNA

C

	Cytosol			Nuclei			
	–	+	+	–	+	+	CpG DNA
	–	–	+	–	–	+	SB203580
IRF-7							
Histone							

IRF-7 was shown in Fig. 8*B* with those of IFN-α/CXCL10/CCL3. The inhibition by SB203580 and/or chloroquine of the CpG DNA induction of IFN-α and/or IRF-7 is shown in Figs. 3 and 4, as well as in our previous report (14). Therefore, as an alternative to biochemical technique or gene manipulation, both of which require many phenotypically stable cells, the working design shown in

Fig. 8*Ba* could be useful for the assessment of the IRF-7 function for the induction of IFN-α in human pDC.

When stimulated with CpG DNA, pDC pretreated with IFN-β were capable of producing a substantial amount of IFN-α even in the presence of the inhibitors, although the extent of increase was low compared with that in the culture without inhibitors (Fig.

8Ab). Similar results were observed when PDTC was used instead of DEX and when the expression of IFN-α mRNA was analyzed regarding the IFN-α-treated pDC (data not shown). Control ODN did not show any effect, and chloroquine abrogated this production. These results suggest that following the stimulation with CpG DNA, IRF-7 is most likely activated upstream of the NF-κB/p38 MAPK activation, probably in chloroquine-sensitive machinery, where CpG DNA is recognized by its specific receptor TLR9 (46, 47).

To verify the activation of IRF-7, we examined the nuclear translocation of IRF-7, as an alternative to phosphorylation or ubiquitination of IRF-7. In this experiment, 20 μM of SB203580 was used to completely inhibit p38 MAPK/NF-κB pathway, and consequently, to prevent the de novo synthesis of IRF-7. Fig. 8C shows that IRF-7 moved to the nuclei independently of the activation of NF-κB/p38 MAPK. Densitometry analysis showed that the combined amount of cytoplasmic and nuclear IRF-7 was as high as 1.9-fold in the CpG DNA-stimulated pDC, but remained unchanged in the presence of SB203680. The rate of nuclear translocation increased from 20 to 67% following the stimulation with CpG DNA and also increased to 62% even when the CpG DNA induction of IRF-7 was inhibited by SB203580. We propose that CpG DNA induces human pDC not only to express but also to activate IRF-7, and that the former is NF-κB-dependent, whereas the later is independent.

Discussion

In this study, we investigated the mechanisms by which CpG DNA induces pDC to express IFN-α, CXCL10, and CCL3, and found that 1) human pDC express constitutively activated NF-κB p65 and p50, and the constitutively activated NF-κB appears to be involved in the constitutive expression of IRF-7 and CCL3; 2), the CpG DNA-activated NF-κB p65/p50 seems to play a crucial role in the type I IFN-independent induction of IRF-7, CXCL10, and CCL3, in collaboration with the p38 MAPK; 3) the signals generated upstream of the activation of NF-κB/p38 MAPK is involved

in the expression of IFN-α, most likely through the activation of IRF-7 in chloroquine-sensitive machinery.

We demonstrate for the first time that pDC express constitutively activated NF-κB family proteins p65 and p50. Although we were unable to define for what purpose NF-κB is constitutively activated in pDC, this activation may render the genes, which are strongly regulated by NF-κB, pre-expressed, and thus efficiently up-regulated by the stimuli that exclusively activate the NF-κB pathway. A representative case is IRF-7, because IRF-7 was constitutively expressed (Figs. 2A, 5, C and D, 7, B and C, and 8, B and C); its expression level decreased in the culture with NF-κB inhibitor (Figs. 5, C and D, and 8B); IRF-7 was directly induced by CpG DNA (Fig. 7C), which activated NF-κB (Fig. 5B); and pDC failed to up-regulate IRF-7 when pretreated with NF-κB inhibitor (Figs. 5, C and D, and 8B). The facile expression of IRF-7 via NF-κB may cause pDC to more efficiently participate in an early defense system against the microbe infection. Another example is CCL3. CCL3 was constitutively expressed (Figs. 1-3), and its expression was inhibited by NF-κB inhibitors (Fig. 5C). This machinery may be important for the participation of pDC in early protection against HIV infection, because the CCL3 receptor, CCR5, acts as a coreceptor for macrophage-tropic HIV-1 strains (48). In this context, the constitutively activated NF-κB seems to represent one of the intrinsic characteristics of pDC.

The second point demonstrated in this study is that, in CpG DNA-stimulated pDC, the inductions of IRF-7, CXCL10, and CCL3, which can be induced by type I IFN stimulation (Fig. 2A), all seem to be mediated by the activation of NF-κB (Fig. 5C) independently of type I IFN signaling (Fig. 2). Of particular interest is that the induction of IRF-7 and CXCL10 by CpG DNA, whose gene expressions have been reported to require the activation of ISRE in other cells (11-13, 28) and the type I IFN signaling in mouse DC (49), was dependent on NF-κB, whereas the contribution of NF-κB was only partial when these gene expressions (IRF-7 and CXCL10) were induced by IFN-α (Fig. 5D). It is not clear at present whether the signal transduction
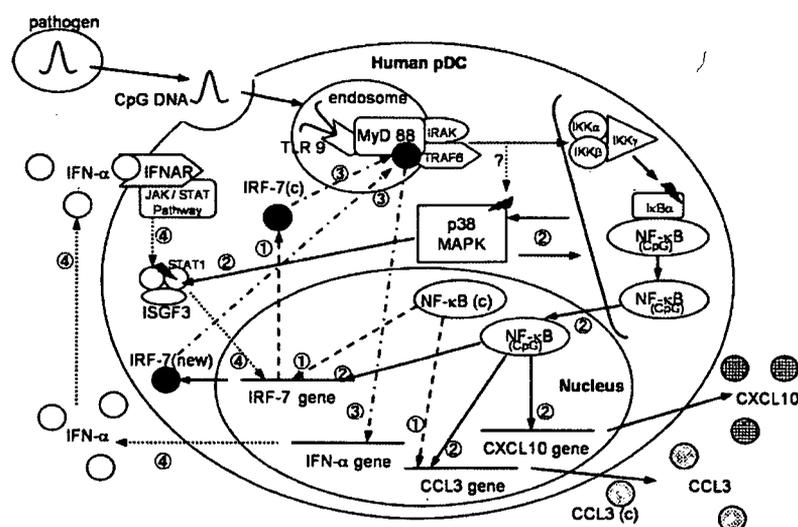

FIGURE 9. Proposed pathways which lead to the expression of IFN-α, CXCL10, and CCL3 following stimulation of human pDC with CpG DNA. The signaling pathway depicted is based on the finding of the current and previous studies (14). ①, Human pDC express constitutively activated NF-κB (NF-κB(c)), which would be involved in the constitutive expression of IRF-7 (IRF-7(c)) and CCL3 (CCL3(c)). ②, When pDC are exposed to a bacterial component CpG DNA, NF-κB (NF-κB(CpG)) and p38 MAPK are activated through the TLR9 signaling pathway, leading to the de novo expression of IRF-7, CXCL10, and CCL3. ③, The signal(s) generated upstream of NF-κB/p38 MAPK activation is involved in the expression of IFN-α, most likely through the activation of IRF-7(c) and/or newly expressed IRF-7(new), in chloroquine-sensitive machinery. ④, The IFN-α, once secreted, returns to pDC via the IFN-αβ receptor and also participates in inducing the expression of IRF-7 gene through the classical type-I IFN signaling pathway. How TLR9 signaling and the IFN signaling pathway affect each other in response to CpG DNA/type I IFN still remains to be clarified.

pathway for IRF-7/CXCL10 induction differ depending on the stimuli. HSV and LPS were demonstrated, quite recently, to up-regulate IRF-7 expression in a manner dependent on NF-κB in human pDC (31). NF-κB-dependent pathway would be important for human pDC in promptly inducing IFN-α-inducible genes without a preceding activation of IFN-αβ receptor.

p38 MAPK regulates NF-κB-dependent gene expression by modifying the activation process, the transcriptional function, and/or the recruitment of NF-κB, in various cells (50–52). The reverse pathway has been recently reported with respect to the expression of cyclooxygenase-2 in *Candida albicans*-infected monocytes (53). In CpG DNA-stimulated pDC, NF-κB and p38 MAPK appeared to collaborate with each other (Fig. 6). As depicted in Fig. 4, the IC_{50} of SB203580 in CpG DNA-stimulated pDC was ~10-fold higher than that in LPS-stimulated monocytes (our personal observation with TNF-α production). The activity of JNK or protein kinase B kinase was reported to be inhibited at higher concentrations of SB203580 in other types of cells (54, 55). These observations might suggest the involvement of other molecule(s) in CpG DNA-activation of pDC. Further examinations are needed. Comparing the NF-κB and p38 MAPK pathways, however, the degree of inhibition by SB203580 varied among the cytokines, while the NF-κB inhibitors revealed the same efficacy to all the IFN-α/CXCL10/CCL3 production. The activation of NF-κB may compensate the function of p38 MAPK, and this may also explain the reason, in part, as to why the high concentration of SB203580 is required for the inhibition of cytokine production in CpG DNA-stimulated pDC. We have previously demonstrated that p38 MAPK pathway is involved in CpG DNA, but not type I IFN, -induced STAT1 phosphorylation in pDC (14). We provide no direct evidence showing that CpG DNA-activated STAT1 participates in IFN-α/chemokine expression. Nevertheless, considering that STAT1 phosphorylation was also inhibited by a NF-κB inhibitor (Fig. 6B), the NF-κB pathway is strongly suggested to form a stem route even for the expression of the genes, which require the ISRE to be activated, such as IRF-7 and CXCL10, in CpG DNA-stimulated pDC. The collaborative action of NF-κB and p38 MAPK may facilitate the up-regulation of these gene expressions.

The third finding is the pathway through which IRF-7 is activated and consequently IFN-α is expressed. We propose that, from the results shown in Fig. 8, the signaling event upstream of NF-κB/p38 MAPK activation, most likely in chloroquine-sensitive machinery, is important for the activation of IRF-7. There are some circumstantial pieces of evidence showing, in mice or cell lines, that 1) IRF-7 is a dominant transcription factor necessary for IFN-α gene expression (56, 57), 2) an association of IRF-7 with MyD88 and TRAF6 in the endosomal compartment is a prerequisite for the activation of the IFN promoter (18, 19), and 3) the kinase activity of IL-1 receptor-associated kinase-1 is necessary for transcriptional activation of IRF-7 independently of the activation of NF-κB and MAPK (42). In CpG DNA-stimulated human pDC, IRF-7 moved to the nuclei in a manner independent of NF-κB/p38 MAPK (Fig. 8). Therefore, also in human pDC, IRF-7 would be activated probably through the formation of a complex with MyD88/TRAF6 in the endosomal vesicles, leading to the transcription of IFN-α gene. To what extent constitutive IRF-7 and CpG DNA-induced IRF-7 contributes to the expression of IFN-α remains uncertain, because the working design shown in Fig. 8 does not show a comparison of their levels. However, the data showing that CHX did not prevent the CpG DNA induction of IRF-7 (Fig. 7C), and that CpG DNA, but not type I IFN, -up-regulation of IRF-7 was completely blocked by NF-κB inhibitors as was that of IFN-α (Fig. 5, C and D), suggest that NF-κB-de-

pendent and newly expressed IRF-7 largely contributes to the expression of IFN-α in CpG DNA-stimulated human pDC.

Recently, Honda et al. (47) demonstrated that a long retention of type I IFN-inducing type of CpG DNA within endosomal vesicles in pDC causes a spatiotemporal regulation of TLR9-MyD88-IRF-7 signaling pathway, and this achieves robust production of type I IFN in the mouse. Regarding their findings, the expression of IFN-α can be augmented through the autocrine response. Indeed, culturing with IFN-α induced IRF-7 expression (Figs. 2A and 5D), and neutralization of IFN-αβ receptor decreased IFN-α production (Fig. 2B). However, this cascade was mostly conducted in a NF-κB-independent way (Fig. 5D). IFN-α has been reported to suppress activation of NF-κB (58). It remains to be elucidated how the NF-κB-dependent and -independent pathways coexist in pDC in relation to the gene induction by CpG DNA.

TLR9 is expressed in endoplasmic reticulum and recognize CpG DNA in endosomes. However, the process by which CpG DNA enter into the cells is still unclear. In a separate set of experiments, we detected SR-A in fresh pDC (positively isolated as $BDCA4^+$ cells). In conjunction with the SR-A ligand-inhibition of cytokine induction by CpG DNA (Fig. 3), it is suggested that the SR-A contributes to the responsiveness of pDC to CpG DNA. We hypothesize an overall cascade that is involved in the CpG DNA induction of IFN-α, CXCL10, and CCL3 in Fig. 9.

CpG DNA has a promising potential for therapeutic use, but the action of CpG DNA in pDC differs depending on the sequence (6, 15, 28, 59). The profiles of cytokine/chemokine production by pal-GACGA1010 are similar to those by number 2216 (prototype CpG-A), and the mechanism demonstrated for palGACGA1010, therefore, would have similarities with that of the IFN-α-inducing type of CpG-A. A large number of microbes and microbial products trigger the production of type I IFN in vivo and in vitro (10). From the current study, pDC appear to represent a key cell type that efficiently detects invading pathogens and translates them into signals, which can be linked by innate and adaptive immunity. The rational manipulation of the NF-κB-dependent and NF-κB-independent pathway may improve the efficacy of pDC/CpG DNA- or IFN-treatment of various diseases.

Acknowledgments

Disclosures

The authors have no financial conflict of interest.

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Double-stranded RNA induces production of RANTES and IL-8 by human nasal fibroblasts

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Abstract

Double-stranded RNA (dsRNA) and the viral RNA mimic, polyinosine–polycytidylic acid (poly(I:C)), are recognized by toll-like receptor 3 (TLR3) that mediates the innate immune response to viral infections. In this study, we investigated the effects of poly(I:C) on the production of chemokines (IL-8, RANTES, and eotaxin), Type I IFNs (IFNα and IFNβ), Th1-cytokines (IL-12 and IFNγ), and pro-inflammatory cytokines (TNF-α and IL-1β) by human nasal mucosa-derived fibroblasts. Human nasal fibroblasts were treated with poly(I:C), and levels of cytokines and chemokines were measured by ELISA. Incubation with poly(I:C) significantly enhanced the secretion of RANTES and IL-8. However, eotaxin, IL-1β, TNF-α, IFNα, IFNγ, and IL-12 were not secreted from nasal fibroblasts stimulated with poly(I:C). The JNK inhibitor SP600125 and the PI3-kinase inhibitor LY294002 significantly blocked the poly(I:C)-induced release of RANTES and IL-8, whereas the p38 MAP kinase inhibitor SB203580 suppressed poly(I:C)-induced secretion of IL-8, but not RANTES. Nasal fibroblasts play an important role in initiating antiviral responses and inflammation of the nasal cavity by producing chemokines leading to enhanced inflammatory cell recruitment.
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Keywords: dsRNA; poly(I:C); Chemokine; IL-8; RANTES; Nasal fibroblast

Introduction

Viral infections of mammalian cells result in the activation of innate immune responses mediated by Type I IFNs, IFNα and IFNβ, and other cytokines [1,2]. Most of the viruses causing upper respiratory infections including rhinoviruses, coxsackievirus, echovirus, influenza viruses, and RSvirus are RNA viruses. RNA viruses synthesize double-stranded RNA (dsRNA) during replication [3], and this is a potent stimulus for innate anti-viral responses through the secretion of cytokines [4]. It is known that dsRNA binds only intracellular targets, including the dsRNA-dependent protein kinase (PKR) [5]. However, cells derived from PKR KO mice still responded to the synthetic dsRNA analogue, polyinosine–polycytidylic acid (poly(I:C)), suggesting the existence of another receptor expressed on the cell surface, which recognizes dsRNA [6,7]. Recently, toll-like receptor (TLR) 3-deficient mice have been

shown to have reduced responses to dsRNA and poly(I:C), suggesting that TLR3 is involved in the recognition of dsRNA [8]. Toll-like receptors play a key role in innate immunity by recognizing conserved microbial pathogen-associated molecular patterns (PAMPs) [9–11]. Recognition of the invading pathogen then triggers production of cytokines and chemokines and up-regulation of co-stimulatory molecules in phagocytes and antigen presenting cells, leading to the activation of T cells [8,12–14].

The nasal mucosa is often affected by viral infection. Thus, it is suspected that dsRNA might also be an important stimulus for the synthesis of cytokines and chemokines. Recently, it was proposed that fibroblasts are not passive players in the immune system. Fibroblasts have been considered mainly a physical barrier, but several studies have shown that they may be important modulators of local inflammation due to their capacity to release a variety of pro-inflammatory mediators, including IL-8, RANTES, eotaxin, and GM-CSF [15–17]. Eotaxin, RANTES, and GM-CSF are implicated in the recruitment and enhanced survival of eosinophils [18–20], and IL-8 is a potent chemoattractant for neutrophils [21].

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Infiltration by these inflammatory cells causes acute inflammation of the nasal mucosa and helps to exacerbate chronic inflammation including allergic rhinitis and nasal polyps. Thus, fibroblasts are important sentinel cells in the immune systems.

In this study, we investigated the effects of poly(I:C) on the production of chemokines (IL-8, RANTES, and eotaxin), Type I IFNs (IFNα and IFNβ), Th1-cytokines (IL-12 and IFNγ), and pro-inflammatory cytokines (TNF-α, IL-1β) in human nasal mucosa-derived fibroblasts. Signaling pathways for the activation by poly(I:C) in nasal fibroblasts were also examined by using inhibitors of MAP kinase (U0126, SB203580, SP600125) and LY294002.

Materials and methods

Reagents

The following reagents were used: SP600125 as the specific inhibitor for JNK (BIOMOL, Plymouth Meeting, PA), SB203580 as the specific inhibitor for p38 MAP kinase (Promega, Madison, WI), U0126 as the specific inhibitor for MEK-1 (Promega), LY294002 as the specific inhibitor for PI3 kinase (Promega), poly(I:C) (Amersham Bioscience, Piscataway, NJ), IL-1β (PeproTech EC, England), TNFα (PeproTech EC), IL-4 (PeproTech EC), IFNγ (PeproTech EC), LPS (MERCK bioscience, Germany), CpG, a synthetic oligodeoxynucleotide that contains CpG motifs mimicking bacterial DNA (5′-ACCGATCGTTCGGCCGGTGACGGCACCA-3′) [22], p44/42 MAP Kinase rabbit polyclonal antibody (Ab) (Cell Signaling, Beverly, MA), SAPK/JNK rabbit polyclonal Ab (Cell Signaling), phospho-p44/42 MAPK (E10) mouse monoclonal Ab (Cell Signaling), phospho-SAPK/JNK (G9) mouse monoclonal Ab (Cell Signaling), phospho-p38 MAPK (28B10) mouse monoclonal Ab (Cell Signaling), phospho-AKT (587F11) mouse monoclonal Ab (Cell Signaling), AKT Rabbit polyclonal Ab (Cell Signaling), and p38 (A12) mouse monoclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA).

Human nasal mucosa-derived fibroblast cell culture and stimulation

Nasal mucosa of the inferior turbinate was obtained from patients with chronic sinusitis or allergic rhinitis when they underwent nasal surgery. Nasal specimens were cultured in 10-cm dishes containing RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% heat-inactivated FCS (Gibco, Grand Island, NY), 0.29 mg/ml glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, at 37°C in 5% CO$_2$ and humidified air. Nasal fragments were removed and the first passage was performed. After a period of 3–4 weeks, nasal mucosa-derived fibroblast cell lines were established. The cells were used at passage numbers 3–5. Epithelial cells were confirmed not to be contaminated by immunohistochemical examination using cytokeratin and vimentin markers. The cells were then placed in a 24-well flat-bottomed tissue culture plate (Corning, Corning, NY) at an initial density of 1×10^5 cells/well for cytokine production or 10-cm dish for Western blotting

and RT-PCR. When the cells were growing in sub-confluent conditions, the culture medium was replaced with serum-free RPMI 1640 medium, and then the cells were stimulated by poly(I:C). Where indicated, cells were also pretreated for 1 h with pharmacological inhibitors. Inhibitors were dissolved in dimethyl sulfoxide (DMSO) at 10 mM and further dilutions were made in cell culture medium. DMSO vehicle controls were included in each experiment.

Cytokine and chemokine assay

The cells were cultured in the presence of poly(I:C) for appropriate periods, then culture supernatants were harvested and stored at −80°C. Amounts of cytokines and chemokines in the cell culture supernatant were measured with commercially available ELISA kits. All the kits were purchased from Biosource International (Camarillo, CA) except for the IFNβ ELISA kit, which was purchased from TFB (Tokyo, Japan). Measurements were performed according to the manufacturer's directions. All samples were assayed in duplicate.

Immunoblot analysis

The cells were washed twice with ice-cold PBS and collected by scraping, then centrifuged and pelleted at 4°C. The cells were homogenized in lysis buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM PMSF, 0.5 mM EDTA, 0.6 μM leupeptin, 2 μM pepstatin A, and 1 mM PMSF] by pipetting and sonication. Protein concentrations were measured using the BioRad Protein Assay Kit (BIORAD, Hercules, CA) in all experiments. Lysates were centrifuged at 10000 rpm for 10 min at 4°C and the supernatants were used for immunoblotting. The supernatants were added to a twofold volume of sample buffer [95% laemmli sample buffer (BIORAD) and 5% 2-mercaptoethanol]. After heating at 95°C for 5 min, the samples were electrophoresed. Proteins were transferred electrophoretically onto polyvinylamidedifluoride (PVDF) membranes (Amersham Bioscience). The blotted membranes were rinsed with 5% non-fat dry milk diluted in PBS containing 0.1% Tween 20 for 60 min at room temperature, then incubated with the appropriate antibodies for 16 h at 4°C. After being washed, the membranes were treated with HRP-conjugated anti-mouse immunoglobulin (Ig) Ab or HRP anti-rabbit Ig Ab (DAKO, Carpinteria, CA) for 60 min at room temperature. Peroxidase color visualization was achieved with TMB membrane Peroxidase Substrate (KPL, Gaithersburg, MD).

Real time PCR

Total RNA was extracted using a total RNA isolation NucleoSpin™ RNA II Kit (MACHERY-NAGEL, Düren Germany). The reverse transcription reaction was performed with TaqMan® RT Reagents (Applied Biosystems Japan, Tokyo, Japan) using random hexamer primers. The amplification of TLRs and β2microglobulin-cDNA was performed in a MicroAmp optical 96-well reaction plate (Applied Biosys-

tems). All TaqMan® probe/primer combinations used in this study were TaqMan® Gene Expression Assay products purchased from Applied Biosystems. β2-Microglobulin was chosen as the reference housekeeping gene because it is convenient to assay and highly expressed. Furthermore, in order to select the housekeeping gene, we evaluated it using a TaqMan® Human Endogenous Control Plate, which was most suitable. TaqMan® PCR was performed in a 20-μl volume using TaqMan® Universal PCR master mix (Applied Biosystems). The reaction was performed in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Reaction mixtures were pre-incubated for 2 min at 50°C. The PCR program was 10 min of Taq Gold activation at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C (maximum ramping speed between temperatures). Human cDNA equivalent to 50 ng of total RNA from each sample was assayed in each tube.

The threshold cycle number (Ct) was determined with sequence Detector Software (version 1.1: Applied Biosystems) and transformed using comparative Ct methods as described by the manufacturer with β2microglobulin as the calibrater gene.

Data and statistical analysis

Data in the text and figure legends are expressed as the mean ± SEM of observations obtained from human nasal fibroblasts cultured from 8 donors, if not otherwise specified. Statistical analysis was performed using the Wilcoxon signed-ranks test to assess the difference in cytokine production levels. Macintosh computers (Apple computer, Cupertino, CA) with Statview software (Abacus Concepts, Berkeley, CA) were used for all statistical analyses.

Results

Human nasal fibroblasts express TLRs

We first set out to determine the expression of TLRs on human nasal fibroblast cells. The expression of mRNA for TLRs on fibroblasts was confirmed by real time RT-PCR. To assess the relative expression levels of TLR mRNAs on the cells, a logarithmic scale was used in Fig. 1a. TLR3, 4, and 9 were highly expressed. TLR1, 2, 5, and 6 were also detected,
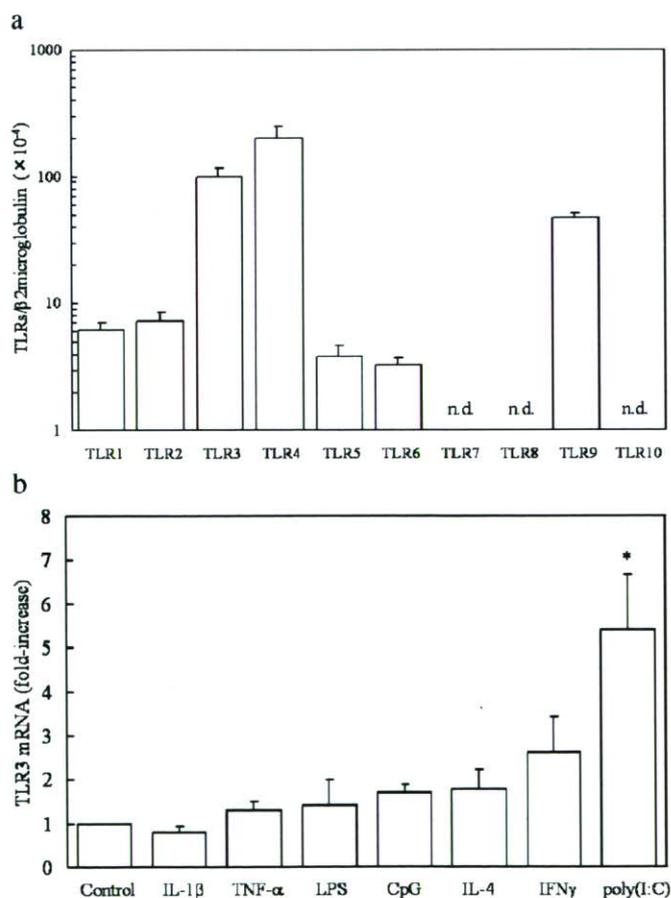

Fig. 1. (a) TLR1-10 mRNA expression levels were assayed by real time RT-PCR using a cDNA template derived by reverse transcriptase from 50 ng of RNA from human nasal fibroblast cells. Reactions were performed in three wells, and results are expressed relative to expression levels of β2microglobulin. Data are presented as the mean ± SEM (n = 12). (b) Analysis of expression of TLR3 mRNA induced by cytokines and PAMPs using real time PCR. Fibroblasts were treated with IL-1β (2 ng/ml), TNF-α (10 ng/ml), LPS (5 μg/ml), CpG (100 μg/ml), IL-4 (10 ng/ml), IFNγ (10 ng/ml), or poly(I:C) (10 μg/ml) for 6 h. Total RNA was isolated and reverse transcribed to cDNA. The cDNAs were used for real time PCR as described in Materials and methods. Data are expressed as the mean ± SEM of the fold increase relative to the control (n = 6). *P < 0.05 compared with control using Wilcoxon's signed-ranks test.

but their expression levels were lower than those of TLR3, 4, and 9. Human nasal fibroblast cells did not express TLR 7, 8, or 10 (Fig. 1a).

We examined the effect on TLR3 mRNA expression of the TLR ligands LPS, CpG, and poly(I:C), pro-inflammatory cytokines IL-1β, and TNFα, Th1 cytokine IFNγ, and Th2 cytokine IL-4. Fibroblasts were treated with the agonists for 6 h, and TLR3 mRNA expression was assessed by real time PCR. The expression of TLR3 mRNA was increased 5.4-fold ($P < 0.05$) in nasal fibroblasts by poly(I:C). However, other PAMPs and cytokines had no significant effect on the level of mRNA for TLR3.

Human nasal fibroblasts produce IL-8 and RANTES when stimulated with poly(I:C)

Production of IL-8, RANTES, eotaxin, IL-1β, TNF-α, IFNα, IFNβ, IFNγ, and IL-12 in poly(I:C)-stimulated nasal fibroblasts was determined in the supernatants by ELISA. Culture supernatants from the fibroblast cells stimulated with various concentrations of poly(I:C) were collected 48 h after the stimulation. A significant release of IL-8 and RANTES was observed with 0.1 µg/ml poly(I:C) (Fig. 2a), and a release of IFNβ was observed with 10 µg/ml poly(I:C), but the amount of IFNβ was very small (Fig. 2b). The rank order for maximal release stimulated by poly(I:C) from human nasal fibroblast cells was IL-8 > RANTES >> IFN-β. The release of eotaxin, IL-1β, TNF-α, IFNα, IFNγ, and IL-12 could not be detected by ELISA (<1–10 pg/ml) at 8, 24, 48, 72, and 148 h after treatment with poly(I:C). Since IL-8 production reached a plateau on stimulation with 10 µg/ml of poly(I:C), 10 µg/ml of poly(I:C) was used in all subsequent experiments. The time course of IL-8 and RANTES production was determined. Concentrations of IL-8 and RANTES in the supernatants changed with time with significant increases detected at 8, 24, 48, and 72 h (Fig. 2c). Optimal harvest time was 48 h after stimulation with 10 µg/ml of poly(I:C).

Poly(I:C) induces phosphorylation of p38 MAP kinase, JNK, and AKT

To determine poly(I:C)-induced intracellular signaling, p38 MAP kinase, JNK, and ERK in nasal fibroblast cells were immunoblotted after stimulation with 10 µg/ml of poly(I:C). The exposure of the cells to poly(I:C) triggered a slow phosphorylation of p38 MAP kinase and JNK. Amounts of phosphorylated threonine and tyrosine of p38 MAP kinase and JNK in poly(I:C)-stimulated cells increased at 30 min (Fig. 3a) and 60 min (Fig. 3b), respectively. The activation of ERK in poly(I:C)-stimulated cells was unchanged during 2 h (Fig. 3c). In addition to MAP kinase, AKT, regulated by the phosphoinositide products of PI3-kinase, was examined. Amounts of phosphorylated serine of AKT in poly(I:C)-stimulated cells increased at 120 min (Fig. 3d). The upper panels of the figure show that equal amounts of p38 MAP kinase, JNK, ERK, and AKT were immuno-
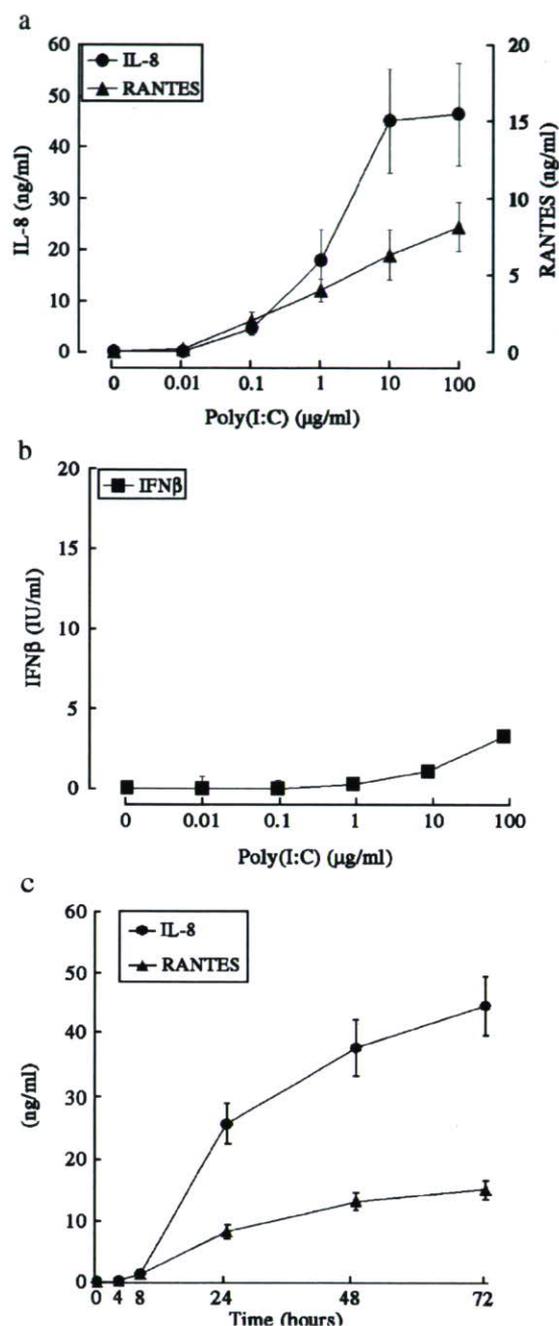
Fig. 2. Poly(I:C) induces production of IL-8, RANTES, and IFNβ by human nasal fibroblasts. Human nasal fibroblast cells were cultured either with medium or with various concentrations of poly(I:C) (0.01, 0.1, 1, 10, and 100 µg/ml) for 48 h. The concentrations of IL-8 (circles), RANTES (triangles) (a), and IFNβ (b) in the culture supernatants were determined by ELISA. Human nasal fibroblast cells were cultured with either medium or poly(I:C) (10 µg/ml) and the concentrations of IL-8 (circles) and RANTES (triangles) in the culture supernatants were determined at 4, 8, 24, 48, and 72 h after stimulation (c). The results are expressed as the mean ± SEM.

blotted with phosphorylation-independent specific antibodies to p38 MAP kinase, JNK, ERK, and AKT regardless of the time course, indicating that poly(I:C)-induced p38 MAP kinase, JNK, ERK, and AKT phosphorylation occurred in
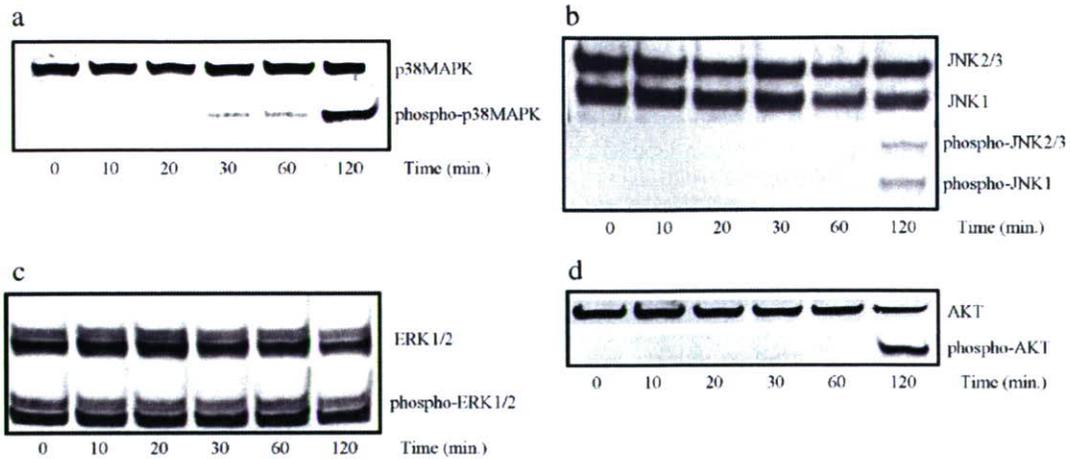
Fig. 3. Poly(I:C) induced the threonine and tyrosine phosphorylation of p38 MAP kinase and JNK but had no effect on the phosphorylation of ERK. Poly(I:C) also induced the serine phosphorylation of AKT. Human nasal fibroblast cells were separated by 10–20% SDS–PAGE, transferred to membranes and blotted with a specific antibody to phosphorylated p38 MAP kinase (phospho-p38) (a, bottom), p38 MAP kinase (a, top), phospho-JNK (b, bottom), JNK (b, top), phospho-ERK (c, bottom), ERK (c, top), phospho-AKT (d, top), or AKT (d, bottom).
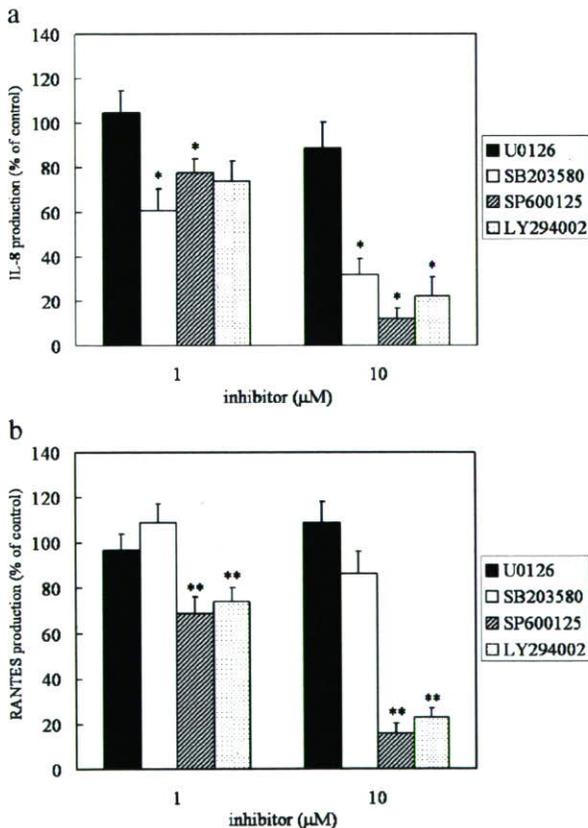

Fig. 4. Human nasal fibroblasts were pre-incubated with SB203580, an inhibitor of p38 MAP kinase; U0126, an inhibitor of MEK; SP600125, an inhibitor of JNK; or LY294002, an inhibitor of PI3 kinase. Data in the graphs show the effects of U0126, SB203580, SP600125, and LY264002 on IL-8 (a) and RANTES (b) production in cells stimulated with poly(I:C) (10 μg/ml) for 48 h. Data are presented as mean values ± SEM (IL-8 $n = 7$, RANTES $n = 10$). *$P < 0.05$, **$P < 0.01$ compared with the levels without inhibitor using Wilcoxon's signed-ranks test.

the absence of changes in ERK, p38 MAP kinase, JNK, and AKT protein levels (Figs. 3a–d).

Effect of MAP kinase inhibitors and PI3 kinase inhibitor on IL-8 production in nasal fibroblast cells stimulated with poly(I:C)

The p38 MAP kinase inhibitor SB203580, the JNK inhibitor SP600125, the ERK inhibitor U1026, and the PI3-kinase inhibitor LY294002 were examined to determine whether they inhibit production of IL-8 in nasal fibroblasts stimulated with poly(I:C) (Fig. 4a). Pre-incubation with SB203580, SP600125, and LY294002 suppressed the poly(I:C)-induced production of IL-8 in a dose-dependent manner. The JNK inhibitor SP600125 had the greatest effect. SP600125 decreased the production of IL-8 in poly(I:C)-stimulated nasal fibroblasts by 87.9% compared to the control level ($P < 0.05$). The ERK inhibitor U0126 had no effect on the levels of IL-8 present in the supernatants of cells stimulated with poly(I:C). There were no differences in cell shape and viability among the four inhibitors (data not shown).

Effect of MAP kinase inhibitors and PI3 kinase inhibitor on RANTES production in nasal fibroblast cells stimulated with poly(I:C)

The four inhibitors were also examined to determine whether they affect the production of RANTES by poly(I:C)-stimulated nasal fibroblasts (Fig. 4b). The same samples used to measure IL-8 levels were examined for RANTES. SP600125 and LY294002 inhibited the production of RANTES by nasal fibroblast cells stimulated with poly(I:C) in a dose-dependent manner. SP600125 had the greatest effect inhibiting production in poly(I:C)-stimulated nasal fibroblasts by 84.3% relative to the control ($P < 0.01$). LY294002 had a similar effect to SP600125 on the production of RANTES by nasal fibroblasts ($P < 0.01$). SB203580 and U0126 had no effect on the level of

RANTES present in the supernatants of cells that were stimulated with poly(I:C).

Discussion

In this study, we demonstrated that nasal mucosa-derived fibroblasts express a large amount of TLR3 mRNA. Stimulation with poly(I:C) directly induced production of IL-8 and RANTES in nasal fibroblasts. The signal pathway for IL-8 and RANTES production was via JNK and PI3 kinase. Additionally, p38 MAP kinase was also important for the production of IL-8.

The nose is the target of a substantial number of infectious agents that produce agonists for TLRs, including gram-negative and positive bacteria, mycobacteria, fungi, viruses, and numerous helminthes. Despite this fact, relatively little is known about the expression of toll-like receptors on nasal fibroblast cells. The presence of TLRs on primary bronchial epithelial cells and BEAS-2B airway epithelial cells [23], as well as alveolar and bronchial epithelial cells [24], has been demonstrated. TLR3 was highly expressed on nasal fibroblast cells as it was on primary bronchial epithelial cells. The expression of TLR3 in primary bronchial epithelial cells was positively regulated by the influenza A virus and by dsRNA [24], and expression of TLR3 in the MRC-5 human lung fibroblast cell line and A549 human lung epithelial cell line was positively regulated by respiratory syncytial virus [25]. We confirmed that poly(I:C) also up-regulated TLR3 expression in human nasal fibroblasts.

Poly(I:C) is a potent and selective stimulus for the secretion of IL-8 and RANTES, but not pro-inflammatory cytokines (IL-1β and TNF-α), Th1 cytokines (IFNγ and IL-12), or eotaxin. We previously showed that pro-inflammatory cytokines and IFNγ induced production of RANTES by nasal fibroblasts [17,26,27]. However, these cytokines were not expressed in nasal fibroblast cells stimulated with poly(I:C). Thus, RANTES was produced as a direct effect of poly(I:C), not in an autocrine manner. Type I IFNs, IFNα and IFNβ, are key effectors in the innate immune responses to viral infections. However, in this study, significant production of IFNα and IFNβ by nasal fibroblasts was not induced by a viral mimic, poly(I:C). We also confirmed that IFNα and IFNβ were not detected in the culture supernatants even if the incubation was extended to 7 days after poly(I:C) stimulation. Recently, several studies showed that plasmacytoid dendritic cells (pDCs) play a pivotal part in antiviral immune responses because of their extraordinary capacity to produce Type I IFNs against viral infection [28,29]. They also sense viral ssRNA or its degradation product via TLR7/8, leading to the production of Type I IFNs [30,31]. In contrast, nasal fibroblasts may not recognize the viral genome of ssRNA viruses because TLR7/8 mRNA was not detected in nasal fibroblasts. Since RNA viruses produce dsRNA during RNA replication, it is considered that in the proliferative phase of the infection, nasal fibroblasts have an antiviral effect.

Poly(I:C), a synthetic dsRNA polymer, was shown to act as an adjuvant [32], and has been used to mimic RNA viral

infections. All TLRs share a common cytoplasmic signaling domain, the toll-interleukin 1 (IL-1) receptor domain (TIR domain). This domain mediates the association between TLRs and adaptors, such as MyD88 and TIRAP [11,33]. MyD88 is used by most TLRs. However, only TLR3 was associated with another adaptor, TRIF (also called TICAM-1) [34–36]. Signal transduction pathways downstream of TRIF lead to NF-κB, IRF-3, and MAP kinases [37–39]. NF-κB is most commonly the transcription factor associated with IL-8 and RANTES production [40,41]. AP-1, which can be activated by p38 MAP kinase and JNK, is also an important positive regulator of IL-8 and RANTES promotor activity [42,43]. Although it is not clear which pathway mediates the signaling of poly(I:C) to MAP kinases, it is suggested that poly(I:C) activates the TLR3-TRIF-MAP kinase pathway in nasal fibroblast cells.

The observation that the p38 MAP kinase inhibitor SB203580 blocks production of IL-8 but not RANTES suggested that there are different signaling pathways involved in the up-regulation of these two chemokines by poly(I:C). There are conflicting reports about the involvement of p38 MAPK and RANTES. In bronchial epithelial cells, Gern et al. reported that SB203580 did not inhibit the production of RANTES induced by dsRNA [44]. Kujime et al. reported that SB203580 did inhibit RANTES production induced by Influenza in vitro [45]. In airway smooth muscle cells, Hallsworth et al. reported that SB203580 did not inhibit the production of RANTES induced by IL-1β [46]. Maruoka et al. reported that SB203580 did inhibit RANTES production induced by PAF [47]. In nasal fibroblasts, the result might change if the stimulation is different. Gern et al. also reported that SB203580 inhibited IL-8 production induced by dsRNA in bronchial epithelial cells [44]. However, the mechanisms activating p38 MAP kinase and producing IL-8 in epithelial cells and fibroblast cells also remain unclear. Further study is necessary.

Our study added the result to Gern's work that JNK and PI3-kinase are also important to the production of IL-8 and RANTES stimulated by poly(I:C). Recently, Sarkar reported that the PI3 kinase-AKT pathway plays an essential role in the TLR3-mediated TBK-1 to IRF-3 pathway [48]. IRF-3 binds to the promotor region of RANTES and plays a crucial role in gene expression [49,50]. There is no report that IRF3 binds to the promotor region of IL-8. It is thought that the pathway to IRF-3 does not lead to IL-8 production though it may lead to RANTES production. NF-κB plays an important role in the gene expression of IL-8 [51]. Xianwu Li reported that PI3-kinase is an important mediator of LPS signaling leading to the activation NF-kB through AKT in human microvascular endothelial cells [52]. The signal transduction pathway downstream of TLR3-TRIF-PI3K may associate with not only IRF3 but also NF-κB.

RANTES is a highly effective chemoattractant for T lymphocytes, monocytes, eosinophils, and basophils [20,53]. Infections with some viruses have been shown to induce RANTES expression in a wide variety of cells [54–59]. Thus, virus-induced RANTES expression could be a major element in the pathogenesis of viral infection. IL-8 is an important

mediator of the inflammatory response to many stimuli, including viruses [60–62]. Most importantly, IL-8 is a major neutrophil chemoattractant and activator [21]. It was confirmed in our laboratory that IL-1β, TNFα, and LPS also induced production of both IL-8 and RANTES by nasal fibroblasts (data not shown). But less RANTES was produced in response to IL-1β, TNFα, and LPS than to poly(I:C). However, the rank order for maximal capacity to stimulate production of IL-8 by human nasal fibroblast cells was IL-1β > TNF-α > LPS > poly(I:C). This difference may contribute to the specific role of nasal fibroblasts in protection against viral infections. T cells and granulocytes recruited to the airways are likely to contribute to antiviral activity. On the other hand, increased cellular inflammation could also add to airway obstruction and dysfunction, leading to symptoms in the upper and lower airway. If the function of chemokines secreted during viral infections was defined further, specific inhibitors of the signal pathway associated with chemokine production might be used to regulate cellular inflammation in the pathogenesis of virus-induced inflammation of the nasal cavity, rhinosinusitis and exacerbations of allergic rhinitis.

Acknowledgments

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PEDIATRIC ALLERGY AND IMMUNOLOGY

Determination of structure and transcriptional regulation of *CYSLTR1* and an association study with asthma and rhinitis

Zhang J, Migita O, Koga M, Shibasaki M, Arinami T, Noguchi E. Determination of structure and transcriptional regulation of *CYSLTR1* and an association study with asthma and rhinitis.
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Pharmacologic studies have revealed that cysteinyl leukotrienes (CYSLTs) act through two receptors, cysteinyl leukotriene receptor 1 (CYSLTR1) and CYSLTR2. CYSLTR1 antagonists are widely used to treat asthma and rhinitis. In this study, we characterized the genomic structure and transcriptional regulation of *CYSLTR1* and examined associations between *CYSLTR1* polymorphisms and asthma/rhinitis. The experiment of rapid amplification of cDNA end revealed that *CYSLTR1* contains three exons and that the entire open reading frame is located in exon 3. Reverse transcriptase-polymerase chain reaction showed that there were multiple splice variants of *CYSLTR1* and that the transcript expression patterns differed from tissues and cell types. The promoter region of *CYSLTR1* is from −665 to −30 bp relative to the transcription start site. We identified four polymorphisms (c.-618-434T/C, c.-618-275C/A, c.-618-136G/A, and 927C/T), and transmission disequilibrium tests revealed that none of these polymorphisms was associated with the development of asthma/rhinitis. However, the TCG and CAA haplotypes in the promoter region caused different transcriptional activity. Our findings indicate that *CYSLTR1* polymorphisms are not likely to be involved in the development of asthma/rhinitis, but it is possible that these polymorphisms could influence drug responses in individuals with atopic diseases.

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Asthma and allergic rhinitis are considered chronic inflammatory diseases characterized by infiltration of inflammatory cells such as T cells, eosinophils and mast cells (1, 2). Among chemical mediators known to be involved in the pathophysiology of bronchial asthma and rhinitis, cysteinyl leukotrienes (CYSLTs) are considered to be some of the most important mediators because they cause bronchoconstriction, vascular hyperpermeability, and mucous hypersecretion (3, 4). CYSLTs act through cell surface receptors. Pharmacologic studies have revealed that CYSLTs activate at least two receptors, cysteinyl leukotriene receptor 1 (CYSLTR1) and CYSLTR2 (5–7). These receptors have different potencies and affinities for endogenous agonists and different selectivities for antagonists (8, 9). In 1999, the *CYSLTR1* was cloned by ligand fishing

for orphan 7-transmembrane (7TM) receptors (10, 11). The cDNA encodes a protein of 337 amino acids and maps to human chromosome Xq21.1. Northern blotting revealed that *CYSLTR1* is expressed in spleen, peripheral blood leukocytes, lung, pancreas, small intestine, and prostate (10, 11). *In situ* hybridization experiments showed that *CYSLTR1* is present in human lung smooth muscle and lung macrophages (12). In intracellular calcium mobilization assays, CYSLTs increased Ca^{2+} concentrations with a rank order of potency of leukotriene D_4 $(LTD_4) > LTC_4 > LTE_4$ (10, 11, 13). CYSLTR1 antagonists, such as montelukast (singulair), zafirlukast (accolate) and pranlukast (onon), have been widely used to treat asthma. CYSLTR1 antagonists have also proven effective for treatment of allergic rhinitis (14). Equilibrium